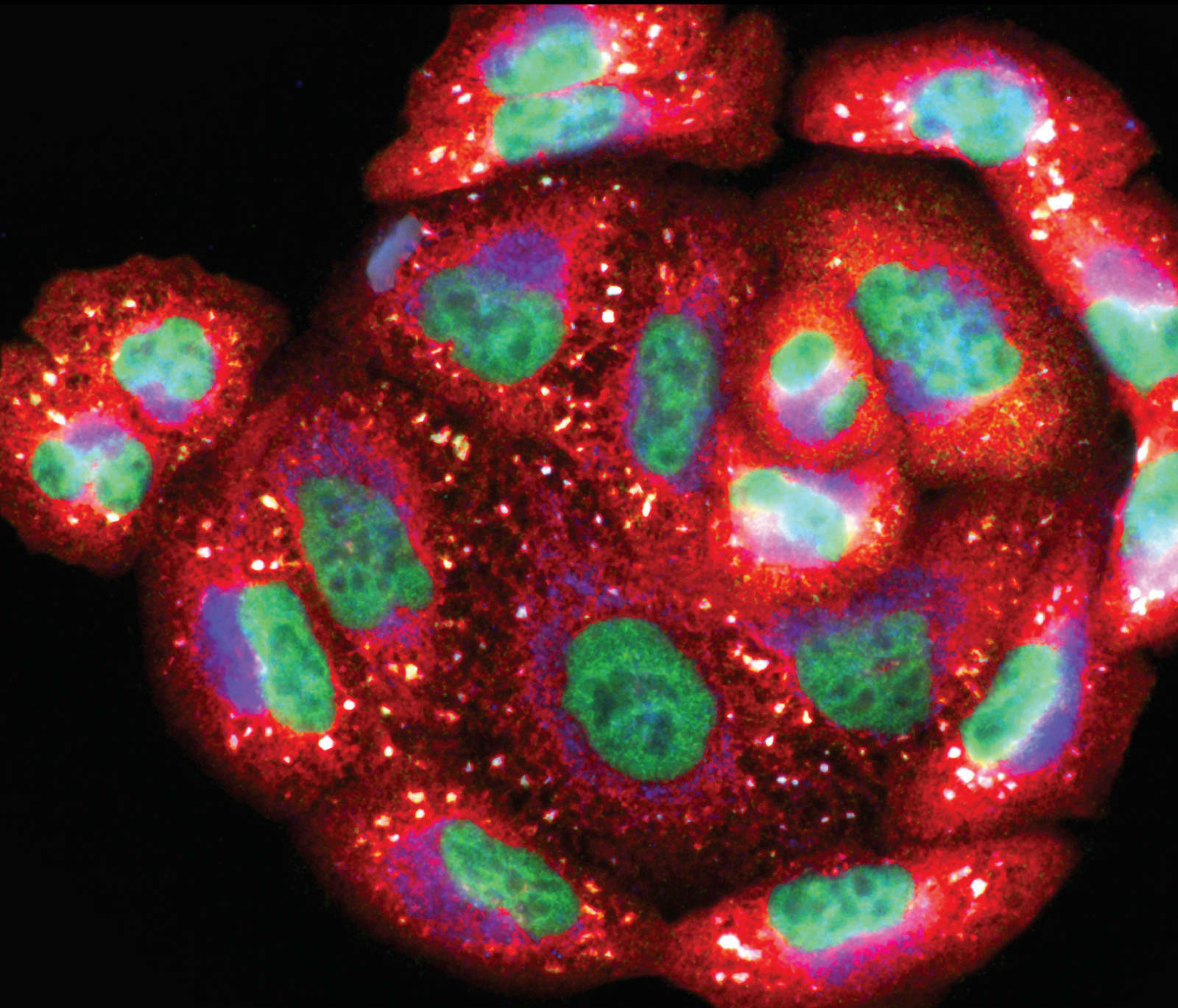# Harmful and Beneficial Role of ROS 2020

Lead Guest Editor: Sergio Di Meo
Guest Editors: Paola Venditti, Victor M. Victor, and Gaetana Napolitano

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Contents

Editorial

Harmful and Beneficial Role of ROS 2020

Sergio Di Meo [iD],[1] **Paola Venditti** [iD],[1] **Victor M. Victor** [iD],[2] **and Gaetana Napolitano** [iD][3]

[1]*University of Naples Federico II, Naples, Italy*
[2]*University of Valencia, Valencia, Spain*
[3]*University of Naples Parthenope, Naples, Italy*

Correspondence should be addressed to Gaetana Napolitano; gaetana.napolitano@uniparthenope.it

Reactive oxygen species (ROS) are molecules deriving from the incomplete reduction of molecular oxygen in the cell. They can be free radicals, atoms, and molecules with an unpaired electron in their outer shell, and because of their chemical structure, ROS are unstable and highly reactive species. Therefore, they are generally short-lived and often leave the subcellular production site after undergoing a reduction process [1]. Thus, ROS may cause oxidative damage to macromolecules such as DNA, proteins, and lipids [2]. More recent findings highlighted that ROS also have important functions in cellular signalling as participants and modifiers of signalling pathways [3]. Although major ROS-sensitive signal transduction pathways have been shown, the research in this field is very active. In our special issue, several research articles identify new involvement of ROS in signalling pathways for triggering pathologies.

A. Nemmar et al. [4] performed *in vivo* experiments to assess the effects of adenine- (0.2% *w/w* in feed for 4 weeks) induced chronic kidney disease (CKD) on heart histology, inflammation, oxidative stress, nuclear factor erythroid 2-related factor 2 (Nrf2) expression, and DNA damage. They showed that adenine intake increases the levels of markers of lipid peroxidation measured by malondialdehyde production and 8-isoprostane and the activities of the antioxidant enzymes superoxide dismutase and catalase. Moreover, the immunohistochemical analysis of the hearts also showed an increase in the expression of Nrf2 in cardiomyocytes. The authors concluded that the administration of adenine in mice induces CKD which is associated with cardiac inflammation, oxidative stress, Nrf2 expression, and DNA damage. These results are consistent with another study that

reports that pretreatment with nicorandil (K_{ATP} channel opener) causes upregulation of Nrf2 mRNA in the aortic tissues of rats fed with an adenine-rich diet, suggesting that the improvement of oxidative stress in the aortic tissue could reduce the CDK-associated aortic calcifications [5].

Z. Zhao et al. [6] demonstrated that in *in vitro* experiments, the involvement of ROS in the differentiation of cancer stem-like sphere cells enriched the Hep G2 human hepatocellular carcinoma cell line into endothelial cells forming functional blood vessels. In particular, H_2O_2 activates the Akt/IKK signalling pathway thus inducing the differentiation process of the tumour blood vessel. This study indicates a possible mechanism of resistance to antiangiogenic agents. The differentiation of cancer stem cells into tumour endothelial cells and, ultimately, into tumour angiogenesis involves the interaction among ROS, the inactivation of the pentose phosphate metabolic pathway, and the activation of autophagy [7]. Knowledge of the origin of tumour blood vessel helps design powerful cancer therapies and suggests that the use of conventional radiotherapy and chemotherapy-based therapies should be reconsidered [7].

Y. Zhao et al. [8] evaluated oxidative stress, mitochondrial function, osteogenic function, and bone formation in *in vivo* and *in vitro* experiments using lipopolysaccharide- (LPS-) induced inflammation models. *In vivo* experiments were performed using 10-week-old C57BL/6J mice characterized by an alveolar bone defect. They were treated with LPS in the absence and presence of cyclosporine A (CsA) for three weeks. *In vitro* experiments were performed using LPS-treated murine osteoblasts in the presence of CsA or an inhibitor of extracellular signal-regulated kinase 1/2

(ERK1/2). *In vivo* results showed that LPS inhibits bone remodelling and promotes the accumulation of oxidative stress in alveolar bone defects. These biochemical changes are reduced by CsA treatment. *In vitro* experiments identified mitochondria as responsible for increased ROS production and oxidative stress in LPS-treated osteoblasts. Oxidative stress reduced the expression of osteogenic differentiation genes by activating the ROS/ERK signalling pathway. Treatment with CsA improved bone remodelling by alleviating oxidative stress caused by LPS. This finding was associated with inhibition of the ERK signalling pathway, suggesting that inflammatory bone diseases can be treated by preserving mitochondrial function and reducing ROS production. In line with these results, it has been reported that mitochondria are a major source of LPS-stimulated ROS generation in microglia and that regulation of ROS production modulates, in turn, the production of proinflammatory mediators by preventing activation of the MAPK pathway induced by LPS and activation of NF-κB in microglia [9].

Lu et al. [10] identified the involvement of ROS in the macrophage's polarization in the *Helicobacter pylori* infection. The study was carried out *in vitro* by coculturing EAW-364.7 cells with *H. pylori* at various multiplicities of infection (MOIs). They evaluated the macrophage polarization in M1 and M2 phenotypes, ROS production, and hypoxia-inducible factor 1α (HIF-1α). Macrophages were also treated with the ROS inhibitor NAC or HIF-1α inhibitor YC-1. The authors concluded that *H. pylori* enhances the ROS production and HIF-1α expression in macrophages and the MOI of *H. pylori* affects macrophage polarization state. These results rely on the crosstalk between ROS and HIF-1α that regulates *H. pylori*-induced macrophage polarization via the Akt/mTOR pathway. This study increases the current knowledge about the mechanisms involved in the macrophage polarization and gets the basis to the development of drugs able to modulate this process in the view of treatments of diseases such as atherosclerosis, enteritis, nephritis, tumour disease, and disorders of the nervous and skeletal system [11].

M. A. Lillo et al. [12] described in their research article the involvement of the superoxide anion in the signalling pathway dependent on pannexin-1 (Panx-1) for the control of endothelial cell function and NO-dependent relaxation. The authors demonstrate that blockade of Panx-1 channels leads to activation of Na$_v$ channels and parallel recruitment of Panx-1 in caveolae, in association with Cav-1. The depolarizing current is mediated by the Na$_v$ channel and is coupled to the opening of Cav$_{3.2}$ and the subsequent entry of Ca^{2+}. The concomitant depolarization of endothelial cells and the increase in [Ca^{2+}]$_i$ results in the further activation of NADPH oxidase/O$_2$$^-$ signalling, which triggers the PI3K/Akt pathway and the consequent increase in NO-mediated vasodilation through the modulation of eNOS activity of enzyme phosphorylation.

The signalling regulation of the signalling pathway by ROS is also relevant for glucose homeostasis, and it has been reported that it is involved not only in alterations in insulin signalling and the onset of insulin resistance [13] but also in glucagon secretion and the onset of type II diabetes, as summarized by A. N. Onyango in his review article [14]. In normal pancreatic islets, when plasmatic glucose concentration exceeds 7 mM, glucagon secretion is suppressed by the paracrine action of insulin and somatostatin produced by beta and delta cells, respectively. This paracrine suppression is lost in diabetes because alpha cells are resistant to insulin and somatostatin. Chronic exposure of alpha cells to elevated glucose levels upregulates SGLT-1 expression and activates the signalling pathway involving PI3K-Akt, PKC-δ, Src, and ROS in the islets of diabetic subjects. Oxidative stress and mitochondrial abnormalities cause reduced ATP production in alpha cells. The author reported that in addition to glucose, hydrogen peroxide promotes glucagon secretion and, if in excess, can induce oxidative stress and reduce ATP which are relevant in glucagon dysregulation in diabetes.

An interesting review of E. Maldonado et al. [15] describes the molecular mechanisms and the signalling pathways through which ROS produced in *Trypanosoma cruzi* infection (Chagas disease) induces beneficial effects on the pathogen and harmful effects on the host. The authors hypothesize that at the beginning of the infection, ROS produced by macrophages in response to *T. cruzi* infection can activate the MAPK transduction pathway in the pathogen that allows its growth and proliferation. In the later stages of infection, mitochondrial ROS are produced by infected cardiomyocytes that contribute to the oxidative damage that persists at the chronic phases of the disease. Oxidative damage leads to functional impairment of the heart. In this way, ROS trigger the growth and proliferation of the parasite and produce long persisting damage to the cardiomyocytes even in the absence of the parasite.

Involvement of ROS in signalling pathways is not only harmful to cells. ROS also play an important role in many homeostatic processes involving metabolism, immunity, growth, and differentiation; they are essential for proper cell development and proliferation, can have mitogenic effects, can mimic and amplify action of growth factors, and can induce the activation of antioxidant systems in response to environmental stimuli. The biological specificity of the actions is obtained through the quantity, duration of production, and location of ROS. This issue is examined in an interesting way by S. Di Meo and P. Venditti [16]. They point out that free radicals play a dual role in living systems: they are toxic by-products of aerobic metabolism, because they cause oxidative damage and tissue dysfunction, and they act as molecular signals by activating beneficial stress responses at low concentrations. From this point of view, the use of antioxidant molecules is generally considered useful to counteract the harmful effects of free radicals, but it is sometimes harmful as it can block adaptive responses induced by low radical levels [17–19].

In our opinion, the articles included in this special issue represent an important contribution to the knowledge concerning the cellular role of ROS, particularly referring to the involvement of these species in the regulation of signalling pathways.

Conflicts of Interest

The authors declare no conflicts of interests.

Sergio Di Meo
Paola Venditti
Victor M. Victor
Gaetana Napolitano

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Research Article

Novel Pannexin-1-Coupled Signaling Cascade Involved in the Control of Endothelial Cell Function and NO-Dependent Relaxation

Mauricio A. Lillo ,[1] **Pablo S. Gaete** ,[1] **Mariela Puebla** ,[2] **Pía C. Burboa** ,[1] **Inés Poblete**,[1] and **Xavier F. Figueroa** [1]

[1]*Departamento de Fisiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago 8330025, Chile*
[2]*Centro de Fisiología Celular e Integrativa, Facultad de Medicina-Clínica Alemana, Universidad del Desarrollo, Santiago, Chile*

Correspondence should be addressed to Xavier F. Figueroa; xfigueroa@bio.puc.cl

Deletion of pannexin-1 (Panx-1) leads not only to a reduction in endothelium-derived hyperpolarization but also to an increase in NO-mediated vasodilation. Therefore, we evaluated the participation of Panx-1-formed channels in the control of membrane potential and $[Ca^{2+}]_i$ of endothelial cells. Changes in NO-mediated vasodilation, membrane potential, superoxide anion ($O_2^{\cdot-}$) formation, and endothelial cell $[Ca^{2+}]_i$ were analyzed in rat isolated mesenteric arterial beds and primary cultures of mesenteric endothelial cells. Inhibition of Panx-1 channels with probenecid (1 mM) or the Panx-1 blocking peptide ^{10}Panx (60 μM) evoked an increase in the ACh (100 nM)-induced vasodilation of KCl-contracted mesenteries and in the phosphorylation level of endothelial NO synthase (eNOS) at serine 1177 (P-eNOS^{S1177}) and Akt at serine 473 (P-Akt^{S473}). In addition, probenecid or ^{10}Panx application activated a rapid, tetrodotoxin (TTX, 300 nM)-sensitive, membrane potential depolarization and $[Ca^{2+}]_i$ increase in endothelial cells. Interestingly, the endothelial cell depolarization was converted into a transient spike after removing Ca^{2+} ions from the buffer solution and in the presence of 100 μM mibefradil or 10 μM Ni^{2+}. As expected, Ni^{2+} also abolished the increment in $[Ca^{2+}]_i$. Expression of $Na_v1.2$, $Na_v1.6$, and $Ca_v3.2$ isoforms of voltage-dependent Na^+ and Ca^{2+} channels was confirmed by immunocytochemistry. Furthermore, the Panx-1 channel blockade was associated with an increase in $O_2^{\cdot-}$ production. Treatment with 10 μM TEMPOL or 100 μM apocynin prevented the increase in $O_2^{\cdot-}$ formation, ACh-induced vasodilation, P-eNOS^{S1177}, and P-Akt^{S473} observed in response to Panx-1 inhibition. These findings indicate that the Panx-1 channel blockade triggers a novel complex signaling pathway initiated by the sequential activation of TTX-sensitive Na_v channels and $Ca_v3.2$ channels, leading to an increase in NO-mediated vasodilation through a NADPH oxidase-dependent P-eNOS^{S1177}, which suggests that Panx-1 may be involved in the endothelium-dependent control of arterial blood pressure.

1. Introduction

Control of blood flow distribution relies on coordinated changes in the diameter of resistance arteries through a complex interplay between the vasoconstrictor and vasodilator signals that determines the degree of smooth muscle constriction (i.e., vasomotor tone). Endothelial cells play a critical role in this process by the activation of several signaling pathways that mediate the response initiated by different stimuli, which are, consequently, known as endothelium-dependent vasodilators [1, 2]. Nitric oxide (NO) has been recognized as the major vasodilator signal generated by endothelial cells; however, in small resistance arteries (i.e., feed arteries and arterioles), an additional vasodilator pathway associated with the NO-independent hyperpolarization of smooth muscle cells has also been described [2]. As this vasodilator pathway relies on the gap junction-mediated transmission to smooth muscle cells of a hyperpolarizing current initiated in the endothelium by the opening of Ca^{2+}-activated K^+ channels (K_{Ca}) of small (SK_{Ca}) and

intermediate (IK_{Ca}) conductance [2–4], this vasodilator component was termed as endothelium-derived hyperpolarization (EDH) [5]. In addition, to mediate the EDH signaling, gap junction communication has also been shown to provide a preferential signaling pathway for NO and contribute to the coordination of vasomotor tone in the microcirculation [6]. Although gap junctions play a central role in the coordination of vascular function, a functional association in endothelial cells between voltage-dependent Na^+ channels (Na_v) and T-type, voltage-dependent Ca^{2+} channels (Ca_v3) of the subtype $Ca_v3.2$ has also been proposed to participate in this process by supporting the conduction of vasodilator signals [2, 7, 8].

Gap junctions are intercellular channels formed by the serial docking of two hemichannels, each one provided by each neighboring cell, and, in turn, hemichannels are made up by the association of six protein subunits known as connexins. Interestingly, individual hemichannels might be functional, providing a pathway to connect the intra- and extracellular compartments [9–11]. Connexin-based channels may allow the release (hemichannels) or intercellular transfer (gap junction channels) of current, ions, and small signaling molecules (<1.4 nm diameter) such as ATP and IP_3 [9–12]. In addition to connexins, channels formed by pannexins have emerged as an important signaling pathway for the control of vasomotor tone in resistance arteries [13]. Pannexins are a protein family structurally related to connexins; however, apparently, these proteins do not constitute functional gap junction channels in physiological conditions and only form membrane channels with similar characteristics to hemichannels, which have been proposed to function as a preferential pathway for ATP release [11, 14, 15].

Of the three pannexin isoforms described (Panx-1, Panx-2, and Panx-3), only Panx-1 has consistently been found in the vessel wall [16]. In resistance arteries, this pannexin isoform is expressed in endothelial and smooth muscle cells and the sympathetic nerve-triggered vasoconstriction initiated by α1-adrenoceptors has been reported to be mediated, in part, by ATP release from smooth muscle cells through Panx-1 channels [13, 16]. In contrast, in conduit arteries, Panx-1 is only expressed in the endothelium [16] and the activation of a Panx-1 channel-initiated purinergic signaling was found to contribute to the EDH-dependent vasodilation elicited by the endothelium-dependent vasodilator, acetylcholine (ACh) [17]. However, although the EDH-mediated vasodilator pathway was reduced in Panx-1 knockout mice, the NO-dependent vasodilator component was enhanced in these animals, suggesting that Panx-1 may be involved in the tonic regulation of NO production by the endothelial isoform of the enzyme endothelial NO synthase (eNOS) [17].

In the present study, we examined the effect of acute Panx-1 channel inhibition on the NO-dependent vasodilation induced by ACh in mesenteric resistance arteries. To this end, we evaluated the variations in vasomotor responses in relation to the changes in membrane potential and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) observed in endothelial cells during the application of Panx-1 channel blockers. Our results show that the blockade of Panx-1 channels triggered a fast, transient, membrane depolarization-initiated increase in $[Ca^{2+}]_i$, which leads to the activation of a complex signaling cascade that evokes eNOS phosphorylation at serine 1177 (P-eNOS^{S1177}) and the subsequent increment in the NO-mediated vasodilator component.

2. Materials and Methods

Male Sprague-Dawley rats (200-230 g) were bred and maintained in the Research Animal Facility of the Pontificia Universidad Católica de Chile. All experimental protocols were conducted according to the Helsinki Declaration and the Guiding Principles of Care and Use of Laboratory Animals endorsed by the American Physiological Society. In addition, the study was approved by the Institutional Bioethics Committee.

2.1. Perfusion of the Isolated Mesenteric Arterial Bed. Rats were anesthetized with xylazine and ketamine (10 and 90 mg/kg, i.p., respectively), and the mesenteric arterial bed was isolated as described by Lillo et al. [18]. Briefly, the superior mesenteric artery was cannulated and the mesentery was perfused at 2 mL/min with a warmed (37°C) Tyrode buffer solution (in mM: 118 NaCl, 5.4 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 23.8 $NaHCO_3$, and 11.1 glucose) that was bubbled with 95% O_2-5% CO_2 to yield pH 7.35-7.45. Immediately thereafter, the aorta was cut to ensure a fast killing by exsanguination and mesenteries were severed from the intestinal wall. Isolated mesenteric arterial beds were placed in a perfusion chamber, and the experiments were started after an equilibration period of 20 min. Changes in perfusion pressure were recorded by means of a pressure transducer (P23Db Statham) connected at the entrance of the superior mesenteric artery and the WinDaq software (DataQ Instruments Inc., USA). All drugs were applied dissolved in the perfusion solution.

2.2. Vasomotor Responses. In the isolated mesenteric preparation, the intraluminal pressure of resistance arteries is low and does not reach the threshold to activate a myogenic response. Therefore, vessels were constricted with 70 mM KCl, since the ACh-elicited vasodilation depends exclusively on NO in KCl-constricted mesenteries [6, 19]. High-KCl solutions were prepared by equimolar substitution of Na^+ ions for K^+ ions. The response to ACh was evaluated 15 min after blocking Panx-1 channels with 1 mM probenecid, and the results were expressed as a percentage of reduction in the perfusion pressure (% relaxation) or as a percentage of change in the perfusion pressure along the time (% baseline).

2.3. Primary Cultures of Mesenteric Endothelial Cells. Microvascular endothelial cells were isolated as described by Ashley et al. [20]. Briefly, after removing the blood from the vessels by perfusing a sterile Tyrode buffer solution containing a mixture of antibiotics and antimycotics (Anti-Anti solution, Gibco, Invitrogen, NY, USA), mesenteries were incubated in a physiological saline solution containing 0.2% collagenase type I and 0.1% BSA at 37°C. After 1 h, the collagenase/BSA solution was removed by two successive applications of M-199 media and centrifugation. Pelleted cells were

resuspended in M-199 media containing $20 \mu g/mL$ endothelial cell growth supplement from bovine pituitary (ECGS) and 20% fetal bovine serum (FBS) and seeded onto sterile glass coverslips. Nonadherent cells were removed 4 h later, and the remaining adherent endothelial cells were kept at 37°C in a 5% CO_2-95% air atmosphere at nearly 100% relative humidity. To carry out the experiments, the culture media of endothelial cells of 70 to 80% of confluence (~2 days of culture) were replaced by a MOPS-buffered Tyrode saline solution (pH 7.4).

2.4. Measurements of Intracellular Ca^{2+} Concentration. Changes in $[Ca^{2+}]_i$ were detected using the fluorescent Ca^{2+} indicator, Fluo 4 (Life Technologies, OR, USA), as described previously [18]. Fluo 4 was uploaded by incubating the primary cultures of endothelial cells with $10 \mu M$ Fluo 4-acetoxymethyl ester (Fluo 4-AM) for 1 h at room temperature (~25°C), and time-lapse measurements of $[Ca^{2+}]_i$ were started after 20 min of equilibration using an Olympus BX50 WI microscope and an intensified CCD camera (Retiga Fast 1394, QImaging) controlled by the IPLab software (Scanalytics, Inc.). Variations in fluorescence intensity were expressed as F/F_0, where F is the fluorescence observed during the recording period and F_0 is the baseline fluorescence value. Fluo 4-AM was prepared in DMSO and diluted to the working concentration in MOPS-buffered Tyrode solution.

2.5. Membrane Potential Recordings. Changes in membrane potential were recorded in primary cultures of endothelial cells and in smooth muscle cells of intact, isolated mesenteric resistance arteries (120–180 μm inner diameter) using glass pulled microelectrodes filled with 3 M KCl (pipette resistance: 30–60 MΩ) connected to an electrometer DUO 773 (World Precision Instruments, Inc., FL, USA), as described by Lillo et al. [18]. In the case of smooth muscle recordings, resistance arteries were pinned down on a Sylgard® (Dow Corning Corporation, MI, USA) surface at the bottom of a chamber containing MOPS-buffered Tyrode solution (pH 7.4) and, to recognize the cell type impaled, the microelectrode filling solution also included $10 \mu M$ dextran-FITC (MW: 3000 Da) in addition to 3 M KCl. The preparation was grounded with an Ag-AgCl reference electrode placed in the buffer solution, and, with the assistance of a microscope (Nikon Eclipse), the recording microelectrode was guided using an electronic micromanipulator (Burleigh TS-5000-I50, NY) to impale an endothelial cell or the isolated artery. Successful cell impalement was recognized by a rapid negative deflection of potential, stable membrane potential in basal conditions, and positive deflection on exit. Changes in membrane potential were recorded at 1000 Hz unless otherwise indicated, using the data acquisition software LabScribe (iWorx Systems, Inc., NH, USA).

2.6. Superoxide Anion Measurements. The superoxide anion ($O_2^{\cdot-}$) probe dihydroethidine (DHE) was used to detect $O_2^{\cdot-}$ formation in intact resistance arteries and in primary cultures of mesenteric endothelial cells [21]. To measure $O_2^{\cdot-}$ in resistance arteries, isolated mesenteric arterial beds were perfused for 15 min with a Tyrode buffer solution containing $10 \mu M$ DHE alone or in combination with Panx-1 channel blockers, probenecid (1 mM) or [10]Panx (60 μM). The effect of the vehicle of the blockers was also evaluated as a control. DHE and blockers were washed out for 10 min, and a small resistance artery (120–180 μm inner diameter, ~1.0 cm length) was isolated and pinned down on a Sylgard® surface at the bottom of a 35 mm dish containing MOPS-buffered Tyrode solution (pH 7.4). A similar protocol was used in cultured endothelial cells, but, in this case, the time course of the DHE-generated signal was recorded. DHE diffuses into the cell and is oxidized by reactive oxygen species (ROS) to form ethidium, which produces nuclear fluorescence after intercalating with DNA. The fluorescent signal was examined by epifluorescence (exciter: 530-550 nm, band-pass filter; emission: 590 nm, long-pass filter) using an intensified CCD camera (Retiga Fast 1394, QImaging) and the IPLab software (Scanalytics, Inc.). The analysis of the fluorescence intensity was performed using the software ImageJ. As the DHE fluorescent signal is not specific for $O_2^{\cdot-}$, these measurements were complemented with the direct $O_2^{\cdot-}$ detection using the analysis by emitted light (ABEL®) assay, which is based on the intense luminescence emitted upon the reaction of $O_2^{\cdot-}$ radicals with the prosthetic group of pholasin, the photoprotein responsible for luminescence in the bivalve *Pholas dactylus* [22, 23] (see Supplementary Materials (available here)).

2.7. Western Blot. Mesenteries were homogenized, and proteins were separated by 12% SDS-PAGE and transferred onto a PVDF membrane (Pierce, Rockford, IL, USA), as described previously [18]. The Signal Enhancer HIKARI (Nacalai Tesque, Inc., Japan) was used to incubate the primary (BD Transduction Labs, Lexington, KY, USA) and secondary antibodies (Pierce, Rockford, IL, USA), the molecular mass was estimated with prestained markers (Bio-Rad, Hercules, CA, USA), and the protein bands were detected with the SuperSignal® West Femto (Pierce, Rockford, IL, USA). Blots were developed for eNOS phosphorylation at serine 1177 (P-eNOS^{S1177}) and then stripped three times successively to reprobe the membranes for total eNOS, Akt phosphorylation at serine 473 (P-Akt^{S473}), and total Akt. Protein bands were analyzed using the ImageJ software, and changes in eNOS and Akt phosphorylation were expressed as the ratio of phosphorylated protein over total protein. In an additional experimental series, a group of mesenteries was perfused for 30 min with a Ca^{2+}-free Tyrode buffer solution equilibrated with 95% O_2-5% CO_2 (pH 7.35-7.45) at 37°C as described above, but containing 0.5 mg/mL collagenase type II (Worthington, Lakewood, NJ, USA) to remove the endothelium. Then, mesenteric arteries were perfused with a control Tyrode buffer solution for an additional 5 min and the tissue was homogenized and prepared for Western blot analysis. In addition, expression of $Na_v1.2$, $Na_v1.6$, and $Ca_v3.2$ channels in the plasma membrane was analyzed by biotinylation of cell surface proteins (see Supplementary Materials (available here)).

2.8. Immunocytochemistry Analysis. Mesenteric arterial beds were first perfused for 10 min and then incubated overnight
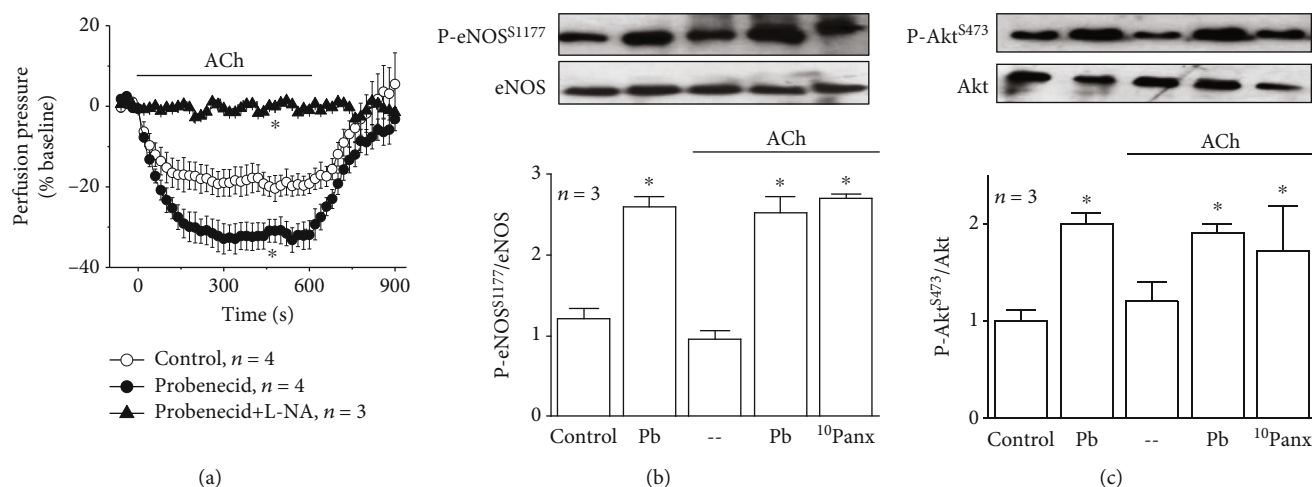
Figure 1: Blockade of Panx-1 channels enhances the NO-mediated vasodilation activated by ACh. (a) Time course of the vasodilation induced by 100 nM ACh in KCl-contracted arterial mesenteric beds in control conditions and in the presence (15 min) of 1 mM probenecid alone or in addition to the treatment for 45 min with 100 μM N^G-nitro-L-arginine (L-NA), a blocker of NO production. Probenecid was applied during the last 15 min of the treatment with L-NA. The horizontal bar indicates the period of stimulation. (b, c) Representative Western blots and densitometric analysis of eNOS (b) and Akt (c) expression as well as the phosphorylation of eNOS at serine 1177 (P-eNOS^{S1177}, (b)) and Akt at serine 473 (P-Akt^{S473}, (c)) observed in basal conditions (control) and after the treatment with 1 mM probenecid (Pb) or 60 μM ^{10}Panx. In the densitometric analysis, the changes in eNOS and Akt phosphorylation are expressed as the ratio of phosphorylated protein over total protein. Values are means ± SEM. *$P < 0.05$ vs. the control by one-way ANOVA plus the Newman-Keuls post hoc test.

with Bouin's solution to fix and postfix the tissue, respectively. Subsequently, mesenteric arteries were prepared for immunohistochemistry or immunofluorescence analysis, as described previously [7, 18]. Tissues were dehydrated, embedded in paraffin, sectioned (10 μm), placed on charge-coated slides, and deparaffinized using standard procedures to analyze the expression of Na$_v$1.2, Na$_v$1.6, and Ca$_v$3.2 channels by immunohistochemistry or Panx-1 and caveolin-1 (Cav-1) by immunofluorescence. The expression of Na$_v$1.2, Na$_v$1.6, and Ca$_v$3.2 channels was also assessed by immunofluorescence in cultured endothelial cells fixed with 4% paraformaldehyde. For immunohistochemistry, tissue sections were blocked with 0.5% BSA in TBS (pH 7.4) for 1 h at room temperature and prepared as indicated by the Mouse/Rabbit ImmunoDetector System (Bio SB, Santa Barbara, CA, USA) protocol. After blocking the endogenous peroxidase activity, sections were incubated overnight at 4°C with anti-Na$_v$1.2, anti-Na$_v$1.6, or anti-Ca$_v$3.2 rabbit primary antibodies (Alomone Laboratories, Israel) and the signal was developed using the biotin link secondary antibody (10 min), HPR label, and DAB chromogen of the Mouse/Rabbit ImmunoDetector System. For immunofluorescence, sections and endothelial cell monolayers were blocked with 0.5% BSA in PBS and incubated with the anti-Panx-1 rabbit primary antibody (Sigma-Aldrich, St. Louis, MO, USA) or anti-Cav-1 mouse primary antibody (BD Transduction Labs, Lexington, KY, USA) in the case of tissue sections or anti-Na$_v$1.2, anti-Na$_v$1.6, or anti-Ca$_v$3.2 primary antibodies in the case of cultured cells and then with the appropriate Alexa Fluor 568-labeled goat anti-rabbit or anti-mouse secondary antibody (Molecular Probes, OR, USA) using the Signal Enhancer HIKARI (Nacalai Tesque, Inc., Japan) as indicated by the manufacturer. The fluorescent signal was examined using

an Olympus BX41 WI microscope and a CCD camera (Jenoptik ProgRes C5).

2.9. Analysis of Panx-1 Subcellular Distribution. The potential subcellular localization of Panx-1 channels in caveolae was evaluated by assessing the spatial association between Panx-1 and Cav-1 using the Proximity Ligation Assay (PLA, Duolink II, Olink Bioscience, Sweden) as described previously [18]. Tissue sections (10 μm) were blocked with 0.5% BSA and incubated with rabbit polyclonal anti-Panx-1 (Sigma-Aldrich) and mouse monoclonal anti-Cav-1 (BD Transduction Labs) primary antibodies, which were detected using oligonucleotide-conjugated secondary antibodies as described in the manufacturer's protocols. The oligonucleotides can meet each other if the proteins are closer than 20 nm and thus can be used as a template for DNA ligase-mediated joining of additional oligonucleotides to form a circular DNA molecule, which was amplified using hybridizing fluorophore-labeled oligonucleotides. Primary antibodies were omitted as a negative control. Images were visualized with an Olympus LSM FLUOVIEW 1000 confocal microscope.

2.10. Chemicals. All chemicals of analytical grade were obtained from Merck (Darmstadt, Germany). MOPS, EGTA, ECGS, BSA, DHE, ACh, probenecid, dextran-FITC (MW: 3000 Da), mibefradil, Ni^{2+}, 18β-glycyrrhetinic acid (β-GA), methyl-β-cyclodextrin (MβCD), and N^G-nitro-L-arginine (L-NA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Apocynin and TEMPOL were obtained from Calbiochem (La Jolla, CA, USA), tetrodotoxin (TTX) from Affix Scientific, and collagenase type I from Worthington (Lakewood, NJ, USA). The Panx-1 channel blocking peptide

[10]Panx was synthesized by GenScript (Israel). β-GA and apocynin were dissolved in DMSO and probenecid in 0.5 M NaOH. These inhibitors were then diluted in a buffer solution to reach the final working concentration (final amount of DMSO < 0.1% and 1.4 mM NaOH). DMSO did not have an effect per se (data not shown). The pH of the final solution of probenecid was checked before the experiments, and the effect of the vehicle was also evaluated.

2.11. Statistical Analysis. Values are represented as mean $\pm$ standard error. Comparisons between groups were made using paired or unpaired Student's t-test, one-way ANOVA plus the Newman-Keuls post hoc test, or two-way ANOVA as appropriate. $P < 0.05$ was considered significant.

3. Results

The endothelium-dependent vasodilation was analyzed in mesenteric resistance arteries precontracted with 70 mM KCl. Perfusion pressure of mesenteric arterial beds was 3.7 $\pm$ 0.8 mmHg in resting conditions and increased during the stimulation with KCl to 20.9 $\pm$ 2.3 mmHg in 2-3 min ($n = 6$). Application of 100 nM ACh for 10 min evoked the relaxation of mesenteric resistance arteries, which was reflected in a rapid reduction in perfusion pressure that reached a maximum after ~2 min of stimulation and gradually returned to the KCl preconstriction level after the end of ACh application (Figure 1(a)).

3.1. Control of NO-Mediated Vasodilation by Panx-1 Channels. Mesenteric arteries were treated with probenecid to evaluate the participation of Panx-1-formed channels in response to ACh. Although probenecid application did not affect the basal perfusion pressure of mesenteric arterial beds (see Supplementary Fig. S1a and S1b), treatment with this blocker attenuated the vasoconstriction evoked by KCl (see Supplementary Fig. S2) and enhanced the ACh-induced vasodilation (Figure 1(a)). In KCl-contracted arteries, the endothelium-dependent vasodilation relies exclusively on NO production [6, 19] (see Supplementary Fig. S3) and, consistent with this notion, inhibition of NO production with 100 μM L-NA abolished the vasodilation activated by ACh in the presence of probenecid (Figure 1(a)). Then, we evaluated the effect of the Panx-1 channel blockade on P-eNOS[S1177], which is a regulatory mechanism that enhances Ca^{2+}-mediated NO production. A basal P-eNOS[S1177] was detected in control conditions, and the larger vasodilation observed in the presence of probenecid was associated with an increment in the P-eNOS[S1177] level (Figure 1(b)) and also in Akt phosphorylation at serine 473 (P-Akt[S473], Figure 1(c)), suggesting that the eNOS phosphorylation triggered by the Panx-1 channel blockade was mediated by the activation of the PI3K/Akt signaling pathway, as previously observed in response to different stimuli such as shear stress or bradykinin [24–26]. In line with previous reports [19], stimulation with ACh did not alter the level of P-eNOS[S1177] and P-Akt[S473] observed in basal conditions or after inhibiting Panx-1 channels with probenecid or the Panx-1 blocking peptide [10]Panx (Figures 1(b) and 1(c)). Altogether, these

results suggest that Panx-1 channels present a basal activity that may be involved in the regulation of endothelial cell function.

3.2. Voltage-Dependent Ca^{2+} Signaling Triggered by the Panx-1 Channel Blockade. Control of membrane potential plays an important role in the regulation of endothelial cell signaling and in the Ca^{2+}-dependent eNOS activation; thus, we evaluated the effect of the Panx-1 channel blockade on endothelial cell membrane potential. Surprisingly, in primary cultures of mesenteric endothelial cells, application of Panx-1 channel blockers, probenecid or [10]Panx, evoked a fast tetrodotoxin- (TTX-) sensitive membrane depolarization that reached a maximum in ~40 ms and returned to the control level after a plateau phase of ~7 s (Figures 2(a)–2(c)). This response was also observed in smooth muscle cells of intact mesenteric resistance arteries (Figures 2(d)–2(f)). However, endothelial and smooth muscle cells are connected through gap junctions (i.e., myoendothelial gap junctions) and the inhibition of these intercellular channels with 18β-glycyrrhetinic acid (50 μM, β-GA) completely prevented the smooth muscle cell depolarization elicited by probenecid (Figures 2(e) and 2(f)). In contrast, β-GA did not affect the response recorded in endothelial cells (Figure 2(c)), indicating that the depolarizing signal was triggered in the endothelium by the activation of TTX-sensitive, voltage-dependent Na^{+} channels (Na$_v$) and thus transmitted to smooth muscle cells via myoendothelial gap junctions.

The activation of Na$_v$ channels is anticipated to be transient, which suggests the possible contribution of a second component in the endothelium-dependent depolarization triggered by the Panx-1 channel blockade, such as a Ca^{2+} influx. Consistent with this hypothesis, the plateau phase of the probenecid-activated depolarization was not observed after removing Ca^{2+} ions from the buffer solution (i.e., Ca^{2+}-free solution) or in the presence of 100 μM mibefradil or 10 μM Ni^{2+} (Figures 3(a) and 3(b)), suggesting that the initial Na$_v$-mediated depolarization was coupled to the subsequent activation of Ca$_v$3 channels, most likely, the subtype Ca$_v$3.2. In addition, the plateau of the depolarization was paralleled by a prominent increase in [Ca[2+]]$_i$ (Figures 3(c) and 3(d)) that showed the same temporal characteristics of the change in membrane potential (Figure 2(a)) and, as expected, was abolished by Ni^{2+}, but also by TTX (Figure 3(d)), supporting the notion that the activation of the Ca^{2+} signal was triggered by TTX-sensitive Na$_v$ channels.

These findings suggest that TTX-sensitive Na$_v$ channels and Ca$_v$3.2 channels are present in endothelial cells and the expression of the isoforms Na$_v$1.2 and Na$_v$1.6 of TTX-sensitive Na$_v$ channels as well as the isoform Ca$_v$3.2 of Ca$_v$3 channels has been detected in the endothelium [7, 27], which we confirmed by immunocytochemistry analysis in mesenteric resistance arteries. Both Na$_v$1.2 and Na$_v$1.6 channels were found to be expressed in endothelial cells as well as in smooth muscle cells, but, in contrast, the staining for Ca$_v$3.2 channels was confined exclusively to the endothelium (Figure 4(a)). In addition, the expression of Na$_v$1.2, Na$_v$1.6, and Ca$_v$3.2 channels in the endothelium was corroborated in primary cultures of mesenteric endothelial cells by
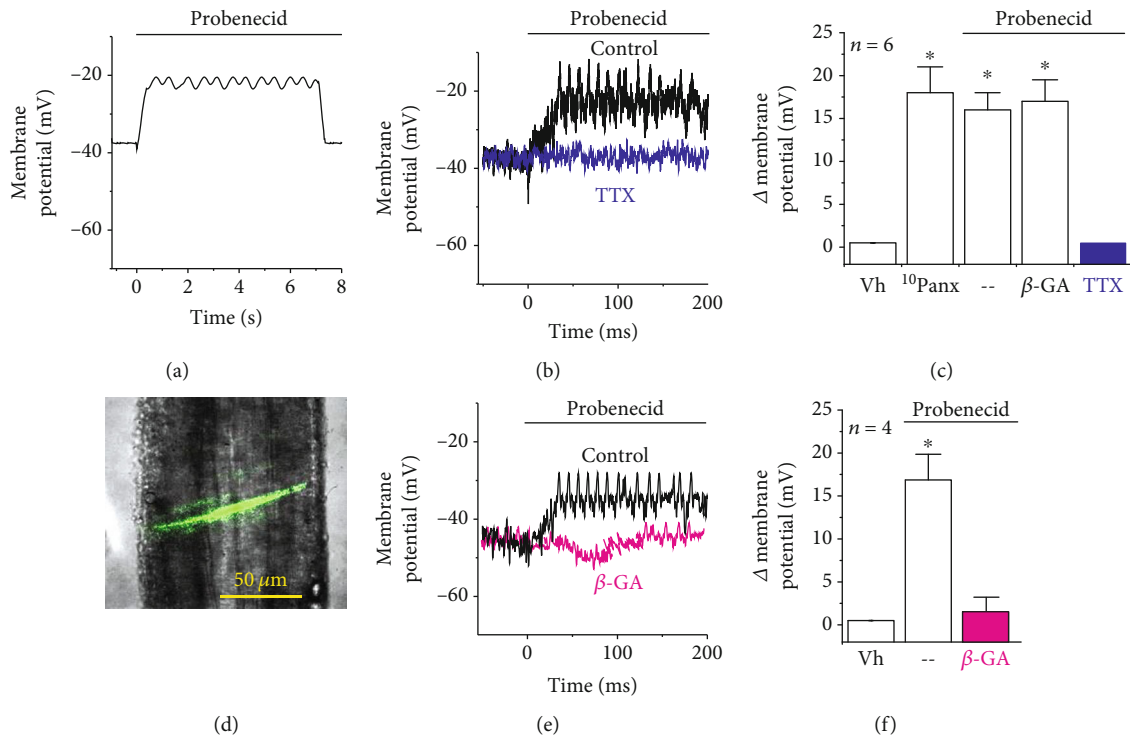
FIGURE 2: Blockade of Panx-1 triggers a rapid tetrodotoxin- (TTX-) sensitive depolarization of endothelial cell membrane potential. (a) Time course of the changes in membrane potential evoked by the Panx-1 channel blockade with 1 mM probenecid in primary cultures of endothelial cells. Membrane potential was recorded at 100 Hz. (b) Representative recordings of the membrane depolarization observed in primary cultures of endothelial cells in response to probenecid in control conditions and in the presence of 300 nM TTX. (c) Analysis of the maximum depolarization evoked by the application of 60 μM ^{10}Panx or 1 mM probenecid and its vehicle (Vh). Resting membrane potential was not affected by the vehicle of probenecid or ^{10}Panx. The effect of 50 μM 18β-glycyrrhetinic acid (β-GA) and TTX is also shown. (d) Representative image of a smooth muscle cell microinjected with dextran-FITC (3000 Da) during the recording of the changes in membrane potential from the smooth muscle layer of an isolated mesenteric resistance artery. (e) Representative recordings of the changes in membrane potential observed in intact arteries in response to the Panx-1 channel blockade with probenecid in control conditions and in the presence of β-GA. (f) Analysis of the maximum smooth muscle depolarization activated by probenecid in control conditions and in the presence of β-GA. The effect of the vehicle (Vh) of probenecid is also shown. Values are means ± SEM. $^*P < 0.05$ vs. the vehicle by one-way ANOVA plus the Newman-Keuls post hoc test.

immunofluorescence (Figure 4(b)) and in intact mesenteric resistance arteries with (E$^+$) or without endothelium (E$^-$) by Western blot (Figure 4(c)). Consistent with the immunocytochemistry analysis, removal of the endothelium by perfusing resistance vessels with collagenase resulted in a striking reduction in the Western blot signal for Na$_v$1.2 and Na$_v$1.6 channels, but the presence of Ca$_v$3.2 channels was practically undetectable in endothelium-denuded vessels (Figure 4(c)), confirming the preferential endothelial cell expression of these channels in the wall of resistance arteries. In this experimental series, the reduction in the eNOS signal after endothelial cell removal was also evaluated as a control (Figure 4(c)). Na$_v$1.2, Na$_v$1.6, and Ca$_v$3.2 channels must be found at the plasma membrane to be functional, which we further analyzed by biotinylation of surface proteins of the endothelial cell luminal membrane in intact mesenteric resistance vessels (see Supplementary Fig. S4). As expected, all three channels, Na$_v$1.2, Na$_v$1.6, and Ca$_v$3.2, were found in the biotin-labeled protein fraction, in addition to the whole vessel sample (see Supplementary Fig. S4), indicating that these channels are expressed at the endothelial cell plasma membrane. It should be noted that perfusion of biotin did

not reach intracellular proteins of endothelial cells or plasma membrane proteins of smooth muscle cells because this treatment did not target eNOS or L-type voltage-dependent Ca^{2+} channels, Ca$_v$1.2, respectively. In contrast, a relevant membrane protein of endothelial cells, such as the Na$^+$-Ca^{2+} exchanger, was also found in the biotin-labeled protein fraction (see Supplementary Fig. S4).

3.3. Panx-1 Subcellular Distribution in Resistance Arteries.
Signaling microdomains, such as caveolae, play a central role in the control of vascular function and in the regulation of NO production; therefore, we analyzed the cellular distribution of Panx-1 and Cav-1, a structural protein of caveolae, in mesenteric resistance arteries by immunofluorescence analysis and Proximity Ligation Assay (PLA). The fluorescent signal for Panx-1 and Cav-1 was detected in endothelial cells as well as in smooth muscle cells (Figure 5(a)), and the analysis of PLA revealed that both proteins are found in close spatial proximity mainly in endothelial cells (Figure 5(b)). The association of Panx-1 with Cav-1 was confirmed in primary cultures of mesenteric endothelial cells, and, interestingly, the blockade of Panx-1 channels with ^{10}Panx evoked

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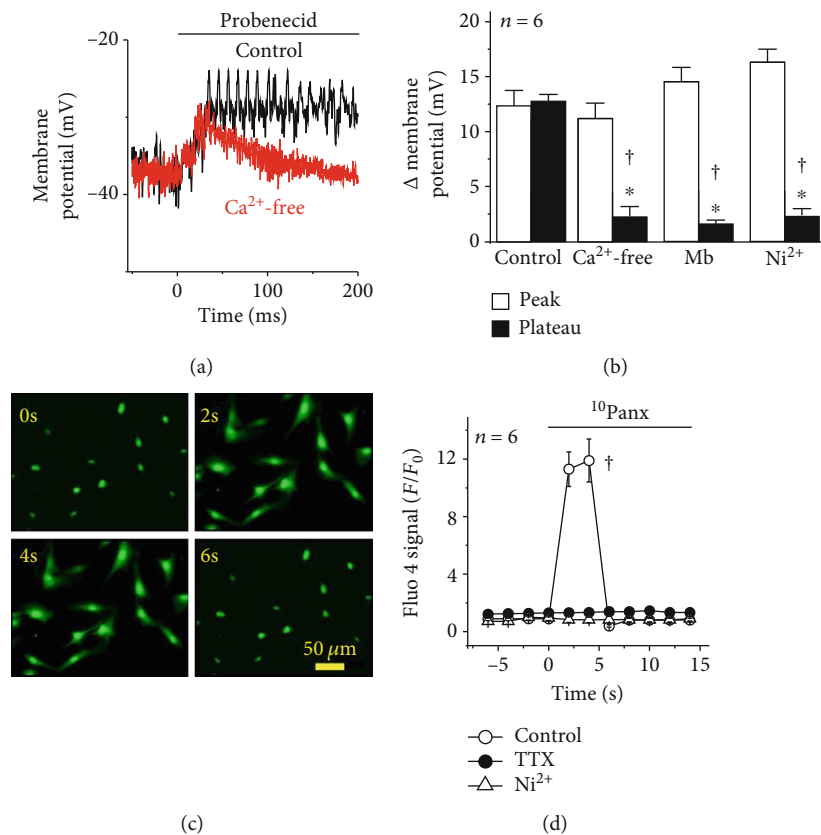
FIGURE 3: T-type voltage-dependent Ca^{2+} channels (T-type Ca_v) are involved in the endothelial cell depolarization evoked by the Panx-1 channel blockade. (a) Representative recordings of the changes in endothelial cell membrane potential evoked by 1 mM probenecid in control conditions or after removing Ca^{2+} ions from the buffer solution (Ca^{2+}-free solution plus 2 mM EGTA). Note that the absence of extracellular Ca^{2+} ions unmasked two components: an initial peak and a Ca^{2+}-dependent plateau phase. Treatment with the Ca^{2+}-free solution was initiated 5 min before probenecid application. (b) Analysis of the peak and plateau phase of the depolarization evoked by probenecid in control conditions and during the treatment with a Ca^{2+}-free solution, 100 μM mibefradil (Mb), or 10 μM Ni^{2+}. (c, d) Representative images (c) and quantitative analysis (d) of the changes in $[Ca^{2+}]_i$ observed in response to the Panx-1 channel blockade with 60 μM ^{10}Panx in primary cultures of endothelial cells. Note that tetrodotoxin (TTX, 300 nM) and Ni^{2+} abolished the increase in $[Ca^{2+}]_i$ activated by ^{10}Panx. Values are means ± SEM. $^*P < 0.05$ vs. the peak by paired Student's t-test. $^\dagger P < 0.05$ vs. the control by one-way ANOVA plus the Newman-Keuls post hoc test.

an increase in the level of spatial interaction between these two proteins (Figure 5(c)), which suggests that the organization of the signaling mechanism initiated by the Panx-1 channel blockade is orchestrated in caveolae. Consistent with this hypothesis, disruption of cholesterol-rich microdomains by treating the cultures of endothelial cells with 5 mM methyl-β-cyclodextrin (MβCD) for 30 min fully prevented the endothelial cell depolarization and the increase in $[Ca^{2+}]_i$ observed in response to ^{10}Panx application (Figures 6(a)–6(c)).

3.4. Panx-1 Channel Blockade Leads to NADPH Oxidase-Derived Superoxide Formation.

As depolarization of membrane potential as well as an increase in $[Ca^{2+}]_i$ may trigger the activation of NADPH oxidase in endothelial cells [28–30], we used DHE to assess $O_2^{\cdot-}$ production in intact mesenteric resistance arteries and primary cultures of mesenteric endothelial cells. The blockade of Panx-1 channels with probenecid or ^{10}Panx in resistance vessels resulted in a strong increment in the DHE-generated fluorescent signal

(Figures 7(a) and 7(b)) that was fully prevented by 10 μM TEMPOL (Figure 7(c)), a $O_2^{\cdot-}$ dismutase mimetic, confirming that the increase in the DHE signal reflected $O_2^{\cdot-}$ production. Additionally, the rise in $O_2^{\cdot-}$ levels observed in response to the Panx-1 channel blockade was also corroborated using the photoprotein pholasin (see Supplementary Fig. S5). As expected, stimulation with ACh did not change the $O_2^{\cdot-}$ levels observed in control conditions or after the treatment with probenecid (Figures 7(a) and 7(b)). The activation of $O_2^{\cdot-}$ formation was rapid, since, in cultured endothelial cells, the increase in the DHE signal started immediately after probenecid application (Figure 7(d)). In agreement with the participation of NADPH oxidase in the response, the increase in $O_2^{\cdot-}$ was abolished by 100 μM apocynin, an inhibitor of NADPH oxidase, in both resistance arteries (Figure 7(c)) and endothelial cell cultures (Figures 7(d) and 7(e)). In addition to TEMPOL and apocynin, $O_2^{\cdot-}$ formation was also inhibited by 10 μM Ni^{2+} (Figure 7(f)), supporting the involvement of $Ca_v3.2$ channels in the NADPH oxidase activation.

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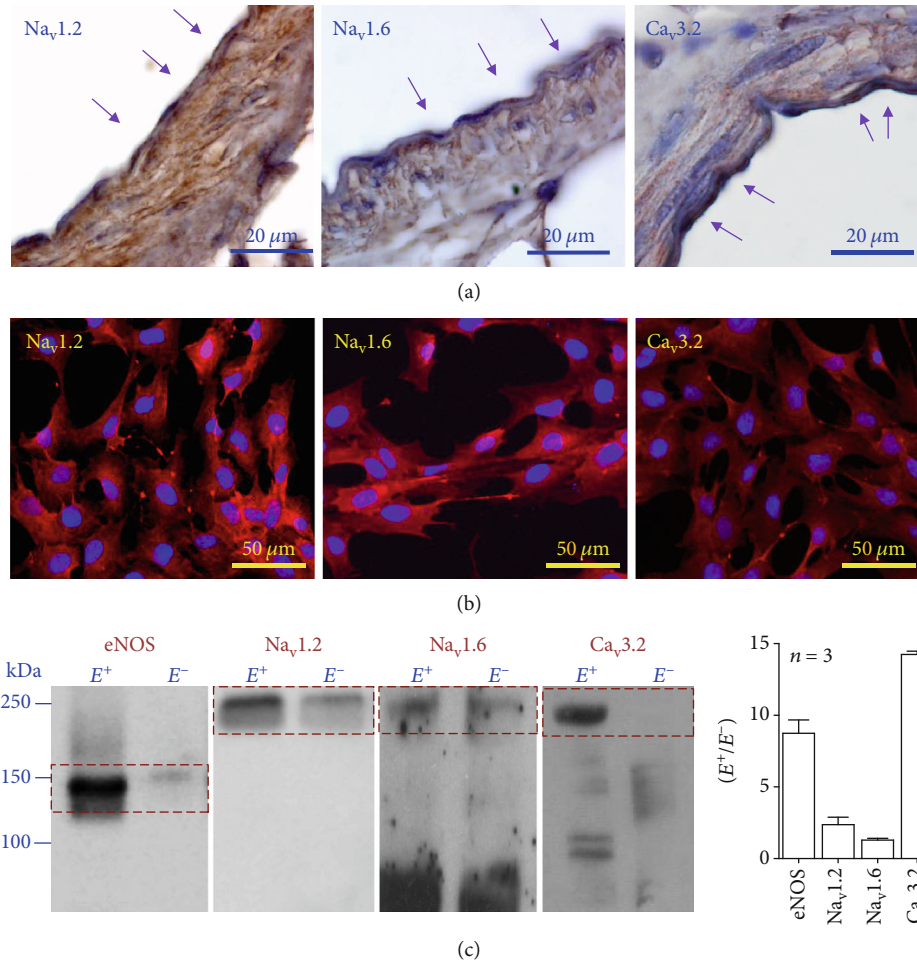
FIGURE 4: Expression of voltage-dependent Na^+ (Na_v) and Ca^{2+} (Ca_v) channels in endothelial cells of mesenteric resistance arteries. (a) Immunohistochemistry analysis of the cellular distribution of Na_v and Ca_v channel-specific isoforms $Na_v1.2$, $Na_v1.6$, and $Ca_v3.2$ in the wall of mesenteric resistance arteries. Note that $Na_v1.2$ and $Na_v1.6$ channels are present in both endothelial cells and smooth muscle cells, but $Ca_v3.2$ channels are expressed exclusively in the endothelium. Arrows highlight the staining observed in endothelial cells. (b) Immunofluorescence detection of the expression of $Na_v1.2$, $Na_v1.6$, and $Ca_v3.2$ channels in primary cultures of mesenteric endothelial cells. (c) Representative Western blots and densitometric analysis of the expression of eNOS and $Na_v1.2$, $Na_v1.6$, and $Ca_v3.2$ channels in intact mesenteric arteries before (E^+) and after (E^-) removing the endothelium by the treatment with collagenase.

3.5. NADPH Oxidase Mediates the eNOS Activation Triggered by the Panx-1 Channel Blockade.

The NADPH oxidase-derived $O_2^{\cdot-}$ production initiated by the Panx-1 channel blockade may lead to the activation of Akt-mediated signaling [31]; therefore, we evaluated if the NADPH oxidase/$O_2^{\cdot-}$/Akt pathway was involved in the P-eNOS^{S1177}-associated increase in NO-dependent vasodilation. Consistent with this hypothesis, the increase in the level of P-Akt^{S473} and P-eNOS^{S1177} observed after probenecid application was not evident in the presence of $10\,\mu M$ TEMPOL (Figures 8(a) and 8(b)). In line with these results, treatment with TEMPOL or apocynin ($100\,\mu M$) completely inhibited both the increment in the ACh-induced vasodilation (Figures 8(c) and 8(d)) and the reduction in the KCl-evoked vasoconstriction (see Supplementary Fig. S6a and S6b) attained after blocking Panx-1 channels with probenecid, which strongly support the involvement of the NADPH oxidase/$O_2^{\cdot-}$ pathway in the Panx-1-mediated regulation of NO-dependent vasodilation.

4. Discussion

The relevance of Panx-1 channels in the control of endothelium-mediated vasomotor signaling is not clear. In spite of this cavity in the Panx physiology, deletion of Panx-1 was reported to attenuate the EDH pathway, and, interestingly, a compensatory increase in the NO-mediated vasodilation was also observed in the absence of this protein, suggesting that EDH or directly Panx-1 channels may be involved in a negative feedback mechanism that restrains NO production [17]. However, in contrast to this notion, our results show that the acute blockade of Panx-1 channels triggers a complex signaling pathway that leads to P-eNOS^{S1177} in response to a NADPH oxidase-mediated increase in $O_2^{\cdot-}$ formation. Interestingly, this mechanism is triggered by a TTX-sensitive depolarization that is coupled to a Ca^{2+} influx through Ca_v3 channels, apparently, the subtype $Ca_v3.2$.

Blood flow distribution is controlled by the fine regulation of the diameter of small resistance arteries. In these

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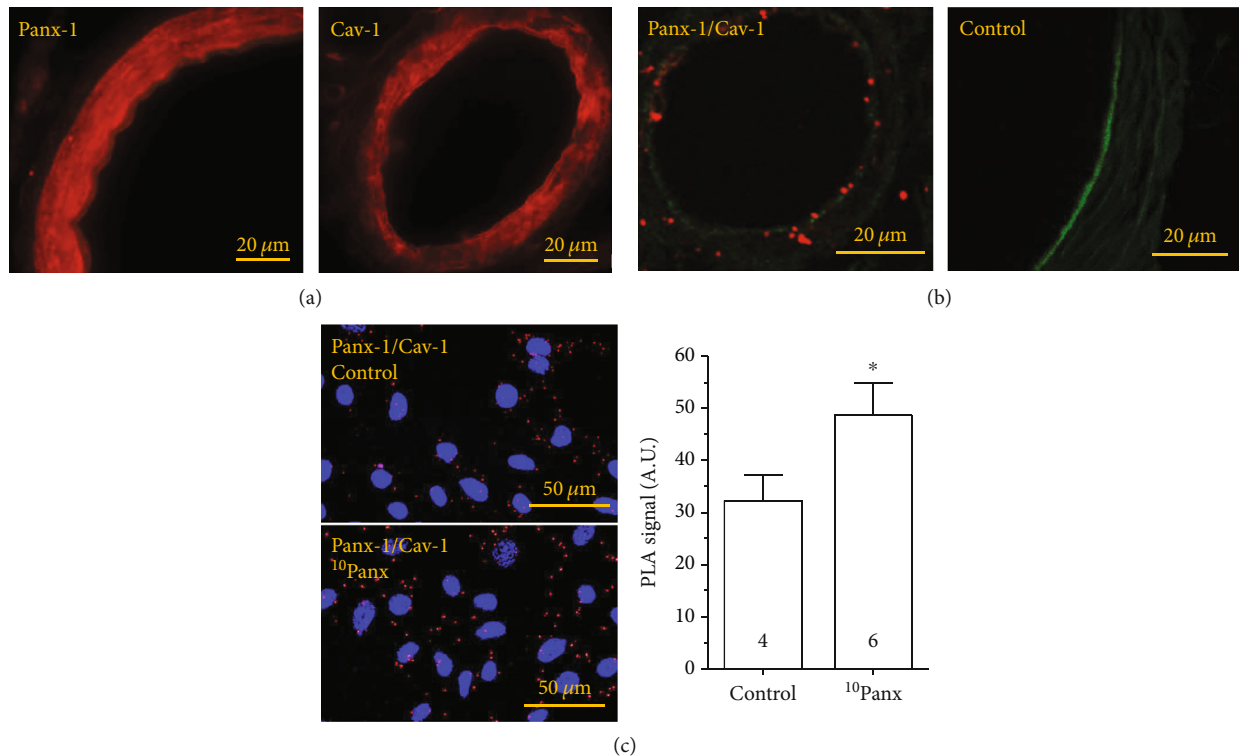
FIGURE 5: Panx-1 is associated with caveolin-1 (Cav-1) in endothelial cells. (a) Immunofluorescence analysis of the cellular distribution of Panx-1 (left) and Cav-1 (right) in mesenteric resistance arteries. (b) Analysis performed by Proximity Ligation Assay (PLA) of the spatial association of Panx-1 with Cav-1 (left) and the negative control (right) in which primary antibodies were omitted. Note that although Panx-1 and Cav-1 are expressed in endothelial cells and smooth muscle cells, the association between these two proteins is mainly observed in the endothelium. The green fluorescent signal corresponds to the internal elastic lamina. (c) Representative images (left) and fluorescence intensity analysis (right) of the PLA-detected association between Panx-1 and Cav-1 in primary cultures of mesenteric endothelial cells in control conditions and 5 min after the application of the Panx-1 blocking peptide [10]Panx. Changes in the PLA signal are expressed in arbitrary units (A.U.). Numbers inside the bars indicate the n value. Values are means ± SEM. $^{*}P < 0.05$ vs. the control by unpaired Student's t-test.

arteries, Panx-1 is expressed not only in endothelial cells but also in smooth muscle cells [16] (Figure 5). Although the physiological relevance of Panx-1-mediated signaling in the control of vascular function is just beginning to be understood, channels formed by Panx-1 have been shown to be involved in the vasoconstriction initiated by the stimulation of α1-adrenoceptors in smooth muscle cells of resistance arteries [13] and in the EDH-mediated vasodilator component activated by ACh in conduit arteries [17]. Furthermore, Panx-1 has also been associated with the regulation of NO signaling, but the mechanism involved in this process has not been determined [17]. Therefore, to focus on the NO-dependent vasodilator component elicited by ACh, without interfering with the Panx-1-mediated vasoconstrictor signaling initiated by α1-adrenoceptors in smooth muscle cells, we used KCl-contracted resistance arteries to disable the EDH signaling and evoke a receptor-independent contraction. The ACh-induced vasodilation depends exclusively on NO in these conditions [19, 32] (Supplementary Fig. S3), and, therefore, the increase in response to ACh observed after the treatment with probenecid (Figure 1) confirmed that the blockade of Panx-1 channels enhances the endothelium-mediated NO signaling, as further demonstrated by the inhibition of NO production with L-NA

(Figure 1). Consistent with increased NO production, probenecid also attenuated the KCl-evoked vasoconstriction (see Supplementary Fig. S2). Interestingly, the increase in the response was associated with an increment in P-eNOS^{S1177} (Figure 1), which is consistent with the upregulation of the relaxation, since eNOS is a Ca^{2+}-dependent enzyme and P-eNOS^{S1177} is a well-characterized regulatory mechanism that enhances Ca^{2+}-activated NO production [33, 34]. Activation of the PI3K/Akt signaling pathway leads to P-eNOS^{S1177} [24, 25, 35], and, in line with the participation of this pathway in the phosphorylation of eNOS, the Panx-1 channel blockade was also coupled to an increase in P-Akt^{S473} (Figure 1).

NO production and eNOS activity have been reported to be modulated by changes in endothelial cell membrane potential, and Panx-1 may work as a Cl⁻-selective channel in basal conditions [36], which may contribute to depolarize the endothelial cell membrane potential [37]. Then, we hypothesized that disruption of Panx-1 channel function may lead to an increase in NO production by triggering the hyperpolarization of membrane potential. Unexpectedly, the application of probenecid or [10]Panx evoked a rapid membrane potential depolarization in primary cultures of mesenteric endothelial cells as well as in smooth muscle cells of

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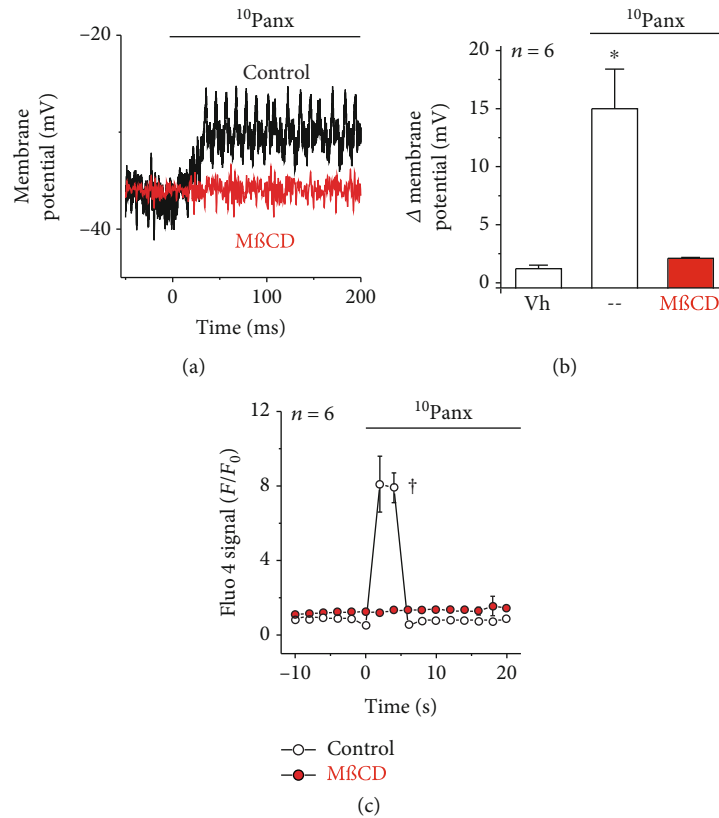
FIGURE 6: The depolarization and Ca^{2+} signaling activated in endothelial cells by the Panx-1 channel blockade depend on the integrity of cholesterol-rich microdomains. (a) Representative recordings of the changes in endothelial cell membrane potential evoked by $60\,\mu M$ [10]Panx in control conditions and after disrupting the cholesterol-rich signaling microdomains by the treatment with 5 mM methyl-β-cyclodextrin (MβCD) for 30 min. (b) Analysis of the maximum depolarization evoked by [10]Panx in control conditions and after the treatment with MβCD. (c) Time course of the changes in $[Ca^{2+}]_i$ observed in primary cultures of endothelial cells in response to [10]Panx application before (control) and after the treatment with MβCD. Values are means ± SEM. $^*P < 0.05$ vs. the vehicle by one-way ANOVA plus the Newman-Keuls post hoc test. $^\dagger P < 0.05$ vs. the control by two-way ANOVA.

intact mesenteric resistance arteries (Figure 2). In resistance arteries, endothelial and smooth muscle cells are communicated through myoendothelial gap junctions [2, 38] and inhibition of connexin-formed channels with β-GA prevented the smooth muscle depolarization but did not affect the response in endothelial cells (Figure 2), confirming that the depolarizing signal was triggered in the endothelium and was subsequently transmitted to smooth muscle cells via myoendothelial gap junctions. Therefore, these results suggest that, in endothelial cells, Panx-1 channels may be coupled to the regulation of a depolarizing mechanism, such as Na_v channels. Although voltage-dependent channels are not generally thought to be present in the endothelium, functional expression of Na_v channels has been detected in endothelial cells [7, 39–41]. In this context, Na_v channels have been reported to be involved in the endothelial response to shear stress [41] and to mediate the endothelium-dependent conducted vasodilation activated by depolarizing electrical stimulation of mouse cremaster arterioles [7]. Consistent with the functional association of these channels with Panx-1, the change in membrane potential triggered by the Panx-1 channel blockade was fully prevented by TTX (Figure 2). As expected, the activation of Na_v channels was

transient (i.e., ~40 ms) and only contributed to the uprising phase of the depolarization, but, in addition, this initial phase triggered a Ca^{2+} influx that accounted for the sustained phase of the response, as demonstrated by the removal of Ca^{2+} ions from the bathing solution or the treatment with mibefradil or low concentrations of Ni^{2+} ($10\,\mu M$). In agreement with these results, application of the Panx-1 blocking peptide [10]Panx elicited a rapid, strong increase in $[Ca^{2+}]_i$ that paralleled the depolarization and was sensitive to TTX and Ni^{2+} (Figure 3). Mibefradil is a blocker of Ca_v3 channels, and Ni^{2+}, at low concentrations such as $10\,\mu M$, is a preferential inhibitor of the subtype $Ca_v3.2$ of Ca_v3 channels [42, 43]. Interestingly, in the wall of resistance arteries, these channels are expressed exclusively in the endothelium [8, 27] and were shown to be essential for normal relaxation of the murine coronary arteries [44]. Furthermore, our results confirmed the expression of $Na_v1.2$, $Na_v1.6$, and $Ca_v3.2$ channels in endothelial cells of rat mesenteric resistance arteries (Figure 4 and Supplementary Fig. S4). Taken together, these data indicate that the blockade of Panx-1 channels leads to the activation of a complex mechanism based on the functional coupling between TTX-sensitive Na_v channels and $Ca_v3.2$ channels, as that previously proposed to mediate the
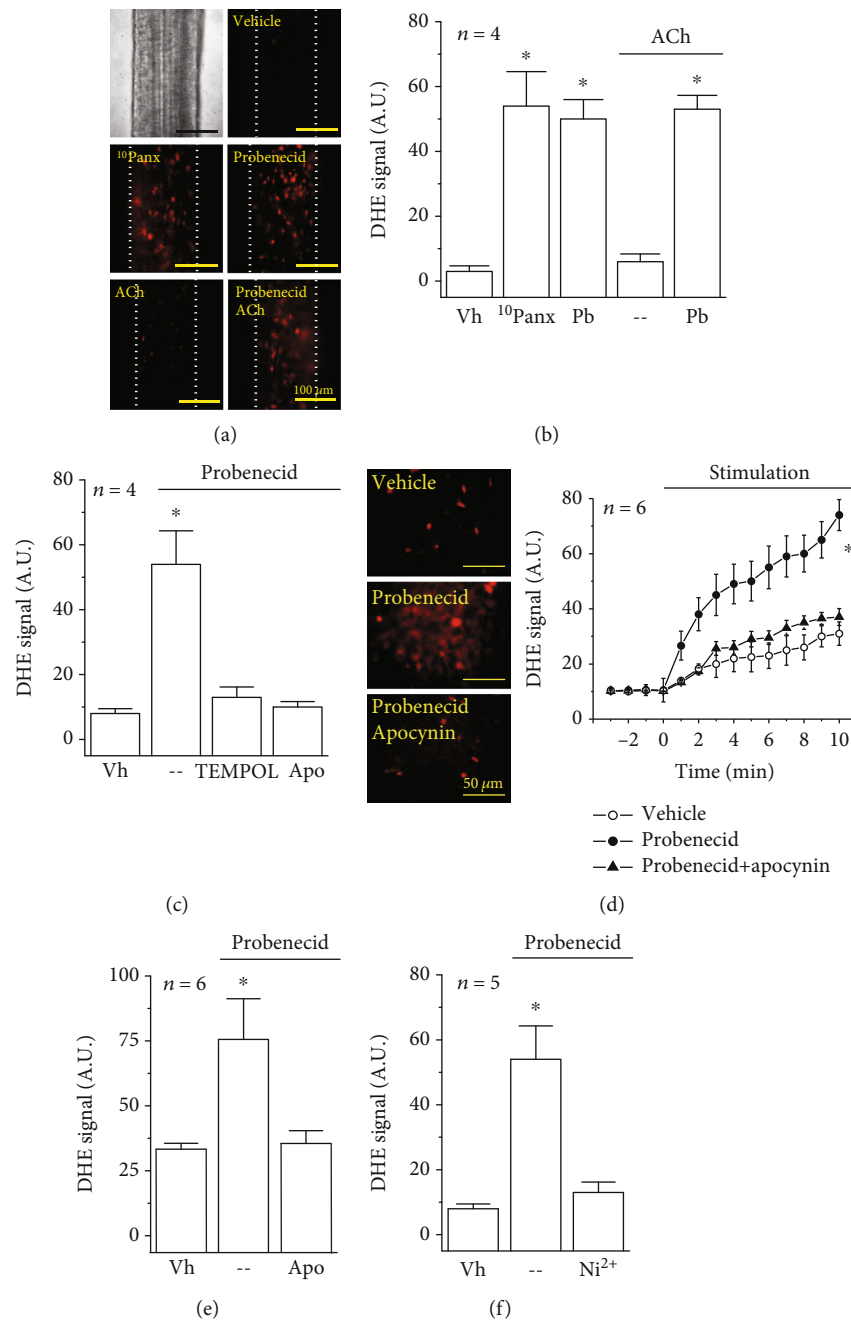
FIGURE 7: Blockade of Panx-1 channels leads to a NADPH oxidase-mediated increase in $O_2^{\cdot-}$ formation. (a) Representative images of the $O_2^{\cdot-}$-generated fluorescent signal observed in intact resistance arteries after 15 min application of 10 μM dihydroethidine (DHE) alone (control, the vehicle (Vh) of probenecid) or with 60 μM ^{10}Panx, 1 mM probenecid (Pb), 100 nM ACh, or probenecid plus ACh. Dotted lines depict the outer edge of vessel walls. (b) Analysis of the $O_2^{\cdot-}$ formation observed in the experiments shown in (a). (c) $O_2^{\cdot-}$ production in response to probenecid in control conditions and in the presence of 10 μM TEMPOL or 100 μM apocynin (Apo). (d) Representative images and time course of the changes in the DHE signal observed in primary cultures of mesenteric endothelial cells in response to probenecid application in control conditions and in the presence of apocynin. The effect of the vehicle of probenecid is also shown. The horizontal bar indicates the period of stimulation. (e, f) Analysis of the maximum DHE signal attained in endothelial cell cultures in response to probenecid in control conditions and during the treatment with apocynin (e) or Ni^{2+} (f). Changes in the DHE-derived fluorescent signal are expressed in arbitrary units (A.U.). Values are means ± SEM. $^*P < 0.05$ vs. the vehicle by one-way ANOVA plus the Newman-Keuls post hoc test.

electrically induced endothelium-dependent conducted vasodilation in mouse cremaster arterioles [2, 7].

The mechanism by which the blockade of Panx-1 channels triggers the activation of Na_v channels requires further investigation. However, it is interesting that although Panx-1 is expressed in both endothelial cells and smooth muscle cells of mesenteric resistance arteries (Figure 5), the analysis of protein association by PLA revealed that Panx-1 is found

(a)

(b)

(c)

(d)
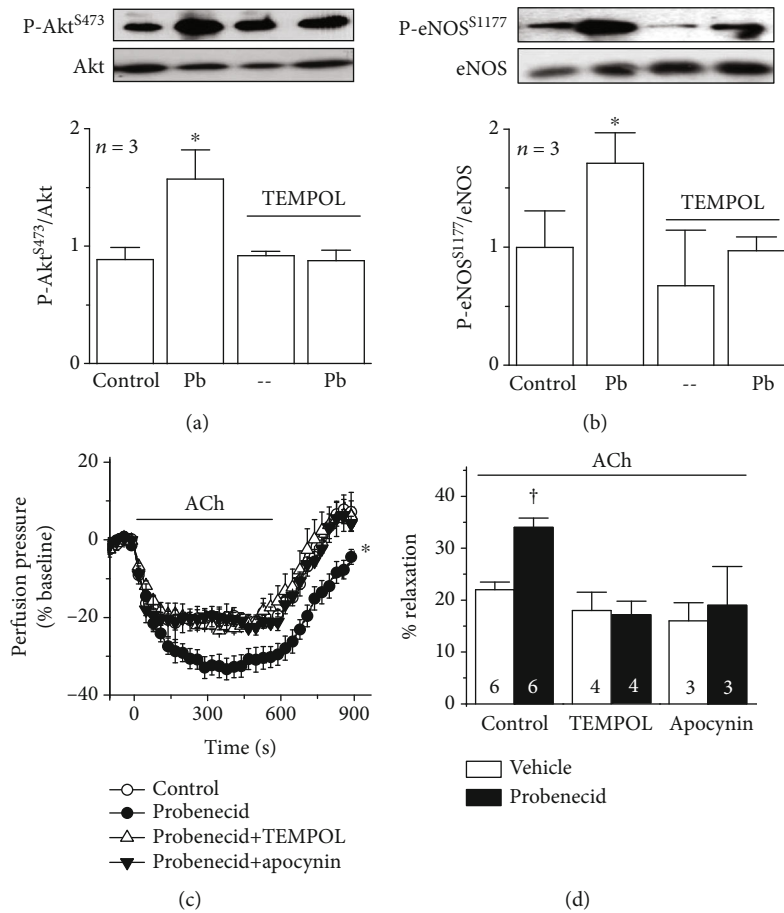
FIGURE 8: The activation of Akt signaling and eNOS phosphorylation initiated by the Panx-1 channel blockade depends on NADPH oxidase-mediated $O_2^{\cdot-}$ production. (a, b) Representative Western blots and densitometric analysis of Akt (a), eNOS (b), Akt phosphorylation at serine 473 (P-Akt^{S473}, (a)), and eNOS phosphorylation at serine 1177 (P-eNOS^{S1177}, (b)) observed in basal conditions (control) and during the treatment with 1 mM probenecid (Pb) in the absence or presence of 10 μM TEMPOL. The effect of TEMPOL alone is also shown. In the densitometric analysis, the changes in eNOS and Akt phosphorylation are expressed as the ratio of phosphorylated protein over total protein. (c) Time course of the vasodilation induced by 100 nM ACh in control conditions and in the presence of 1 mM probenecid alone or in combination with 10 μM TEMPOL or 100 μM apocynin. Arterial mesenteric beds were constricted with 70 mM KCl. The horizontal bar indicates the period of stimulation. (d) Maximum ACh-induced vasodilation attained during the application of probenecid or its vehicle in control conditions and in the presence of TEMPOL or apocynin. Numbers inside the bars indicate the n value. Values are means ± SEM. *$P < 0.05$ vs. the control by one-way ANOVA plus the Newman-Keuls post hoc test. †$P < 0.05$ vs the vehicle by paired Student's t-test.

in close spatial relation with Cav-1 mainly in the endothelium (Figure 5). Cav-1 is a structural protein of caveolae [45], and the special location of Panx-1 in these signaling microdomains may provide the functional organization required to trigger the coordinated activation of Na$_v$ and Ca$_v$3.2 channels by Panx-1 channel blockers, as these channels have been detected to be expressed in caveolae [46–48]. Interestingly, the application of Panx-1 channel blockers evokes an increase in the spatial association of Panx-1 with Cav-1 (Figure 5), which further supports the involvement of these microdomains in the response activated by the blockers. Consistent with this notion, disruption of cholesterol-rich microdomains, such as caveolae, by the treatment with MβCD [49] fully prevented the [10]Panx-activated depolarization and [Ca^{2+}]$_i$ increase in cultured endothelial cells (Figure 6). These results indicate that caveolae play a central role in the signaling mechanism triggered by

Panx-1 channel blockers and suggest that the analysis of protein-protein direct molecular interactions may help us to elucidate the functional connection between the Panx-1 and Na$_v$ channels, but the activation of a Panx-1-mediated receptor-like signaling cascade by probenecid or [10]Panx cannot be ruled out.

In endothelial cells, both depolarization of membrane potential and an increase in [Ca^{2+}]$_i$ have been shown to lead to a rise in $O_2^{\cdot-}$ production by the activation of NADPH oxidase [28–30, 50, 51]. In addition, an increment in $O_2^{\cdot-}$ may lead to the activation of the PI3K/Akt signaling pathway and, thereby, to the further increase in P-eNOS^{S1177} [31, 52]. Three NADPH oxidase isoforms are expressed in the endothelium of rats: NOX1 oxidase, NOX2 oxidase, and NOX4 oxidase. Interestingly, of these three isoforms, only NOX1 and NOX2 generate $O_2^{\cdot-}$, since NOX4 mainly releases hydrogen peroxide (H$_2$O$_2$) [31, 53, 54]. Although the DHE-

based analysis used in this study does not distinguish between O_2^{-} and H_2O_2, these measurements were complemented with the direct O_2^{-} detection by emitted light (ABEL®) assay (Supplementary Fig. S5), which supports the participation of NOX1- or NOX2-mediated O_2^{-} formation in the response activated by the Panx-1 channel blockade. Consistent with this notion, the increase in the DHE signal was fully blocked by both apocynin and TEMPOL (Figure 7). In this context, it should be noted that the effect of apocynin relies on the inhibition of the p47phox subunit association with the membrane-bound heterodimer of the NADPH oxidase complex (NOX and p22phox), which is a critical process for NOX1 and NOX2 activation, but not for NOX4 function [53]. Likewise, TEMPOL is a superoxide dismutase mimetic, and thus, it buffers the increase in O_2^{-} with the consequent H_2O_2 production [55]. Altogether, these data indicate that the response triggered by Panx-1 blockers was associated with an increase in O_2^{-} formation in endothelial cells of resistance arteries (Figure 7), although the involvement of NOX1 or NOX2 in this process must be confirmed in future investigations. Furthermore, the treatment with apocynin or TEMPOL prevented the increase in P-Akt[S473] and P-eNOS[S1177] and the increment in the magnitude of the ACh-induced vasodilation (Figure 8), confirming the participation of the NADPH oxidase/O_2^{-} in the Panx-1-mediated control of vasomotor tone by the regulation of NO production.

It is interesting to note that Panx-1-mediated signaling may be involved in the endothelium-dependent control of peripheral vascular resistance and, consequently, arterial blood pressure. In this context, probenecid treatment *in vivo* has been shown to elicit a reduction in systolic blood pressure in spontaneously hypertensive rats and an increment in leg vascular conductance in humans [56, 57]. As ATP release through Panx-1 channels has been reported to contribute to the vasoconstrictor response initiated by α1-adrenoceptors in smooth muscle cells of resistance arteries [13], the effects of Panx-1 blockers on peripheral vascular resistance have mostly been attributed to the disruption of the purinergic-mediated component of sympathetic nerve-triggered α1-adrenoceptor activation, although a possible direct interaction of probenecid with α-adrenoceptors has been also proposed [56]. However, in view of our results, an increase in the NO-mediated vasodilator component through an increment in P-eNOS[S1177] may also be involved in the hypotensive response evoked by Panx-1 channel blockers, which highlights the potential relevance of endothelial cell Panx-1 in the control of vascular function.

5. Conclusions

Panx-1 channels have typically been described as a transmembrane pathway for ATP release, and accordingly, acute inhibition of the channel function is associated with the disruption of purinergic signaling [58]. However, at least in endothelial cells of resistance arteries, Panx-1 signaling seems to be more complex and is linked to eNOS phosphorylation through a NADPH oxidase/O_2^{-}-mediated pathway. The results of the present work indicate that the blockade of Panx-1 channels leads to the activation of TTX-sensitive Na_v channels and the parallel recruitment of Panx-1 into caveolae, in association with Cav-1. The Na_v channel-mediated depolarizing current is coupled to the $Ca_v3.2$ opening and the subsequent Ca^{2+} entry. Apparently, caveolae provide a signaling platform for the functional association between the Panx-1 and Na_v channels. The concomitant endothelial cell depolarization and $[Ca^{2+}]_i$ increase elicit the further activation of the NADPH oxidase/O_2^{-} signaling, which triggers the PI3K/Akt pathway and the subsequent increase in the ACh-induced NO-mediated vasodilation through the modulation of eNOS activity by the phosphorylation of the enzyme at serine 1177. These findings are thus consistent with the discovery of a novel regulation mechanism of NO production and highlight the relevance of Panx-1 and Na_v and Ca_v3 channels in the control of endothelial cell function. This Panx-1-dependent signaling pathway is likely to play a critical role in the tonic, endothelial control of arterial blood pressure and, therefore, may contribute to the design of new therapeutic strategies for the treatment of cardiovascular-related diseases such as hypertension.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

Supplementary Materials

Supplementary Figure S1. Pannexin-1 channel blockade with probenecid (Pb) does not affect the basal perfusion pressure of mesenteric arterial beds. (a) Time course of the perfusion pressure in mesenteric arterial beds before and after probenecid application. (b) Level of perfusion pressure observed in mesenteric arteries during 10 min in control conditions or in the presence of probenecid. The horizontal bar indicates the period of probenecid application. Values are means ± SEM. Supplementary Figure S2. Inhibition of the KCl-evoked vasoconstriction by pannexin-1 channel blockade with probenecid (Pb). (a) and (b) Time course (a) and maximum increase in perfusion pressure (b) elicited by KCl (70 mM) in mesenteric arteries in control conditions and in the presence of probenecid (15 min). The horizontal bar indicates the period of KCl stimulation. Values are means ± SEM. *, $P < 0.05$ vs Control by paired Student's t test. Supplementary Figure S3. The vasodilation induced by

acetylcholine (ACh) depends exclusively on NO production in KCl-contracted resistance arteries. Time course of changes in perfusion pressure elicited by 1 min stimulation with 100 nM ACh in control conditions and after blocking NO production with 100 μM N^G-nitro-L-arginine (L-NA) in arterial mesenteric vessels contracted with 70 mM KCl. The horizontal bar indicates the period of ACh stimulation. Values are means ± SEM. Supplementary Figure S4. Plasma membrane expression of Na$_v$1.2, Na$_v$1.6 and Ca$_v$3.2 channels in mesenteric resistance arteries. The presence of these channels in the plasma membrane was analyzed by biotinylation of cell surface proteins. Mesenteric arteries were perfused with 1 mM sulfosuccinimidyl-2-[biotinamido]ethyl-1,3-dithiopropionate for 40 min at 0.5 mL/min to label membrane proteins of the luminal surface of the vessels. Then, tissues were homogenized and proteins were precipitated with ice-cold acetone. The pellet was suspended in Tris-buffer, the biotinylated proteins were captured with streptavidin–agarose beads and separated by SDS-PAGE to be detected with specific antibodies. A representative Western blot of three independent experiments is shown. The presence of eNOS, Na$^+$-Ca^{2+} exchanger and the channels Na$_v$1.2, Na$_v$1.6, Ca$_v$3.2 and Ca$_v$1.2 was evaluated in the total tissue homogenized (**H**) and in the fraction of biotinylated-plasma membrane proteins (**M**). In addition to Na$_v$1.2, Na$_v$1.6 and Ca$_v$3.2 channels, eNOS and Ca$_v$1.2 were also analyzed as negative control to confirm that the biotin treatment did not label intracellular proteins of endothelial cells or plasma membrane proteins of smooth muscle cells. In addition, Na$^+$-Ca^{2+} exchanger (NCX) was used as a positive control for the detection of plasma membrane proteins of endothelial cells. Supplementary Figure S5. Superoxide anion (O$_2$$^{.-}$) production activated by probenecid (Pb). O$_2$$^{.-}$ levels were measured in isolated mesenteric arterial beds of rats using the analysis by emitted light (ABEL®) assay, which is based on the intense luminescence emitted upon reaction of O$_2$$^{.-}$ radicals with the prosthetic group of Pholasin, the photoprotein responsible for luminescence in the bivalve Pholas dactylus. After treating mesenteric vessels with the pannexin-1 channel blocker, probenecid, or its vehicle, 20 μL of perfusate samples were mixed with 100 μL of adjuvant-K™ solution and, following 1 min of equilibration, the reaction was initiated by the injection of 250 μL of Pholasin solution (10 μg/mL). The resulting luminescence was immediately measured on a Turner TD20e luminometer (Promega). Adjuvant-K™ and pholasin solutions were prepared using the reconstitution buffer (Hank's balanced salt solution with 20 mM HEPES, pH 7.4) as indicated by the manufacturer (Knight Scientific Ltd.). Changes in O$_2$$^{.-}$ formation are expressed in arbitrary units (AU). Numbers inside the bars indicate the n value. All measurements were made in duplicate. Values are means ± SEM. *, $P < 0.05$ vs Vehicle by unpaired Student's t test. Supplementary Figure S6. The inhibition of the KCl-elicited vasoconstriction evoked by pannexin-1 channel blockade with probenecid (Pb) depends on a NADPH oxidase-initiated superoxide anion signaling. (a) and (b) Effect of probenecid on the time course (Left) and maximum increase in perfusion pressure (Right) evoked by KCl (70 mM) in mesenteric arteries treated with TEMPOL (a), a superoxide scavenger, or apocynin (b), a

NADPH oxidase inhibitor. Note that the reduction in the KCl-induced vasoconstriction observed after pannexin-1 channel blockade with probenecid (Supplementary Fig. S2) is fully prevented by TEMPOL. (a) and apocynin (b). Horizontal bars indicate the period of KCl stimulation (Left) or the treatment applied (Right). Values are means ± SEM. *(Supplementary Materials)*

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Research Article

Cardiac Inflammation, Oxidative Stress, Nrf2 Expression, and Coagulation Events in Mice with Experimental Chronic Kidney Disease

Abderrahim Nemmar [1,2] **Suhail Al-Salam,** [2,3] **Sumaya Beegam,** [1] **Nur Elena Zaaba,** [1] **Javed Yasin,** [4] **Naserddine Hamadi,** [5] **and Badreldin H. Ali** [6]

[1]*Department of Physiology, College of Medicine and Health Sciences, United Arab Emirates University, P.O. Box 17666, Al Ain, UAE*
[2]*Zayed Center for Health Sciences, United Arab Emirates University, Al Ain, UAE*
[3]*Department of Pathology, College of Medicine and Health Sciences, United Arab Emirates University, P.O. Box 17666, Al Ain, UAE*
[4]*Department of Internal Medicine, College of Medicine and Health Sciences, United Arab Emirates University, P.O. Box 17666, Al Ain, UAE*
[5]*Department of Life and Environmental Sciences, College of Natural and Health Sciences, Zayed University, P.O. Box 144534, Abu Dhabi, UAE*
[6]*Department of Pharmacology and Clinical Pharmacy, College of Medicine & Health Sciences, Sultan Qaboos University, P.O. Box 35, Muscat, 123 Al-Khod, Oman*

Correspondence should be addressed to Abderrahim Nemmar; anemmar@uaeu.ac.ae

Chronic kidney disease (CKD) is known to be associated with cardiovascular dysfunction. Dietary adenine intake in mice is also known to induce CKD. However, in this experimental model, the mechanisms underlying the cardiotoxicity and coagulation disturbances are not fully understood. Here, we evaluated cardiac inflammation, oxidative stress, DNA damage, and coagulation events in mice with adenine (0.2% w/w in feed for 4 weeks)-induced CKD. Control mice were fed with normal chow for the same duration. Adenine increased water intake, urine output, relative kidney weight, the plasma concentrations of urea and creatinine, and the urinary concentrations of kidney injury molecule-1 and neutrophil gelatinase-associated lipocalin. It also decreased the body weight and creatinine clearance, and caused kidney DNA damage. Renal histological analysis showed tubular dilation and damage and neutrophilic influx. Adenine induced a significant increase in systolic blood pressure and the concentrations of troponin I, tumor necrosis factor-α, and interleukin-1β in heart homogenates. It also augmented the levels of markers of lipid peroxidation measured by malondialdehyde production and 8-isoprostane, as well as the antioxidants superoxide dismutase and catalase. Immunohistochemical analysis of the hearts showed that adenine increased the expression of nuclear factor erythroid-derived 2-like 2 by cardiomyocytes. It also caused cardiac DNA damage. Moreover, compared with the control group, adenine induced a significant increase in the number of circulating platelet and shortened the thrombotic occlusion time in pial arterioles and venules *in vivo*, and induced a significant reduction in the prothrombin time and activated partial thromboplastin time. In conclusion, the administration of adenine in mice induced CKD-associated cardiac inflammation, oxidative stress, Nrf2 expression, and DNA damage. It also induced prothrombotic events *in vivo*. Therefore, this model can be satisfactorily used to study the cardiac pathophysiological events in subjects with CKD and the effect of drug treatment thereon.

1. Introduction

There is a worldwide substantial increase in the prevalence of chronic kidney disease (CKD) attaining as much as 13%, and more than seven million people with end-stage kidney disease are requiring renal replacement therapy [1, 2]. The latter has been associated with the increase of prevalence of ageing, obesity, diabetes mellitus, hypertension, and metabolic syndrome [1, 2].

Kidney and cardiovascular diseases are tightly interconnected, and injury in one of these organs leads to adverse effects on the other one [1, 2]. In fact, it is well established that patients with CKD present cardiovascular complications including hypertension, thromboembolic disorders, cardiac hypertrophy and failure, and their pervasiveness augments with deteriorating kidney function [1, 2].

Animal models of CKD have been shown by several workers to be extrapolatable and useful (albeit imperfect) to the human disease [3]. In order to provide biological plausibility and better understanding on the mechanisms underlying CKD and their extrarenal impact, animal models with CKD are often utilized including the adenine-induced CKD [4]. When adenine is ingested by rodents, it is oxidized to 2,8-dihydroxyadenine by xanthine oxidase, which produces precipitates and crystals in the renal tubules which consequently induce tubular damage, inflammation, and fibrosis [5, 6].

Rats treated with adenine-induced CKD have been shown to develop cardiovascular complications including increase of systolic blood pressure (SBP) starting at week four, and at week 16, the SBP is further augmented and is associated with left ventricular hypertrophy and interstitial and perivascular inflammation and fibrosis and compromised vascular reactivity [4, 7, 8]. However, the mechanisms of action of cardiovascular injury in adenine-induced CKD are not fully understood.

Moreover, a recent study has shown a prolongation in tail bleeding time and delay in thrombus formation in cremaster arterioles following vascular injury in mice with CKD induced by 5/6th nephrectomy- or adenine (0.25% for two weeks)-induced CKD [9]. On the contrary, other studies have reported platelet hyperactivity and increased thrombogenicity in a rat or mouse model of CKD induced by 5/6 ablation/infarction [10, 11].

We have recently shown that administration of adenine (0.2% w/w in feed for 4 weeks) in mouse-induced CKD is accompanied by lung oxidative stress, DNA damage, and fibrosis [12]. However, the impact of the latter model of CKD on the cardiovascular system has received only scant attention [4]. Therefore, the aim of this study conducted in mice was twofold: (1) to assess the effects of adenine (0.2% w/w in feed for 4 weeks)-induced CKD on SBP, heart histology, inflammation, oxidative stress, nuclear factor erythroid 2-related factor 2 (Nrf2) expression, and DNA damage and (2) to evaluate the impact of the adenine-induced CKD on circulating platelets, photochemically induced thrombosis in pial microvessels *in vivo* and prothrombin time (PT), and activated partial thromboplastin time (aPTT) *in vitro*.

2. Material and Methods

2.1. Experimental Animals and Treatments. Male C57BL/6 mice aged between 8 and 10 weeks, weighing in the beginning about 20-25 g (UAEU, College of Medicine and Health Sciences animal house), were housed at the Animal House of the College of Medicine and Health Sciences, UAEU, in light (12 h light : 12 h dark cycle), relative humidity of 50–60%, and temperature-monitored $(21 \pm 2°C)$ rooms. Animals had unrestricted access to tap water and commercial laboratory chow. They were indiscriminately separated into two groups of mice and put in cages. The control mice were given standard food for four weeks. The second group consisting of the adenine-treated group received the same diet in the form of powder containing adenine 0.2% w/w (0.2 g of adenine in 100 g of powder diet) for four weeks. The dose and duration of adenine treatment used in the present study were selected from our previous publications and have been shown to be effective in causing CKD in mice [6, 12–15]. It has been shown that when adenine is consumed by mice or rats, it gets metabolized to 2,8-dihydroxyadenine, which precipitates and produces tubular crystals that consequently induce kidney injury [5, 6, 16]. The weights of the animals were taken at the start of the study and just prior to sacrifice. Mice were relocated in metabolic cages on day 28 and kept there for 24 h to allow the quantification of water intake and urine volume. Twenty-four hours later, numerous renal and cardiovascular parameters were assessed. Mice were cared for under the protocol of the Animal Research Ethics Committee of our college and as per the NIH *Guide for the Care and Use of Laboratory Animals*, NIH publication no. 85-23, 1985.

2.2. Measurement of SBP. A computerized noninvasive tail-cuff manometry system was used to assess SBP in control and adenine-treated mice (ADInstruments, Colorado Springs, USA) [17]. To circumvent technique-induced stress, animals were adapted to the technique and trained for three successive days earlier to the experimental procedure.

2.3. Blood Collection, Histology, Immunohistochemistry, and Biochemical Analysis. After the measurement of SBP, mice were anesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 45 mg/kg, and then, the blood was collected from the inferior vena cava in citrate solution (3.2%). A sample was used for platelet count in a VET ABX Micros with mouse card (ABX, Montpellier, France), and the rest was spun at 4°C for 15 min at a speed of 900 g. The plasma samples acquired after centrifugation were kept at -80°C awaiting analysis.

The kidneys and hearts were excised following sacrifice, washed with ice-cold saline, blotted with filter paper, weighed, and fixed with 10% buffered formalin for 24 h. The latter was followed by dehydration in cumulative concentrations of C_2H_5OH, cleared with xylene, and embedded in paraffin. Sections of 3 μm were prepared from paraffin blocks and stained with hematoxylin and eosin. These were examined by light microscopy by a histopathologist who contributed in this study (SA).

Concerning the detection of Nrf2 by immunohistochemistry, $5\,\mu$m heart sections were made ready and mounted on aminopropyltriethoxysilane-coated slides. Subsequent to dewaxing with xylene and rehydrating with graded alcohol, slides were put in a 0.01 M citrate buffer solution (pH = 6.0) and the pretreatment processes to unmask the antigens were accomplished in a water bath at 95°C (30 min). Then, the sections were treated for 30 min with peroxidase block followed by protein block for 30 min. After that, sections were incubated for one hour at room temperature (RT) with anti-Nrf2 (Rabbit Polyclonal, 1 : 300, Abcam, USA). Following the conjugation with primary antibodies, sections were incubated with secondary antibody (EnVision™ Detection System, DAKO, Agilent, USA) for 20 min at RT followed by DAB chromogen (EnVision™ Detection System, DAKO, Agilent, USA) addition and counter staining achieved with hematoxylin. Suitable positive controls were utilized. Regarding the negative control, the primary antibody was not supplemented to sections. Both controls (negative and positive) were utilized in each set of slides which were stained (not shown in figures). The heart tissue immunohistochemical staining was scored according to the % of staining of heart muscles and endothelial cells of each section of the heart [18].

The concentrations of urea and creatinine in plasma and creatinine in urine were spectrophotometrically measured using commercial kits (Roche Diagnostics, Indianapolis, IN, USA). ELISA kits were utilized to quantify the levels of kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) in the urine (R&D Systems, MN, USA).

2.4. Assessment of Markers of Injury, Inflammation, and Oxidative Stress in Heart Homogenates.

Preparation of heart homogenates for the assessment of markers of injury inflammation and oxidative stress was achieved as previously reported [19]. The levels of troponin I (Life Diagnostics, West Chester, PA, USA), tumor necrosis factor α (TNF-α; R&D Systems, Minneapolis, MN, USA), interleukin-1β (IL-1β; R&D systems, Minneapolis, MN, USA), 8-isoprostane (Cayman Chemicals, Michigan, USA), malondialdehyde (MDA; Sigma-Aldrich Fine Chemicals, St. Louis, MO, USA), superoxide dismutase (SOD; Cayman Chemicals, Michigan, USA), and catalase (Cayman Chemicals, Michigan, USA) were assayed according to the protocols described by the respective manufacturers.

2.5. DNA Damage Evaluation by COMET Assay.

In a separate set of mice, the hearts and kidneys were removed from each mouse immediately after sacrifice and processed for the quantification of DNA injury by COMET assay according to a previously reported technique [17, 20].

2.6. In Vivo Experimental Pial Cerebral Microvessel Thrombosis Model.

In vivo pial arteriolar and venular thrombogenesis was assessed on day 29 of the experiment in control and adenine-treated mice according to a previously described technique [17, 21].

2.7. In Vitro Assessment PT and aPTT.

On day 29, all animals were anesthetized, and the blood was withdrawn from the

TABLE 1: Daily water intake, urine volume, body weight change, and relative kidney weight in control and adenine-treated mice.

Parameters/group	Control	Adenine
Water intake (ml)	9.08 ± 0.55	$22.83 \pm 2.45^{***}$
Urine volume (ml)	3.11 ± 0.05	$14.32 \pm 0.926^{**}$
Body weight (% change)	6.25 ± 0.97	$-22.26 \pm 0.80^{***}$
Relative kidney weight (g)	1.14 ± 0.02	$1.23 \pm 0.03^{*}$

Values in the table are presented as the mean $\pm$ SEM ($n = 6 - 8$). Adenine was added to the feed at a concentration of 0.2% w/w, for 4 weeks. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (control vs. adenine group).

TABLE 2: Plasma concentration of urea and creatinine and creatinine clearance, and urinary concentration of kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL), and kidney DNA damage in control and adenine-treated mice.

Parameters/group	Control	Adenine
Urea (mmol/l)	4.05 ± 0.15	$8 \pm 1.08^{**}$
Creatinine (μmol/l)	9.1 ± 0.72	$17.63 \pm 3.72^{*}$
Creatinine clearance (ml/min)	0.50 ± 0.05	$0.13 \pm 0.03^{***}$
KIM-1 (pg/ml)	86.25 ± 4.32	$762.3 \pm 54.58^{***}$
NGAL (pg/ml)	$4,477 \pm 262$	$6,897 \pm 308.4^{****}$
DNA migration in kidney (mm)	5.23 ± 0.10	$10.43 \pm 0.05^{****}$

Values in the table are presented as the mean $\pm$ SEM ($n = 5 - 8$). Adenine was added to the feed at a concentration of 0.2% w/w, for 4 weeks. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ (control vs. adenine group).

inferior vena cava and placed in citrate solution (3.2%) (ratio of the blood to anticoagulant: 9 : 1). The PT was assayed on freshly collected platelet-poor plasma with human relipidated recombinant thromboplastin (Recombiplastin; Instrumentation Laboratory, Orangeburg, NY, USA) along with a coagulometer (MC 1 VET, Merlin, Lemgo, Germany) [22]. The aPTT was assessed with the automated aPTT reagent from bioMerieux (Durham, NC, USA) with the identical coagulometer [22].

2.8. Statistics.

All statistical analyses were carried out using GraphPad Prism Software version 7. To assess whether the measured parameters were normally distributed, the Shapiro-Wilk normality test was first used. Normally distributed data were tested using the unpaired t-test for differences between the two groups. Data which were not normally distributed (TNF-α, IL-1β, MDA, and thrombotic occlusion time in venules) were tested using the Mann-Whitney test for differences between groups. All the data in figures and table were expressed as the mean $\pm$ SEM. P values < 0.05 are considered significant.

3. Results

3.1. Renal Endpoints.

Tables 1 and 2 depict data related to physiological and biochemical parameters assessed in control and adenine-treated mice. Adenine treatment significantly decreased the body weight ($P < 0.001$) and significantly

(a)

(b)

(c)

(d)

FIGURE 1: Representative light microscopy sections of kidney tissues of control mice and those given with adenine mixed in the feed (0.2% w/w, for four weeks), stained with H&E. (a, b) The control group shows unremarkable morphologic changes in renal tubules (thin arrow) and glomeruli (arrowhead). (c, d) The adenine-treated group shows dilatation of the renal tubules (thin arrow), tubular damage (arrowhead), and neutrophil polymorph infiltration of the affected tubules (thick arrow). Scale bars in (a, c): 200 μm and (b, d): 50 μm.

increased water intake ($P < 0.001$), urine volume ($P < 0.01$), and relative kidney weight ($P < 0.05$) when compared with control mice (Table 1). Table 2 depicts a significant increase in plasma urea ($P < 0.01$) and creatinine ($P < 0.05$) concentrations, and a significant decrease in creatinine clearance ($P < 0.001$) in the adenine group. Moreover, adenine treatment induced a significant increase in the urinary concentrations of KIM-1 ($P < 0.001$) and NGAL ($P < 0.0001$) and DNA damage ($P < 0.0001$) assessed by COMET assay (Table 2). Likewise, the histological analysis of the kidneys collected from the adenine-treated group showed the presence of tubular dilation and damage and neutrophilic influx (Figure 1).

3.2. Cardiovascular Endpoints.

Figure 2 shows that adenine treatment induced a significant increase in SBP ($P < 0.0001$).

Figure 3 illustrates the effect of adenine administration on the concentrations of the marker of cardiac injury, troponin I, and the proinflammatory cytokines TNF-α and IL-1β. Compared with the control group, the concentrations of troponin I ($P < 0.05$), TNF-α ($P < 0.01$), and IL-1β ($P < 0.001$) were significantly increased in the heart homogenates of the adenine-treated group.

Figure 4 shows the impact of adenine treatment on the levels of markers of lipid peroxidation including 8-isoprostane and MDA and the activities of the antioxidants SOD and catalase. Compared with the control group, the levels of 8-isoprostane ($P < 0.01$), MDA ($P < 0.01$), SOD
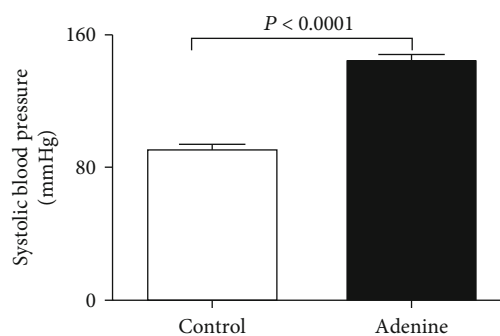
FIGURE 2: Systolic blood pressure in control mice and those given with adenine mixed in the feed (0.2% w/w, for four weeks). Mean $\pm$ SEM ($n = 8$ in each group).

($P < 0.05$), and catalase ($P < 0.01$) were found to be markedly elevated in adenine-treated mice (Figure 4).

The assessment of DNA damage by COMET assay in the heart of control and adenine-treated mice is shown in Figure 5. Compared with the control group, adenine administration induced a significant augmentation of heart DNA injury ($P < 0.0001$).

Light microscopy analysis of the heart sections stained with H&E obtained from control mice exhibited normal structure (Figure 6). Following adenine treatment, no morphological changes have been observed in the hearts collected from the adenine group (Figure 6). However, the
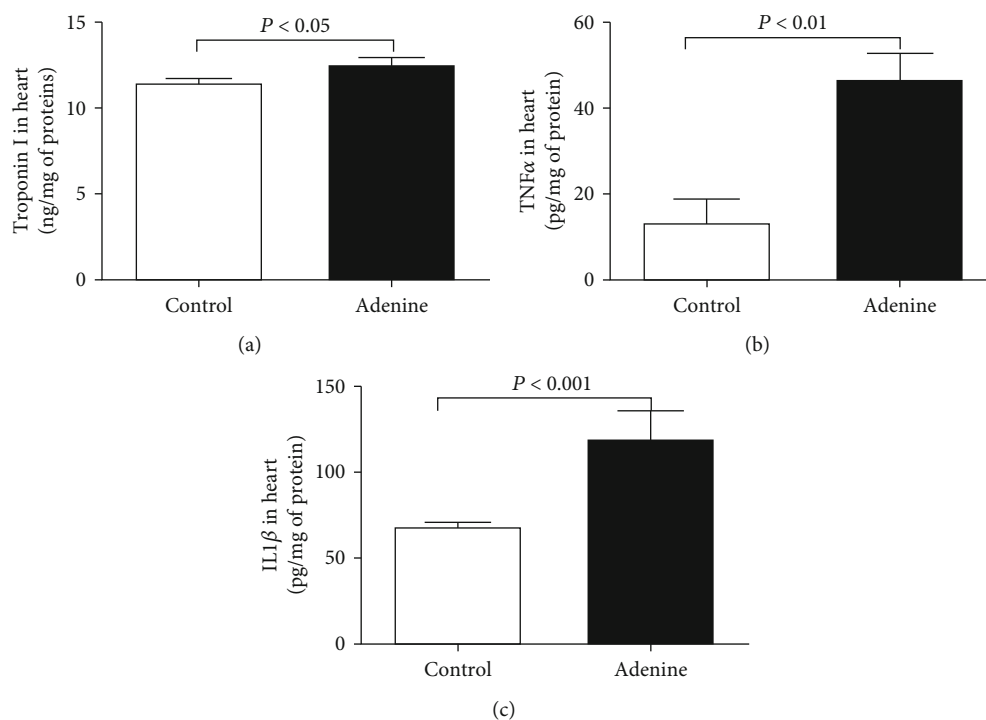
FIGURE 3: Troponin I (a), tumor necrosis factor-α (TNF-α) (b), and interleukin-1β (c) concentrations in heart homogenates of control mice and those given with adenine mixed in the feed (0.2% w/w, for four weeks). Mean $\pm$ SEM ($n = 7$-8 in each group).
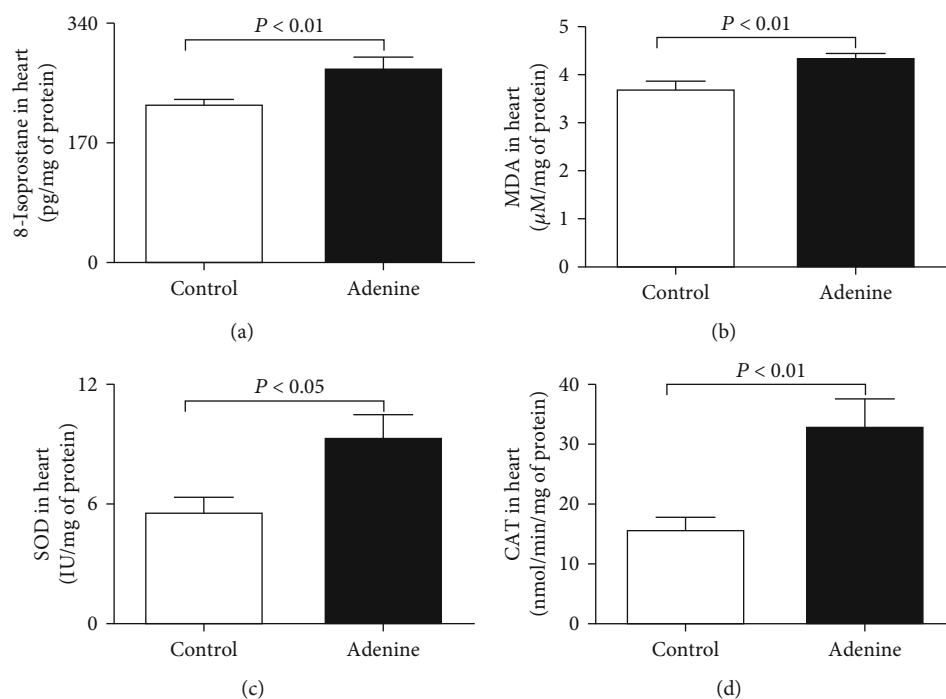
FIGURE 4: 8-Isoprostane (a), malondialdehyde (MDA) (b), superoxide dismutase (SOD) (c), and catalase (CAT) (d) levels in heart homogenates of control mice and those given with adenine mixed in the feed (0.2% w/w, for four weeks). Mean $\pm$ SEM ($n = 8$ in each group).

immunohistochemistry analysis of the heart revealed the presence nuclear expression of Nrf2 by cardiomyocytes in the heart sections of all groups (Figure 7), with different intensity and distribution. The control group shows mild nuclear expression of Nrf2 by cardiomyocytes (Figure 7). The adenine-treated group showed a significant increase ($P < 0.05$) in the expression of Nrf2 by cardiomyocytes when compared to the control group (Figure 7).
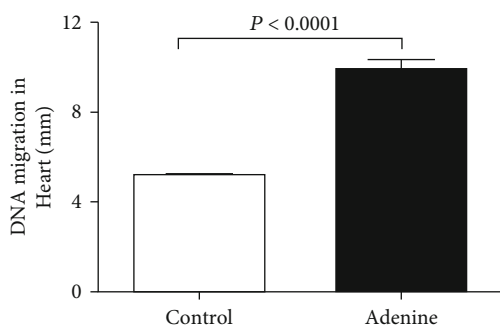
Figure 5: DNA migration (mm) in the heart tissues assessed by COMET assay in control mice and those given with adenine mixed in the feed (0.2% w/w, for four weeks) (a). Data are presented as the means ± SEM ($n = 5$).

Figure 8 shows the effects of adenine on the number of circulating platelets and the thrombotic events in pial microvessels *in vivo*. Compared with the control group, the number of circulating platelets was significantly augmented ($P < 0.0001$), and thrombotic occlusion time measured in pial arterioles significantly shortened ($P < 0.0001$) in adenine-treated mice (Figure 8(a) and Figure 8(b), respectively). Similarly, in pial venules of adenine-treated mice, the thrombotic occlusion time was also significantly shortened ($P < 0.001$) compared with the control group, indicating a prothrombotic effect of adenine treatment (Figure 8(c)).

Figure 9 depicts the impact of adenine administration on PT and aPTT. Compared with the control group, adenine treatment significantly shortened the PT ($P < 0.0001$; Figure 9(a)) and aPTT ($P < 0.0001$; Figure 9(b)).

4. Discussion

In the present study, we showed that feeding adenine to mice (0.2% w/w for four weeks) induced CKD which was associated with cardiac inflammation, oxidative stress, Nrf2 expression, and DNA damage. It also induced prothrombotic events *in vivo*.

Renal and cardiovascular functions are tightly related under physiological and pathophysiological situations [23, 24]. It is well-known that cardiovascular diseases are the main cause of death in patients with impaired renal function [23, 24]. Moreover, patients with CKD develop cardiovascular disease including hypertension, cardiac hypertrophy, and thrombotic complications [23, 24].

Experimental animal models using rats or mice have been widely utilized to enhance our knowledge about the pathophysiology of CKD and develop pharmacological interventions aiming at mitigating or averting renal damage [4]. Although the majority of CKD animals models do not simulate totally the intricacy of human CKD and its accompanying complications, the experimental CKD induced by adenine supplementation to feed of rats or mice have been shown to reproduce gastrointestinal, pulmonary, and cardiovascular complications seen in clinical situations [4, 7, 8, 12, 15, 25]. With respect to cardiovascular effects, it has been shown that adenine treatment in rats (0.75% w/w for four

weeks) increased blood pressure and the absolute volume of left ventricle and it decreased volume density and absolute volume of myocardial capillaries [7, 25]. Moreover, Diwan et al. [4, 8] showed that adenine added in diet at 0.25% for 16 weeks mimicked the cardiovascular changes seen in humans with CKD, including elevation of blood pressure (which was significant at four weeks) along with hypertrophy of the left ventricle and augmentation in interstitial and perivascular inflammation and fibrosis resulting in augmented stiffness of the left ventricular. However, the mechanisms underlying the cardiovascular events seen in CKD are not fully understood.

In line with earlier reports describing the renal effects of adenine treatment in mice [6, 12, 14], we found here that the body weight and creatinine clearance were reduced, whereas the water intake, urine volume, relative kidney weight, the plasma concentrations of urea and creatinine, the urinary concentrations of KIM-1 and NGAL, and renal DNA damage were significantly elevated compared with the control group. Moreover, histological analysis of the kidneys collected from the adenine-treated group showed the presence of tubular dilation and damage and neutrophilic influx.

It has been previously shown that adenine-treated rats (0.25% or 0.75% w/w) induce an increase of blood pressure at four-week time point and continues up to 16 weeks [8, 25, 26]. Likewise, adenine administration in mice (0.2% for four weeks) induced a significant increase of blood pressure [6, 14]. The data of the present study confirmed the elevation of SBP of mice given with adenine and further showed a significant increase of troponin I, a biomarker of myocardial damage, and two proinflammatory cytokines including TNF-α and IL-1β in heart homogenates. The latter findings indicate the occurrence of myocardial injury and cardiac inflammation. Both inflammation and oxidative stress are concurrently found to be elevated in cardiovascular diseases, and each one can be readily triggered and potentiated by the other one [27, 28]. Recently, inflammation and oxidative stress have attracted much interest as crucial pathophysiological players in the cause and progression of various cardiovascular diseases [27, 28]. Despite the fact that reactive oxygen species exert signalling functions under physiological condition, a disproportionate and decontrolled generation of these molecules may trigger oxidative stress and cardiomyocyte injury [27, 28]. Therefore, to gain more insights into the mechanisms underlying the observed cardiac injury and inflammation, we measured various markers of oxidative stress in heart homogenates, including 8-isoprostane, MDA, SOD, and catalase. Our data showed that the hearts of adenine-treated mice showed a significant increase of markers of lipid peroxidation, namely, 8-isoprostane and MDA, and the antioxidant enzymes SOD and catalase. The latter indicate the occurrence of oxidative stress in the heart, and the increase of antioxidants suggests an ongoing compensatory mechanisms taking place in the heart and aiming at mitigating the injurious effects of oxygen radicals. Furthermore, besides heart inflammation and oxidative stress, we, presently, found a significant increase in cardiac DNA damage. These results indicate that the treatment with adenine caused oxidative stress *milieu* which in turns induced DNA

(a)

(b)

(c)

(d)

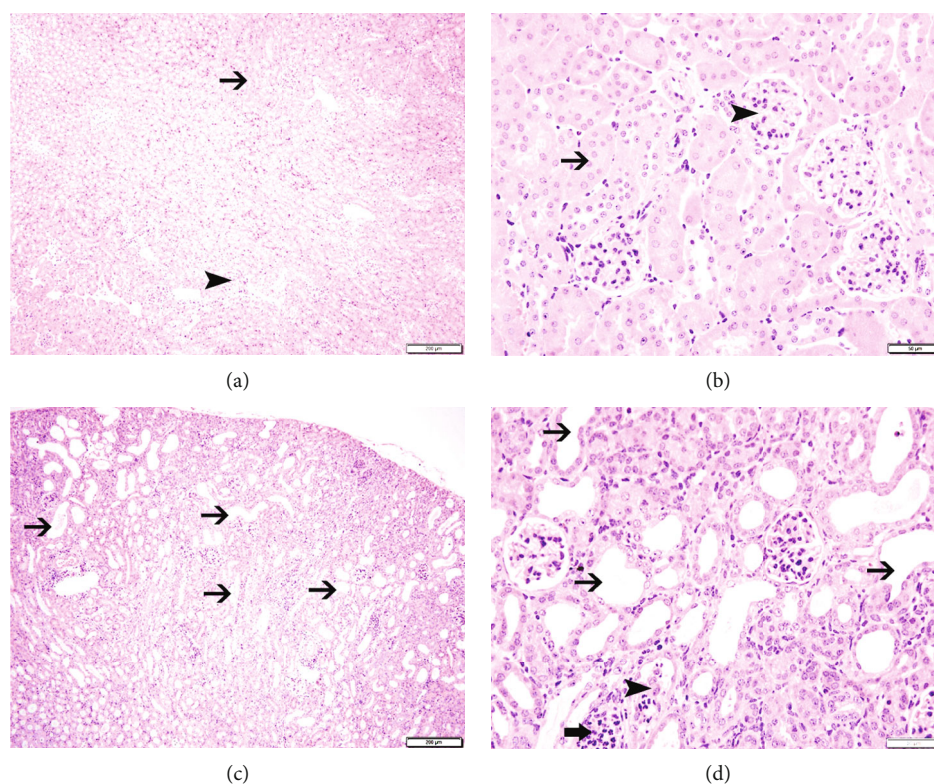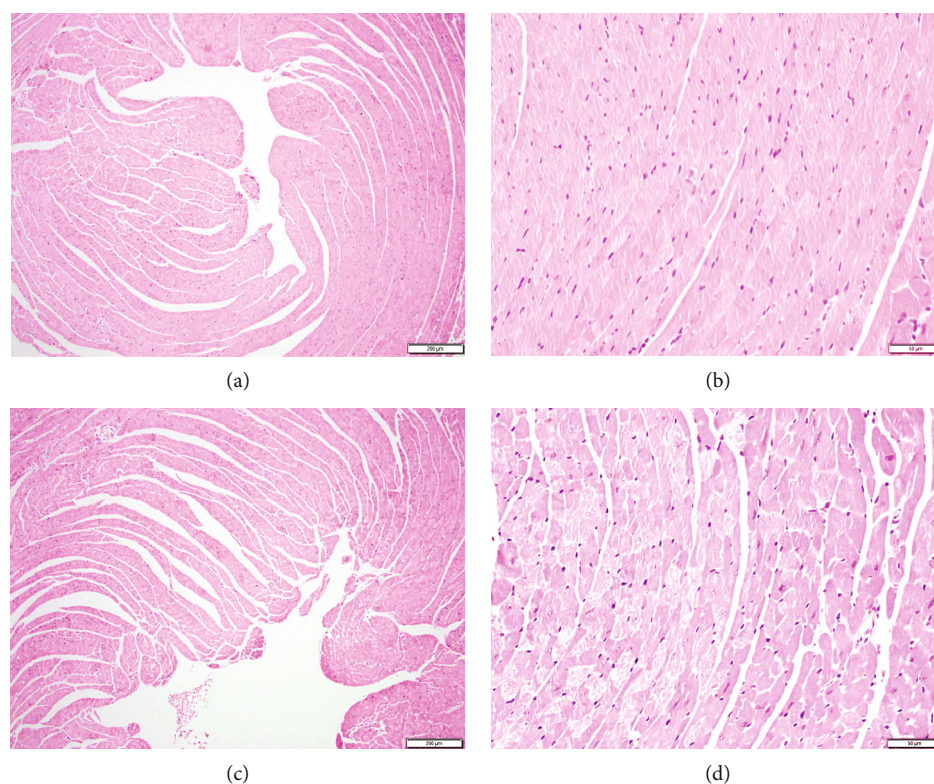FIGURE 6: Representative light microscopy sections of heart tissues of control mice and those given with adenine mixed in the feed (0.2% w/w, for four weeks), stained with H&E. (a, b) The control group shows unremarkable heart morphology and architecture. (c, d) The adenine-treated group shows unremarkable heart morphology and architecture. Scale bars in (a, c): 200 μm and (b, d): 50 μm.

injury. As far as we are aware, these findings have not been reported before. Using the same model of adenine-induced CKD in mice, we have recently reported the occurrence of lung oxidative stress and DNA damage [12].

Despite the occurrence of inflammation, oxidative stress, and DNA damage seen in the adenine group, the histological analysis of the hearts revealed a lack of clear morphologic alterations in H&E-stained sections. This effect can be related to the duration of adenine treatment (4 weeks at 0.2% w/w) applied in this study. Moreover, the apparent absence of morphological alteration is not in disagreement with the occurrence of biochemical changes shown presently in heart tissue of mice treated with adenine (Figures 3–5). The latter biochemical alterations can plausibly pave the way of morphological change of the heart which may be seen after treatment with adenine for a longer period of time [29]. In fact, in rats exposed to adenine for sixteen weeks at 0.25 w/w, various cardiac morphological changes have been reported encompassing compromised vascular responses, elevated left ventricular stiffness, and augmented left ventricular mass [8]. The immunohistochemistry analysis of the heart obtained from the adenine-treated group revealed a significant increase in the nuclear expression of Nrf2 by cardiomyocytes. Nrf2 is an important transcription factor involved in antioxidant enzyme activation after the manifestation of oxidative stress [30, 31]. Following the occurrence of oxidative stress, Nrf2 is freed from the regulatory Keap1-Nrf2 complex and moves from the cytoplasm to the cell nucleus, where it

attaches to the antioxidant response element, a regulatory enhancer region within gene promoters [32]. This attachment triggers the production of antioxidant enzymes that play a protective role against oxidative stress-induced cell injury [32]. We have recently demonstrated the increase of expression of Nrf2 in the lung of mice treated with adenine [12]. Moreover, Nrf2 expression was found to increase in the heart of mice exposed to waterpipe tobacco smoke, and that the treatment with the antioxidant gum Arabic potentiated Nrf 2 expression [18].

Patients with CKD present various defects in hemostasis and coagulation including the elevation platelet aggregation and von Willebrand factor activity, and the plasma concentrations of D-dimer, fibrinogen, and plasminogen activator inhibitor that prevents the activation of the fibrinolytic system [33, 34]. The latter effects induce increase of thrombogenicity in CKD [33, 34]. On the other hand, as the CKD progresses to a greater extent, the bleeding risk augments in relation to platelet defect [33, 34]. Experimental studies using rat and mouse model of CKD induced by 5/6 ablation/infarction showed an increase of platelet activity and a prothrombotic tendency assessed 4 weeks after the surgery [10, 11]. Conversely, a recent study has shown a prolongation of tail bleeding time and delay in thrombus formation in cremaster arterioles following vascular injury in mice with CKD induced by 5/6th nephrectomy or fed with 0.25% adenine (for two weeks) [9]. In the present work, similar to our previous studies using the same dose and duration of treatment

(a)

(b)

(c)
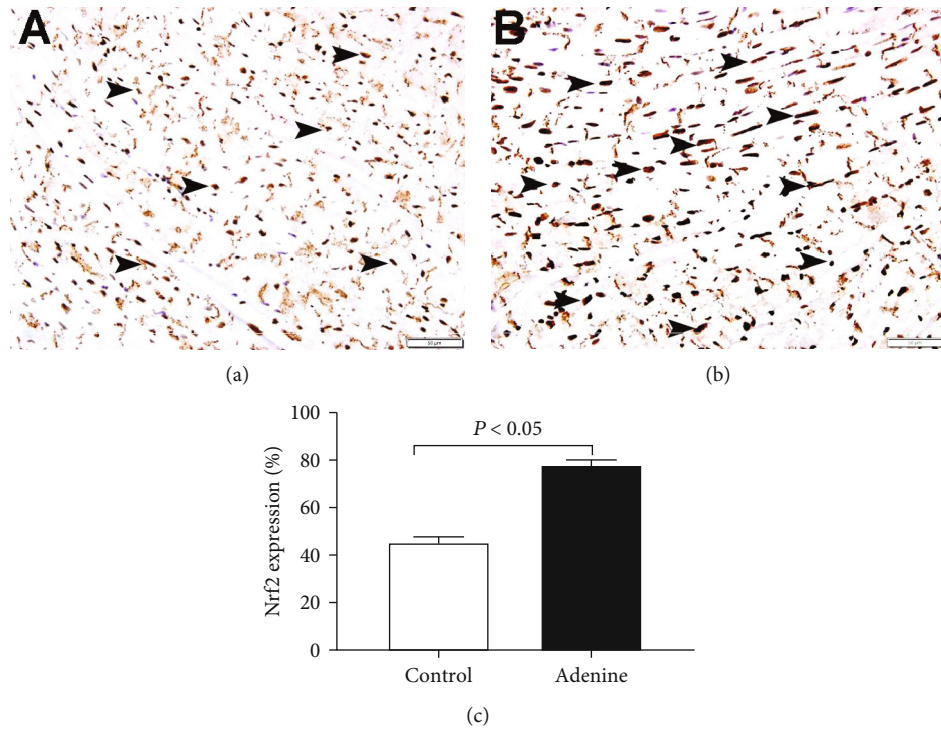
FIGURE 7: Immunohistochemical analysis of the heart tissue sections for the detection of nuclear factor erythroid-derived 2-like 2 (Nrf2) in control mice and those given with adenine mixed in the feed (0.2% w/w, for four weeks). (a) Representative section of the heart of control mice showing mild nuclear expression of Nrf2 by cardiomyocytes (arrow). (b) Representative section of the heart of adenine-treated mice showing a significant increase of nuclear expression of Nrf2 by cardiomyocytes (arrow). (c) Semiquantitative assessment of the % immunohistochemical staining of the heart tissue for Nrf2 in control mice and those given with adenine mixed in the feed. Data are presented as the means $\pm$ SEM ($n = 6$). Scale bars in (a, b): 50 μm.

(a)

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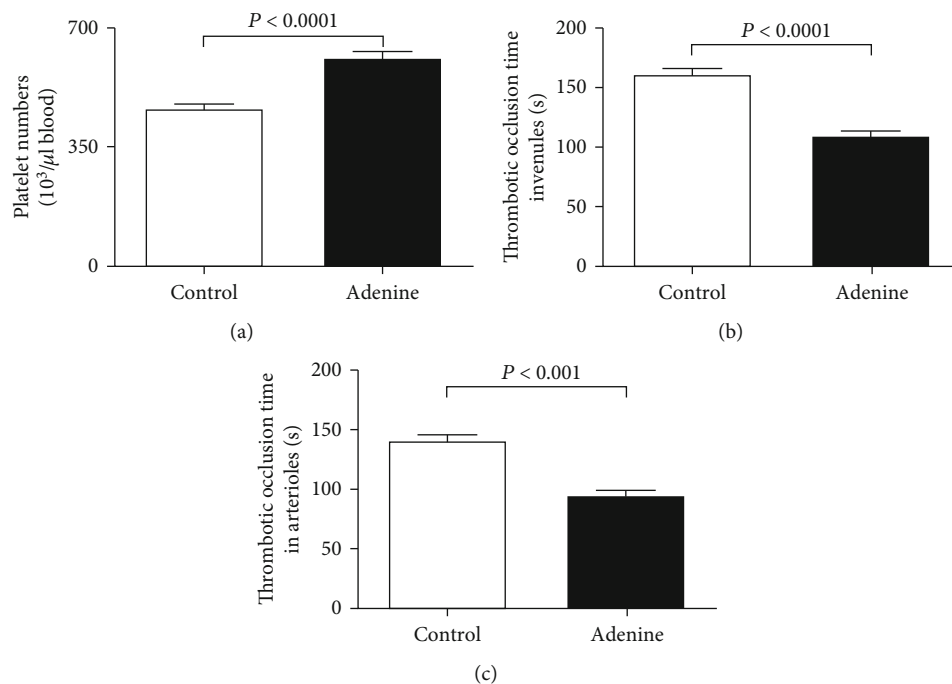
FIGURE 8: Circulating platelet numbers (a) and thrombotic occlusion time in pial arterioles (b) and venules (c) in control mice and those given with adenine mixed in the feed (0.2% w/w, for four weeks). Mean $\pm$ SEM ($n = 7$-8 in each group).
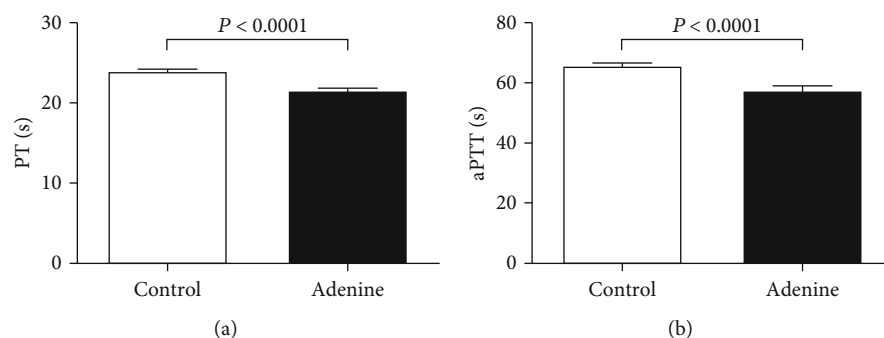
Figure 9: Prothrombin time (PT) (a) and activated partial thromboplastin time (b) in plasma of control mice and those given with adenine mixed in the feed (0.2% w/w, for four weeks). Mean ± SEM ($n = 7$-8 in each group).

(0.2% for four weeks), we did not notice any mortality related to the adenine treatment [6, 12, 14]. Our data show a significant increase in the number of circulating platelet. In patients with CKD, relative thrombocytosis has been associated with the severity of cardiovascular disease [35, 36]. Interestingly, our data also showed a significant shortening in the thrombotic occlusion time in pial arterioles and venules *in vivo* indicating an increased thrombogenicity. Additionally, we assessed the PT and aPTT in plasma of control and adenine-treated mice. PT evaluates the production of the fibrin clot through the activity of the extrinsic and common coagulation pathways, and aPTT assesses the activity of the intrinsic and common pathways of coagulation. We found a significant shortening of the PT and aPTT in the plasma of the adenine-treated group, demonstrating a propensity to hypercoagulability and confirming our in vivo findings. The discrepancy between our study and that of Makhloufi et al. [9] could be ascribed to the difference in the dose and duration of treatment of adenine (4 weeks at 0.2% in our study versus 2 weeks at 0.25%). In the latter study, the reason why the duration of treatment with adenine was limited to 2 weeks was related to high animal mortality observed at 4 weeks of treatment [9]. Additional studies are needed to understand the reason of this discrepancy and to further investigate the mechanism of increased thrombotic tendency in adenine-treated mice.

In conclusion, our data show that administration of adenine in mice induced CKD which is associated with cardiac inflammation, oxidative stress, Nrf2 expression, and DNA damage. It also induced prothrombotic events *in vivo*. Further studies are required to establish whether adenine can have direct harmful effects on the heart.

Data Availability

The data that support the findings of this study are available from the corresponding author, Abderrahim Nemmar, upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Cyclosporine A Promotes Bone Remodeling in LPS-Related Inflammation via Inhibiting ROS/ERK Signaling: Studies *In Vivo* and *In Vitro*

Yuwei Zhao ⬤, **Jing Gao** ⬤, **Yarong Zhang** ⬤, **Xueqi Gan** ⬤, and **Haiyang Yu** ⬤

State Key Laboratory of Oral Disease, National Clinical Research Center for Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, China

Correspondence should be addressed to Xueqi Gan; 522707976@qq.com and Haiyang Yu; yhyang6812@scu.edu.cn

In some inflammatory diseases of bone, osteogenesis and osteoclasis are uncoupled and the balance is usually tipped resulting in bone destruction. The underlying mechanism of osteogenic dysfunction in inflammation still needs further study. This study is aimed at investigating the effects of cyclosporine A (CsA) on bone remodeling in lipopolysaccharide- (LPS-) related inflammation. *In vivo*, an alveolar bone defect model was established using 10-week-old C57BL/6J mice. The mice were divided into phosphate-buffered saline (PBS), LPS, and LPS+CsA groups. After 3 weeks, micro-CT analysis and histomorphometric evaluation were conducted. *In vitro*, murine osteoblasts were treated with vehicle medium, LPS, LPS+CsA, LPS+extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor (LPS+PD98059), and LPS+antioxidant (LPS+EUK134). Cell proliferation, osteogenic behaviors, oxidative stress, and ERK signaling were determined. By these approaches, LPS inhibited bone remodeling and promoted oxidative stress accumulation in alveolar bone defects. When animals were treated with CsA, all LPS-induced biochemical changes ameliorated with a marked protective effect. *In vitro*, the reactive oxygen species (ROS) levels in mitochondria increased in LPS-treated osteoblasts, with decreased expression of osteogenic differentiation genes. The CsA, PD98059, and EUK134 presented remarkable protective effects against LPS treatment. CsA effectively enhanced bone remodeling and attenuated oxidative stress caused by LPS via inhibiting ROS/ERK signaling. Taken together, the protective effect of CsA and the inhibitory effect of ERK signaling on the maintenance of mitochondrial function and reduction of ROS levels hold promise as a potential novel therapeutic strategy for inflammatory diseases in bones.

1. Introduction

The alveolar bone is a dynamic and continuously changing organ, with osteogenesis and osteoclasis, similar to bone tissues in other parts of the body [1]. This continuous remodeling can prevent and eliminate fatigue-related microinjuries and allow adaptation of bone mass and structure. Some stimulatory factors can cause quantitative changes in the alveolar bone. For example, a physiological inflammatory response can promote the formation of new blood vessels, enhance the function of fibroblasts, and accelerate the process of healing and repair of bone defects [2]. However, an overactive inflammatory response not only inhibits osteoid calcification and new bone formation in the alveolar bone but also

promotes osteoclast genesis, resulting in osteolysis and bone resorption [3, 4]. It might be accompanied by inflammatory damage to periodontal tissue destruction in some inflammatory diseases such as osteoporosis, atherosclerosis, diabetes, periodontitis, and chronic apical periodontitis, leading to severe alveolar bone defects that subsequently cause tooth loosening and loss [5, 6]. Controlling inflammation in the alveolar bone might help improve and preserve alveolar ridge height and width.

Cyclosporine A (CsA) was first used to prevent organ rejection after clinical transplantation as a potent immunosuppressant [7]. Later, CsA was regarded as an immunoregulatory and anti-inflammatory drug [8, 9]. Many studies [10, 11] found that CsA inhibited mitochondrial permeability transition pore

(mPTP) opening. Enhanced reactive oxygen species (ROS) levels promote the opening of mPTP and reduce the mitochondrial membrane potential (MMP), leading to irreversible mitochondrial dysfunction. In the pathogenesis of some inflammatory diseases, the opening of mPTP induces mitochondria to release a large number of ROS and calcium ions and then cause damage to cell and mitochondrial functions by activating downstream apoptotic signaling pathways [12, 13]. According to recent studies, CsA could protect cells against $CoCl_2$-induced hypoxic injury and was a potential therapeutic agent for cardiac hypoxic injury [14], and CsA treatment considerably reduced the H_2O_2-induced intracellular generation of ROS [15]. CsA might be developed as a free radical scavenger, but its regulatory mechanism remains unclear. Moreover, the effect of CsA on the healing of bone defect in an inflammatory environment has not been studied.

An increasing number of studies showed that elevated ROS levels could cause oxidative stress-related osteogenesis damage, such as bone remodeling disorders in diabetes, osteoporosis, and periodontitis. Increased levels of oxidative stress impede the proliferation and differentiation of osteoblasts and accelerate cell death. During the pathogenesis of chronic periodontitis, neutrophils produce a large number of ROS in the form of respiratory bursts to kill pathogenic bacteria, while excessive ROS production accelerates cellular senescence, amplifies the inflammatory response, and destroys periodontal tissues [16]. Proinflammatory factors can affect the function of osteoblasts, enhance inflammatory-related bone destruction, and then interfere with the bone remodeling process. Besides, some studies revealed that ROS-mediated oxidative stress was closely related to bone destruction during inflammation [17, 18]. A large number of studies have confirmed that osteoblast apoptosis was related to ROS enhancement. Excessive ROS production caused by external or internal pathways inevitably damaged the lipids and proteins of osteoblasts, leading to fatal cell damage and programmed cell death [19, 20].The normal level of ROS is very important for maintaining bone formation and reconstruction. Whether the ROS is affected and how it is affected during the pathological process of inflammatory diseases need further exploration.

In view of the effect of CsA on antioxidant stress and the close link between ROS and bone remodeling, we hypothesized that CsA promotes the remodeling of alveolar bone defects in the environment of inflammation by suppressing the level of ROS and improving the function of damaged osteoblasts. Using alveolar bone defect model and osteoblasts, we comprehensively evaluated the consequences of changes with CsA treatment on inflammation-specific bone defects. We further delineated the mechanism by which CsA regulates osteoblast function and oxidative stress. Our investigation provides new insight into the role of CsA in inflammatory bone, highlighting the potential the therapeutic application of CsA.

2. Materials and Methods

2.1. Alveolar Bone Defect Model. A total of 18 male C57BL/6J mice (10 weeks), purchased from the Experimental Animal Center of Sichuan University, were randomly divided into three groups. All mice were reared in the State Key Laboratory of Oral Diseases. The rearing environment (25°C, 55% humidity, and 12 h day and night alternation) was strictly controlled, and water and food were supplied according to the standards. Their weight and health status were all under daily monitoring. The experimental procedures were reviewed and approved by the ethics committee of the State Key Laboratory of Oral Diseases, Sichuan University.

The alveolar bone defect model was prepared as described in previous studies [17, 18]. The mice were acclimated in the new rearing environment for at least 7 days. Four days before extraction surgery (Figure 1), the crown of the left mandibular central incisor was cut at the gingival margin under anesthesia and strict disinfection protocol so that the teeth could be smoothly extracted at a later stage. While cutting the crown of the tooth, the central hair on the skin of mice was shaved, and the surgical area was wiped with 10% povidone iodine and deiodinated with 70% ethanol. The mice in the experimental groups received subcutaneous injections of 10 mg/kg LPS (Sigma–Aldrich Corporation, MA, USA) or phosphate-buffered saline (PBS; Solarbio Corporation, Beijing, China) onto the calvaria. Four days after tooth extraction, the residual root of the left mandibular incisor was extracted under anesthesia and strict disinfection protocol. The mice were fed with a sterile soft diet after the extractions for proper wound healing.

The LPS-treated mice were divided into two groups. The residual root was extracted, and the mice received an intraperitoneal injection of 10 mg/(kg · day) CsA (Sigma–Aldrich Corporation) according to the studies by Chen et al. [21] and Li et al. [22] or ethanol solution for seven consecutive days. The three experimental groups were abbreviated as follows: (1) PBS- and EtOH-injected group (PBS), (2) LPS- and EtOH-injected group (LPS), and (3) LPS- and CsA-injected group (LPS+CsA). The mice were sacrificed on day 21 after tooth extraction.

2.2. Micro-CT Imaging. The mandible bone free of the skin and the outer layer muscle was harvested 21 days after the extraction and fixed with 4% formalin buffer at 4°C for 2 days. Micro-CT scans were taken using micro-CT 50 (Scanco Medical, Switzerland) with a voxel size of 20 μm and an energy setting of 60 kV and 667 mA. RAW images were reconstructed and analyzed using the Scanco Medical Evaluation and Visualizer software. The region of interest (ROI) was defined to cover the newly formed bone area and the total area in the tooth socket, and a total of 30 successive images were selected from ROI for reconstruction and analysis. The bone volume fraction (BV/TV, %) and bone mineral density (BMD, mg/cc) were measured at all time points.

2.3. Hematoxylin–Eosin and Immunohistochemical Staining. The fixed samples were subsequently decalcified for 4 weeks in EDTA–glycerol solution, embedded in paraffin, and sectioned in the coronal plane at a thickness of 4 μm. After deparaffinization, hematoxylin–eosin (HE) staining and immunohistochemical staining were performed. Immunohistochemical staining samples were incubated with anti-

(a)

(b)

(c)

(d)

(e)

(f)
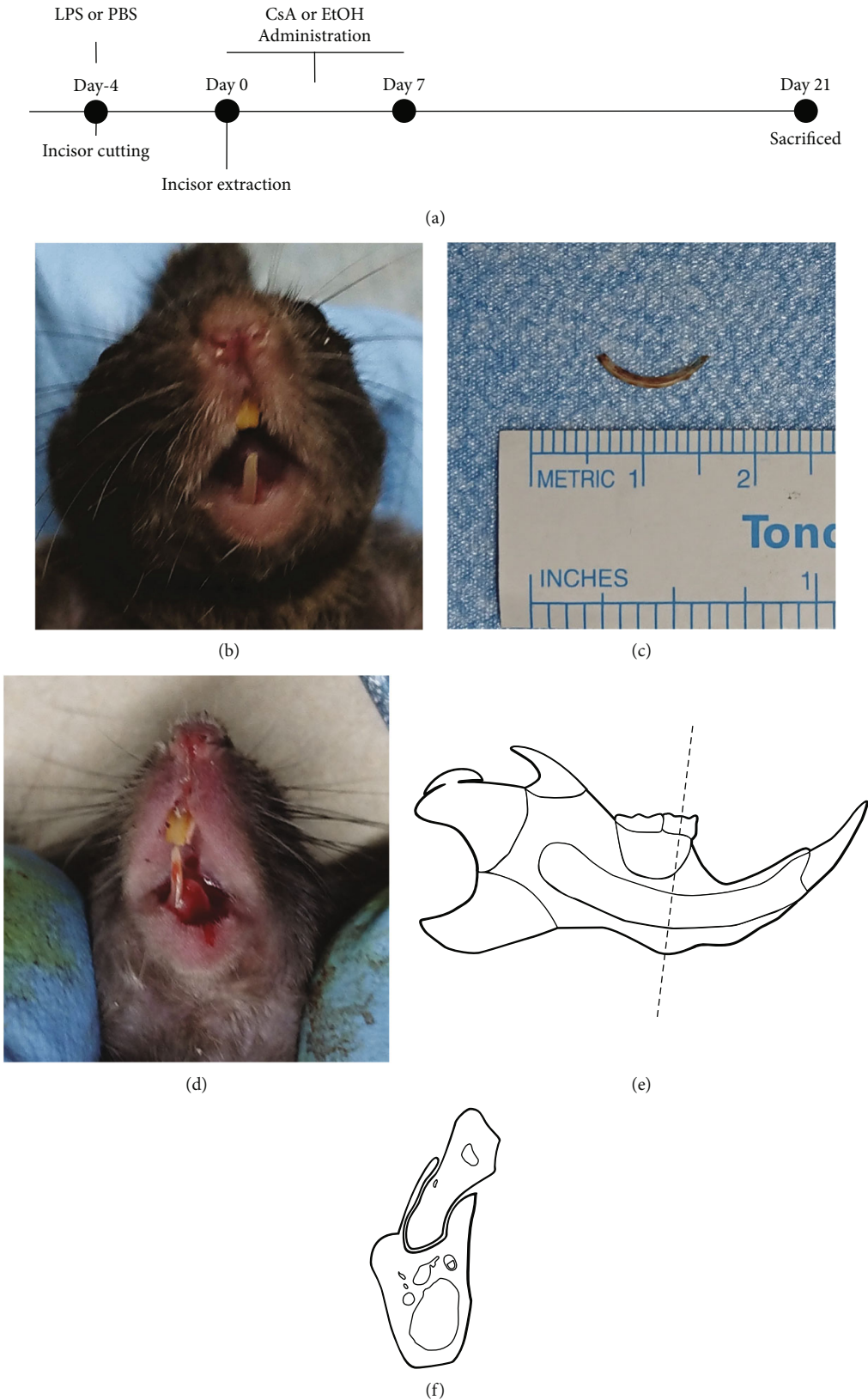
FIGURE 1: The design of *in vivo* study. (a) Time schedule of *in vivo* study. (b) The crown of the mandibular central incisor was cut. (c) The residual root of the left mandibular incisor was lifted out. (d) The alveolar bone defect model. The dotted line of anatomical diagram of (e) mouse mandible indicates the (f) slices that were performed in the area that was set at the first molar.

alkaline phosphatase (ALP) antibody (Abcam, MA, USA; 1 : 2000), anti-osteocalcin (OCN) antibody (Abcam; 1 : 200), anti-osteoprotegerin (OPG) antibody (Abcam; 1 : 200), anti-RANKL antibody (Abcam; 1 : 200), anti-heme oxygenase-1 (HO-1) antibody (Abcam; 1 : 1000), anti-glutathione peroxidase (GPX) antibody (Abcam; 1 : 50), anti-superoxide dismutase 1 (SOD1) antibody (Abcam; 1 : 500), and anti-monocyte chemotactic protein-1 (MCP-1) antibody (Abcam; 1 : 500). Then, the procedures were performed following standard protocols. Quantitative analysis was performed using the ImageJ software (Rawak Software Inc., Germany).

2.4. Cell Culture and Treatment.
Murine osteoblasts were generated as described in previous studies with some modifications [23]. In brief, calvarias from newborn mice were dissected aseptically and treated with 0.1% collagenase and 2.5% trypsin. Six populations (I through VI) were obtained after sequential digestion for ~10, 20, 30, 40, 50, and 60 min, respectively. Populations I and II, containing periosteal fibroblastic cells, were discarded. Populations III and IV, containing osteoblast precursors and/or immature osteoblasts, and populations V and VI, containing mature osteoblasts, were cultured in α-MEM containing 10% fetal bovine serum (FBS), 2 mM glutamine, and 1% penicillin/streptomycin at a final concentration of 100 IU/mL and 100 μg/mL (Life-Technology, CA, USA). This basic medium was replenished every 3 days. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2/95% O_2.

The drugs were prepared as stock solutions and diluted to working concentrations immediately before use according to manufacturers' protocols. The osteoblast cells were treated with or without LPS (100 ng/mL; Sigma–Aldrich Corporation), CsA (1 μM; Sigma–Aldrich Corporation) according to the study by Yeo et al. [24], ERK1/2 inhibitor PD98059 (10 μM, Sigma–Aldrich Corporation), and EUK134 (10 μM, Sigma–Aldrich Corporation) for 24 h in the basic medium or differentiation medium prior to biochemical and molecular assays.

2.5. Cell Viability Assay.
The osteoblast cells were plated at 10^4 cells/well in 96-well plates and cultured under variable conditions as indicated. The cell viability assay was performed using a cell counting kit 8 (CCK-8, Dojindo, Minato-ku, Tokyo, Japan) following the manufacturer's protocol. After LPS stimulation, the cells were transferred to 100 μL of a fresh medium containing 10 μL of reagent mixture and incubated at 37°C for 1.5 h. The plate was gently shaken for 10 s, and the absorbance was measured at 450 nm in a microplate reader (Thermo Scientific, Life Technologies Co., NY, USA).

2.6. Cell Differentiation Assay.
After drug exposure, the cell monolayer was gently scraped off on the ice and lysed by the ultrasound method. After centrifugation at 10,000 rpm for 5 min at 4°C, the supernatant was used for measuring intracellular ALP activity using an ALP activity assay kit (Jiancheng Bioengineering Institute, Nanjing, China). The absorbance of the reactive volume was detected at 520 nm.

For ALP staining assay, the osteoblast cells were plated at 5×10^5 cells/well in 24-well plates and stimulated with a differentiation medium for 1 week. ALP staining was performed using a standard protocol. Briefly, the cells were gently washed with 1% PBS twice and fixed with 4% paraformaldehyde for 10 min. After incubation with 4% paraformaldehyde (PFA), the cells were gently washed with 1% PBS twice and subjected to ALP staining reagent (Sigma–Aldrich Corporation) following the manufacturer's protocol. The staining results were captured using a digital camera (Canon 60D; Canon, Tokyo, Japan).

Regarding the mineralization assay, the cells were seeded at 10^5 cells/well in 12-well plates and stimulated with an osteogenic differentiation medium and indicated reagents for 3 weeks. The medium was replenished every 3 days. For detecting osteogenic differentiation, bone nodule formation was examined using Alizarin red staining. Briefly, the cells were fixed with 4% PFA for 30 min and washed with PBS three times. Alizarin red solution 2%, pH 4.1–4.3, was added to the cells for 30 min at 37°C and then washed with PBS.

2.7. Mitochondrial Function.
The cells were seeded in 24-well plates at a density of 10^5 cells/well. For mitochondrial ROS determination, the cells were costained with 2.5 μM Mito-SOX Red (Molecular Probes) and 150 nM Mitotracker Green (Molecular Probes) for 30 min at 37°C. For determining MMP, the cells were costained with 150 nM tetramethylrhodamine methyl ester (TMRM, Molecular Probes) and 150 nM Mitotracker Green (Molecular Probes) for 30 min at 37°C. After staining, the cells were gently washed with 1× PBS and photographed using an Olympus IX71 fluorescent microscope (400x magnification). The excitation wavelength was 543 nm for MitoSOX and TMRM and 488 nm for Mitotracker Green. The ImageJ software was used for quantification and measurement of fluorescent intensity.

For measuring adenosine triphosphate (ATP) levels, the cells were plated at 10^3 cells/well in 96-well plates, and the ATP level was detected using an ATP assay kit (Millipore) after drug treatment. Subsequently, the cells were treated with 100 μL of nucleotide-releasing buffer for 5 min at room temperature with gentle shaking, 1 μL of ATP-monitoring enzyme was added into the cell lysate, and the sample was read for 1 min in a microplate reader (Thermo Scientific Varioskan Flash).

The mitochondrial respiration complex activity was measured in osteoblast homogenates as described in previous studies [25, 26]. Briefly, the cells under different culture conditions were harvested, homogenized, and sonicated in the isolation buffer (pH 7.2) containing 225 mM D-mannitol, 75 mM sucrose, 2 mM K_2HPO_4, and 5 mM HEPES. Further, 10–50 μg mitochondrial fractions were used for complex activity assay. In a previous report [27], ROS significantly decreased complex III (ubiquinol cytochrome c oxidoreductase) activities in osteoblasts. Therefore, the activity of this respiratory chain component was measured.

2.8. Quantitative Real-Time Polymerase Chain Reaction and Western Blot Analysis.
Total RNA was extracted with the RNA extraction kits (TaKaRa MiniBEST Universal, Takara

TABLE 1: Primer sequences for real-time qPCR analysis of the mRNA expression.

Genes	Primers ($5'$–$3'$)	
Runx2	F: CCCAGCCACCTTTACCTACA	R: TATGGAGTGCTGCTGGTCTG
ALP	F: CCAACTCTTTTGTGCCAGAGA	R: GGCTACATTGGTGTTGAGCTTTT
OPG	F: TTACCTGGAGATCGAATTCTGCTTG	R: GTGCTTTCGATGAAGTCTCAGCTG
RANKL	F: GCAGCATCGCTCTGTTCCTGTA	R: CCTGCAGGAGTCAGGTAGTGTGTC
OCN	F: GGAGGGCAATAAGGTAGTGAACAG	R: ATAGCTCGTCACAAGCAGGGT
COL-I	F: TGACTGGAAGAGCGGAGAGTA	R: GACGGCTGAGTAGGGAACAC
HO-1	F: GCTGGTGATGGCTTCCTTGTA	R: ACCTCGTGGAGACGCTTTACAT
SOD1	F: ATGTGACTGCTGGAAAGGACG	R: CGCAATCCCAATCACTCCAC
GPX	F: CCAGGAGAATGGCAAGAATGA	R: GGAAGGTAAAGAGCGGGTGA
MCP-1	F:GCAGGTCCCTGTCATGCTTCT	R:TGTCTGGACCCATTCCTTCTTG
GAPDH	F: ACTTTGTCAAGCTCATTTCC	R: TGCAGCGAACTTTATTGATG

F: forward; R: reverse.

Bio Inc., Shiga, Japan), and the concentration and purity of extracted RNA were evaluated using a NanoDrop spectrophotometer (Thermo Scientific, USA). After synthesizing cDNA, polymerase chain reaction (PCR) was performed with a real-time PCR system (Applied Biosystems, USA) using SYBR Premix Ex Taq II (TaKaRa MiniBEST Universal). The primer sequences are listed in Table 1. The expression of detected genes was analyzed using the $2^{-\Delta\Delta CT}$ method. The Western blot procedures used were described in a previous study [28]. Primary antibodies used included anti-ERK, anti-p-ERK, and anti-β-actin (Abcam, Cambridge, MA).

2.9. Statistical Analysis. All assays were repeated in three independent experiments. Data were presented as mean ± standard deviation. Significance was determined using the Student t-test for pairwise comparison and one-way analysis of variance with the Bonferroni posttest for multiple comparisons using the GraphPad Prism 7.0 software (GraphPad Software, Inc., CA, USA). A P value <0.05 indicated a statistically significant difference.

3. Results

3.1. CsA Attenuated LPS-Induced Osteoblast Dysfunction. Excessive inflammation can cause cytotoxicity, interfere with cell growth, and induce cell apoptosis. Cytotoxicity was measured using the CCK-8 assay to determine the toxicity profile. As shown in Figure 2(a), the cell viability did not decrease significantly after treatment with 100 ng/mL LPS for 24 h. Also, CsA treatment displayed a nontoxic effect on cells. The survival of cells pretreated with CsA in the presence of LPS significantly increased compared with the survival of LPS-treated cells for 48 and 96 h ($P < 0.05$)(Figure 2(a)), suggesting that CsA suppressed LPS-induced cytotoxicity.

ALP activity and staining and the expression of calcium deposition- and osteogenic differentiation-related genes were determined to assess the effect of CsA on osteoblast differentiation in an inflammation model. CsA significantly enhanced the expression of osteogenic differentiation-related genes (Runx2, COL-I, OCN, and OPG) compared

with that in the group treated with LPS alone (Figures 2(b)–2(e)). When osteoblasts were pretreated with CsA in the presence of LPS, the cellular ALP activity, which was one of the major osteoblast differentiation markers, significantly increased. Consistent with ALP activity assay, the results of ALP staining showed that that the expression of ALP-positive cells was significantly less in the presence of LPS than in controls; however, CsA promoted the expression of ALP (Figures 2(f) and 2(g)). Only slight bone nodule formation was observed in the cells exposed to LPS; however, CsA showed a significant recovery effect on LPS-induced mineralization (Figure 2(h)). These data indicated that CsA attenuated LPS-induced osteoblast dysfunction.

3.2. CsA Attenuated LPS-Induced Osteoblast Mitochondrial Dysfunction. ROS are natural by-products produced during cell metabolism. However, under some pathological conditions such as inflammation, the production and accumulation of ROS increase significantly and cause serious damage to the cellular structure. ROS can participate in mediating osteoblast dysfunction caused by LPS by regulating related signaling pathways [29, 30]. Increased ROS levels in mitochondria can interfere with the normal mitochondrial functions, such as decreased MMP, inhibited oxidative respiratory chain function, and decreased ATP production [31, 32]. MitoSOX Red, a highly specific dye, was used to identify and label ROS in mitochondria so as to evaluate mitochondrial function more carefully. A specific fluorescent dye TMRM and kits were used to measure inner MMP, ATP levels, and complex III activity. The TMRM staining results showed that the staining intensity decreased in the LPS group compared with the vehicle control group (Figures 3(a) and 3(b)) and returned to normal after CsA treatment. In the vehicle control group, cells showed weak red fluorescence in the mitochondria. However, the red fluorescence intensity in the mitochondria increased significantly in the LPS group compared with the vehicle control group. Also, the red fluorescence intensity in the mitochondria significantly reduced after CsA treatment (Figures 3(c) and 3(d)). Moreover, complex III activity and ATP production followed the same trend. They decreased after LPS treatment

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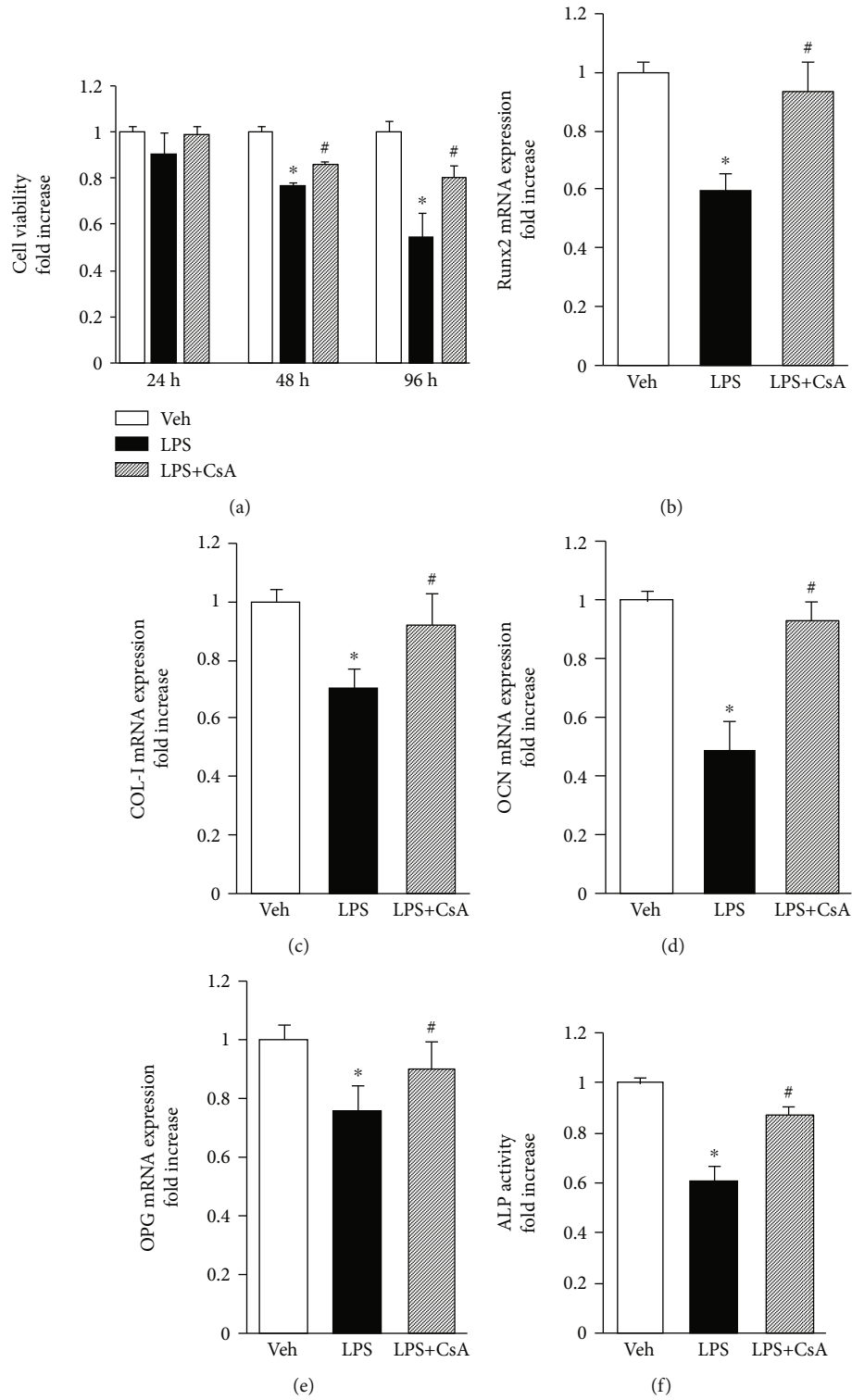
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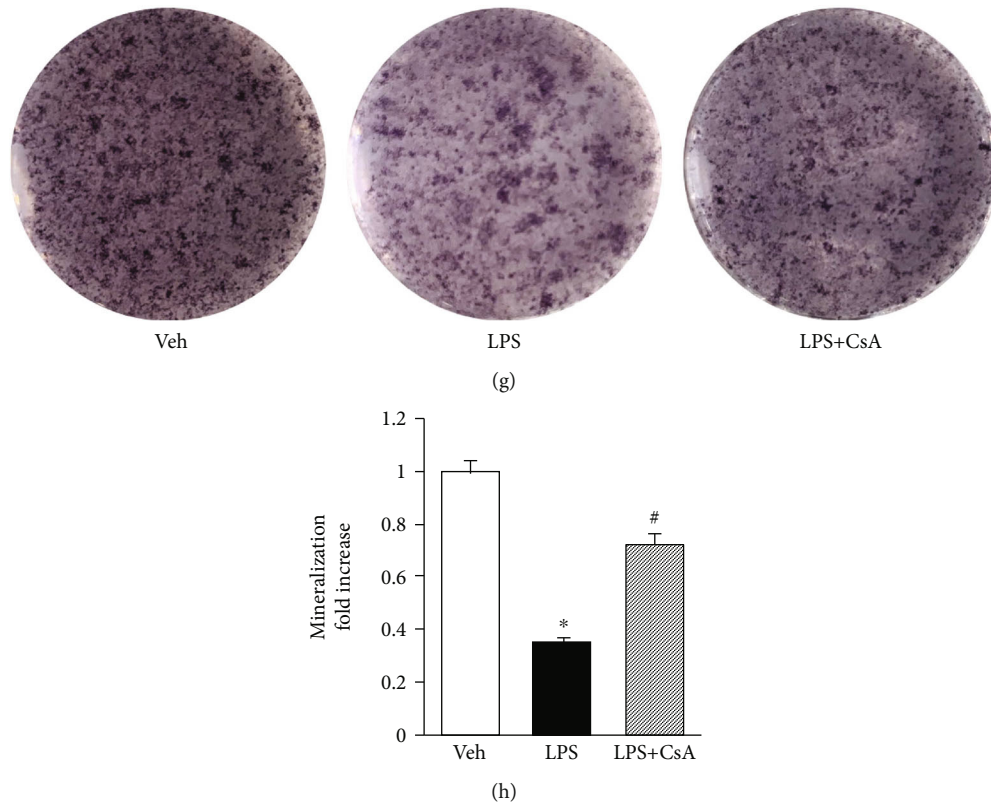
Figure 2: CsA attenuated LPS-induced osteoblasts dysfunction. (a) Cell viability evaluated by the CCK-8 assay at 24, 48, and 96 h, $n = 6$. Relative expression of (b) Runx2, (c) COL-I, (d) OCN, and (e) OPG detected at 24 h by RT-qPCR analysis, $n = 3$. (f) ALP activity was detected by the ALP assay, $n = 3$. (g) ALP staining after an osteogenic induction of 7 days, $n = 3$. (h) Quantification of mineralization nodules in different groups, $n = 3$. $^*P < 0.05$ vs. PBS group; $^{\#}P < 0.05$ vs. LPS group.

and increased to a normal level under the action of CsA (Figures 3(e) and 3(f)). Besides the changes in ROS levels in mitochondria, this study found that LPS could interfere with oxidative metabolism balance in osteoblasts, leading to mitochondrial dysfunction. Moreover, CsA could protect cells from LPS-induced oxidative stress.

3.3. CsA Promoted Bone Formation in Alveolar Bone Defects under an LPS Environment. This study is aimed at exploring the effect of CsA on the healing of bone defect areas in an inflammatory environment in vivo. Therefore, the morphological examination and detection of the expression of bone formation-related proteins were performed. Micro-CT three-dimensional scanning revealed that the bone formation was significantly better in the LPS+CsA group than in the LPS group (Figure 4(a)). The BMD and BV/TV values significantly increased in the LPS+CsA group than in the LPS group and were even better than those in the PBS group (Figures 4(b) and 4(c)). The HE staining results showed a large amount of tissue fragments and necrotic bone tissue in the LPS-treated group, with no obvious granulation tissue. Granulation-like tissues, similar to those in the PBS group, and a few thinner bones were found in the LPS+CsA group (Figure 4(d)). The aforementioned results indicated that inflammation was not conducive to the bone remodeling process in the alveolar defect. The immunohistochemical

staining showed that the staining intensity and mRNA expression levels of runt-related transcription factor 2 (Runx2), ALP, OCN, and OPG were significantly higher in the LPS+CsA group than in the LPS group (Figures 4(e)–4(g)). However, the expression of RANKL was significantly inhibited after cotreatment with CsA (Figures 4(e)–4(g)). The present study supported that inflammation was negative to bone formation in alveolar bone defects, and CsA could promote bone formation in alveolar bone defects.

3.4. CsA Effectively Suppressed LPS-Induced Oxidative Stress Status. This study examined the effect of LPS and CsA on the oxidative stress of alveolar bone defects in vivo by determining the expression of oxidative stress-related proteins. A previous study showed that the products produced by HO-1 by catalyzing the degradation of heme caused oxidative stress damage [33]. The activity of HO-1 had a direct impact on the ability to tolerate oxidative stress. SOD1 and GPX constitute a primary defense against oxidative stress; they are involved in regulating mitochondrial ROS [34–36]. The production of ROS is concomitant with oxidative stress, which is exacerbated by the excessive release of cytokines, such as MCP-1, which is an activator of monocytes and can induce oxidative burst [37]. Therefore, the expression of HO-1, SOD1, GPX, and MCP-1 in the alveolar socket was analyzed in this study. Then, whether LPS caused oxidative

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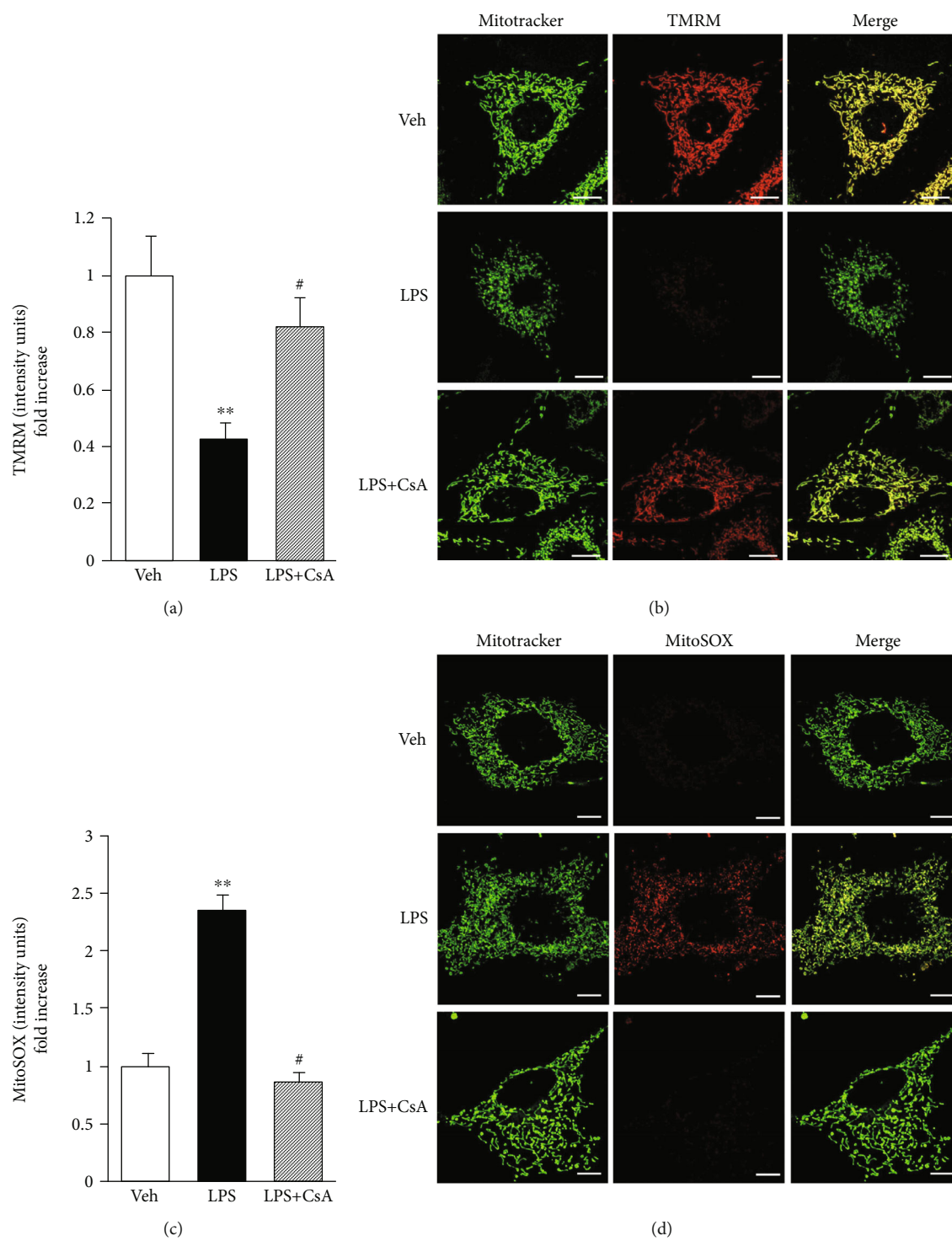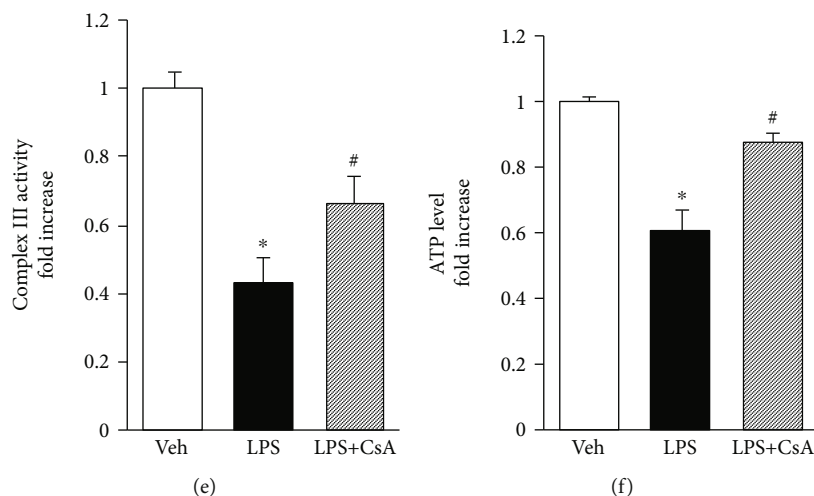
FIGURE 3: CsA attenuated LPS-induced OB mitochondrial dysfunction. (a) TMRM staining intensity, $n = 3$. (b) Representative images of TMRM staining. (c) MitoSOX staining intensity, $n = 3$. (d) Representative images of MitoSOX staining. (e) Complex III activity was detected by complex activity assay, $n = 3$. (f) ATP production was detected by an ATP assay kit, $n = 3$. Scale bar = 5 μm. $^{**}P < 0.01$ vs. PBS group; $^{*}P < 0.05$ vs. PBS group; $^{#}P < 0.05$ vs. LPS group.

stress in the alveolar bone defect area and whether CsA alleviated oxidative stress were explored. The immunohistochemical staining results showed that the HO-1 staining intensity was significantly higher in the LPS treatment group than in the PBS treatment group (Figure 5(a)). The expression of HO-1 increased by about 70% in the LPS group compared with the PBS group (Figure 5(b)). After the action of CsA, the staining intensity and the protein and mRNA levels of HO-1 and MCP-1 in the alveolar socket significantly decreased (Figures 5(a)–5(c)). The concentrations of SOD1 and GPX decreased in the LPS group compared with the PBS group (Figure 5(b)). Also, the concentrations and mRNA levels of SOD1 and GPX increased after CsA treatment (Figures 5(b) and 5(c)). LPS increased the level of oxidative stress, which was relieved after CsA treatment.

3.5. Antioxidant (EUK134) Reversed LPS-Induced Osteoblast Dysfunction and Mitochondrial Dysfunction.

Since mitochondrial oxidative stress is important in osteoblast dysfunction, the study tested whether antioxidant treatment could recover the LPS-induced osteoblast dysfunction. EUK134, an antioxidant, is a biomimetic of SOD2 and catalase. As shown in Figure 6, the osteoblasts treated with EUK134 in the presence of LPS significantly inhibited LPS-induced toxic effect on cell viability (Figure 6(a)) and increased cellular ALP activity (Figure 6(b)), ALP expression (Figure 6(c)), and bone nodule formation (Figure 6(d)). Moreover, EUK134 supplementation significantly enhanced the expression of osteogenic differentiation genes (Runx2, COL-I, OCN, and OPG) compared with that in the group treated with LPS alone (Figure 6(e)). The results demonstrated that the antioxidative treatment attenuated LPS-induced osteoblast dysfunction.

Considering increased mitochondrial ROS in LPS-treated osteoblasts, LPS was an important contributor of oxidative stress to mitochondrial dysfunction. The study next determined whether antioxidant treatment could rescue the altered mitochondrial function. The results showed that deficits in the membrane potential (Figures 6(f) and 6(g)) and complex III activity (Figure 6(h)) in the osteoblast inflammation model were reversed following treatment with EUK134. Moreover, EUK134 administration suppressed ATP production in LPS-treated osteoblasts (Figure 6(i)). Collectively, the data indicated that EUK134 conferred protective effects on mitochondrial function relevant to LPS-derived mitochondrial toxicity in osteoblasts.

3.6. ERK1/2 MAPK Was Involved in LPS-Induced Osteoblast Dysfunction.

Mitogen-activated protein kinase (MAPK) signaling pathway is known to be involved in both oxidative stress and osteoblast differentiation [38–40]. Moreover, previous studies demonstrated that oxidative stress induced MAPK activation, which was linked to abnormal mitochondrial function [25]. Besides, the inhibition of the MAPK signaling pathway downregulated the expression of inflammatory factors, thereby improving the prognosis of inflammatory diseases [41]. The phosphorylation of MAPK was first analyzed by immunoblotting to determine whether the MAPK signaling pathway was also involved in LPS-induced osteoblast dysfunction. A significantly increased ERK1/2 phosphorylation was observed when osteoblasts were stimulated with LPS. However, the addition of CsA (Figure 7(a)), EUK134 (Figure 7(b)), and PD98059 (ERK pathway inhibitor) (Figure 7(c)) largely abolished ERK1/2 phosphorylation. The total ERK1/2 level did not significantly change under each condition. These results demonstrated that CsA attenuated LPS-induced osteoblast dysfunction through ERK signaling transduction.

3.7. ERK1/2 Inhibitor Rescued LPS-Induced Osteoblast Dysfunction and Mitochondrial Dysfunction.

The study evaluated whether the ERK1/2 inhibitor could recover LPS-

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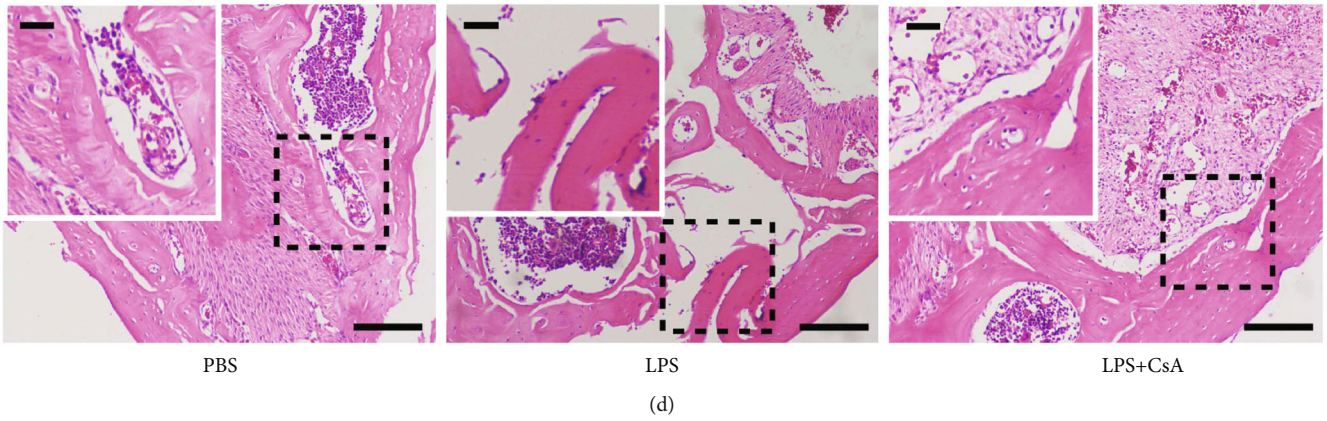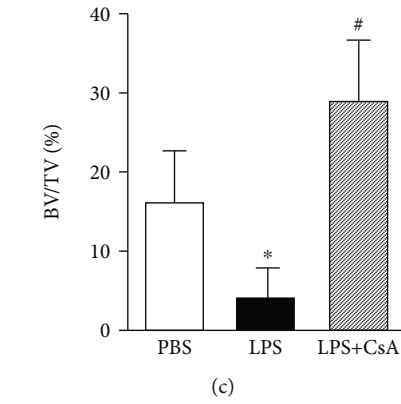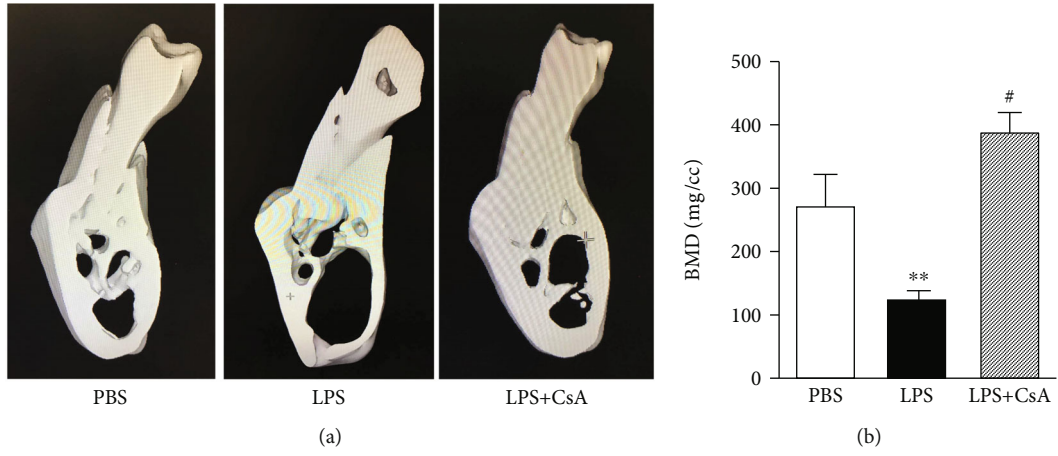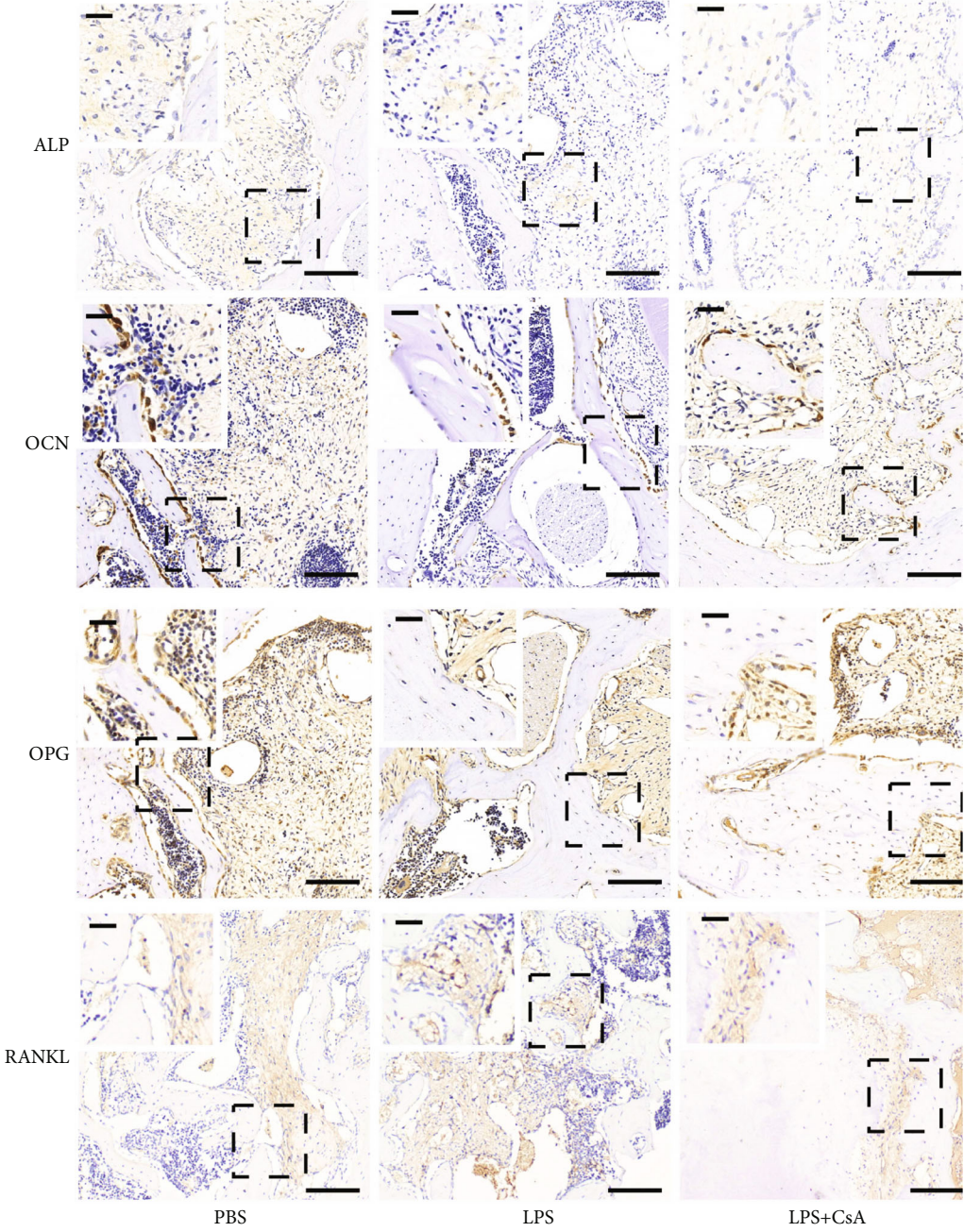
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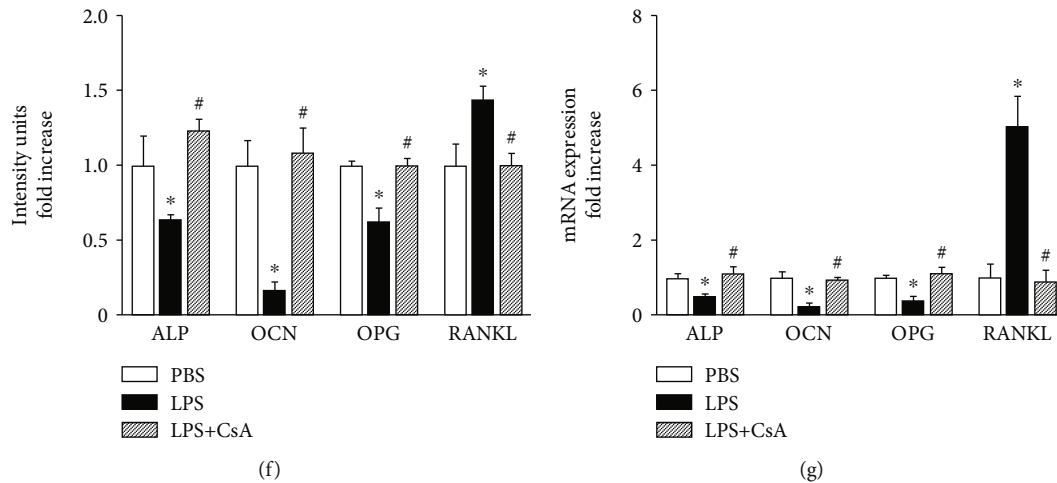
FIGURE 4: CsA promotes bone formation under an LPS environment *in vivo*. (a) Micro-CT of alveolar bone defect. (b) BMD results, $n = 6$. (c) BV/TV results, $n = 6$. (d) HE staining. (e) Immunohistochemistry staining of ALP, OCN, OPG, and RANKL. (f) IOD values in each group, $n = 6$. (g) Relative expression of ALP, OCN, OPG, and RANKL in the alveolar bone defect area by RT-qPCR analysis, $n = 6$. The longer scale bar = 100 μm, and the shorter scale bar = 20 μm. $^{**}P < 0.01$ vs. PBS group; $^{*}P < 0.05$ vs. PBS group; $^{\#}P < 0.05$ vs. LPS group.

induced osteoblast dysfunction to test the important role of the ERK signaling pathway in the oxidative stress model. PD98059 administration significantly reduced the LPS-induced toxic effect on cell viability (Figure 8(a)). It also enhanced cellular ALP activity (Figure 8(b)), ALP expression, and bone nodule formation (Figures 8(c) and 8(d)). Moreover, EUK134 supplementation significantly enhanced the expression of osteogenic differentiation-related genes (*Runx2*, *COL-I*, *OCN*, and *OPG*) compared with that in the group treated with LPS alone (Figure 8(e)). Then, the study determined whether the ERK1/2 inhibitor could rescue the altered mitochondrial function. The results showed that the deficits in the membrane potential (Figures 8(f) and 8(g)) and complex III activity (Figure 8(h)) in the osteoblast inflammation model reversed following treatment with PD98059. Moreover, PD98059 administration suppressed ATP production in the LPS-treated osteoblasts (Figure 8(i)). These results indicated that the disruption of ERK1/2 expression in osteoblasts might be responsible for abnormal osteoblast and mitochondrial functions induced by LPS.

4. Discussion

CsA is a cyclic neutral hydrophobic peptide and a third-generation immunosuppressant. It is often used to treat auto-immune diseases and improve the survival rate of patients after organ transplantation and the survival rate of transplanted organs. Early reports on the role of CsA in bone mineral density were derived from clinical observations, which noted that treatment with CsA and another immunosuppressive drug FK506 was associated with an increased incidence of fractures [42]. However, CsA monotherapy has been reported to significantly increase lumbar vertebral bone density in renal transplant patients [43]. In our study, we studied on the effects of CsA in an alveolar bone defect mouse model under inflammation and investigated whether and how CsA regulates osteogenic function and mitochondrial function

during inflammation using murine osteoblasts. We comprehensively evaluated the oxidative stress, mitochondrial function, osteogenic function, and bone formation *in vivo* and *in vitro*.

Bone remodeling mainly comprises osteoblast-mediated bone formation and osteoclast-mediated bone resorption [16]. This process is strictly regulated by a series of related transcription factors, such as Runx2, BMP-2, and RANKL [1]. Runx2 is an important regulator of osteoblast differentiation. Although Runx2-/- skull cells can normally express BMP-2, osteogenic differentiation cannot be completed *in vivo* and *in vitro* [33]. Runx2 overexpression can accelerate the early differentiation of osteoblasts. The results of the present study showed that Runx2 expression was significantly inhibited in the LPS treatment group. After CsA treatment, the expression level of Runx2 increased significantly. Unlike Runx2, RANKL is an important regulatory factor that promotes osteoclast formation and induces osteoclast differentiation. The overexpression of RANKL in osteoblasts can enhance osteoclast activity and promote bone resorption [44]. In the present study, the expression of RANKL in the alveolar bone defects and osteoblasts was significantly upregulated after LPS treatment, while it was downregulated after treatment with CsA and was close to normal physiological levels. Previous study demonstrated that CsA significantly inhibited the dexamethasone-induced decrease in MMP and cell viability reduction in primary cultured osteoblasts [45]. Some studies suggested that CsA might reduce the risk of glucocorticoid-induced osteopenia in patients undergoing transplantation not only by serving as a glucocorticoid-sparing agent but also transiently stimulating bone formation more than bone resorption [43, 46]. The study indicated that LPS correlated with a reduction in cellular ALP activity, a decrease in calcium mineralization, and reduced production of osteogenic genes, which was consistent with previous results showing that the cytotoxicity of LPS resulted in osteoblast dysfunction. In brief, our study showed that LPS treatment suppressed both the expression of early osteogenic

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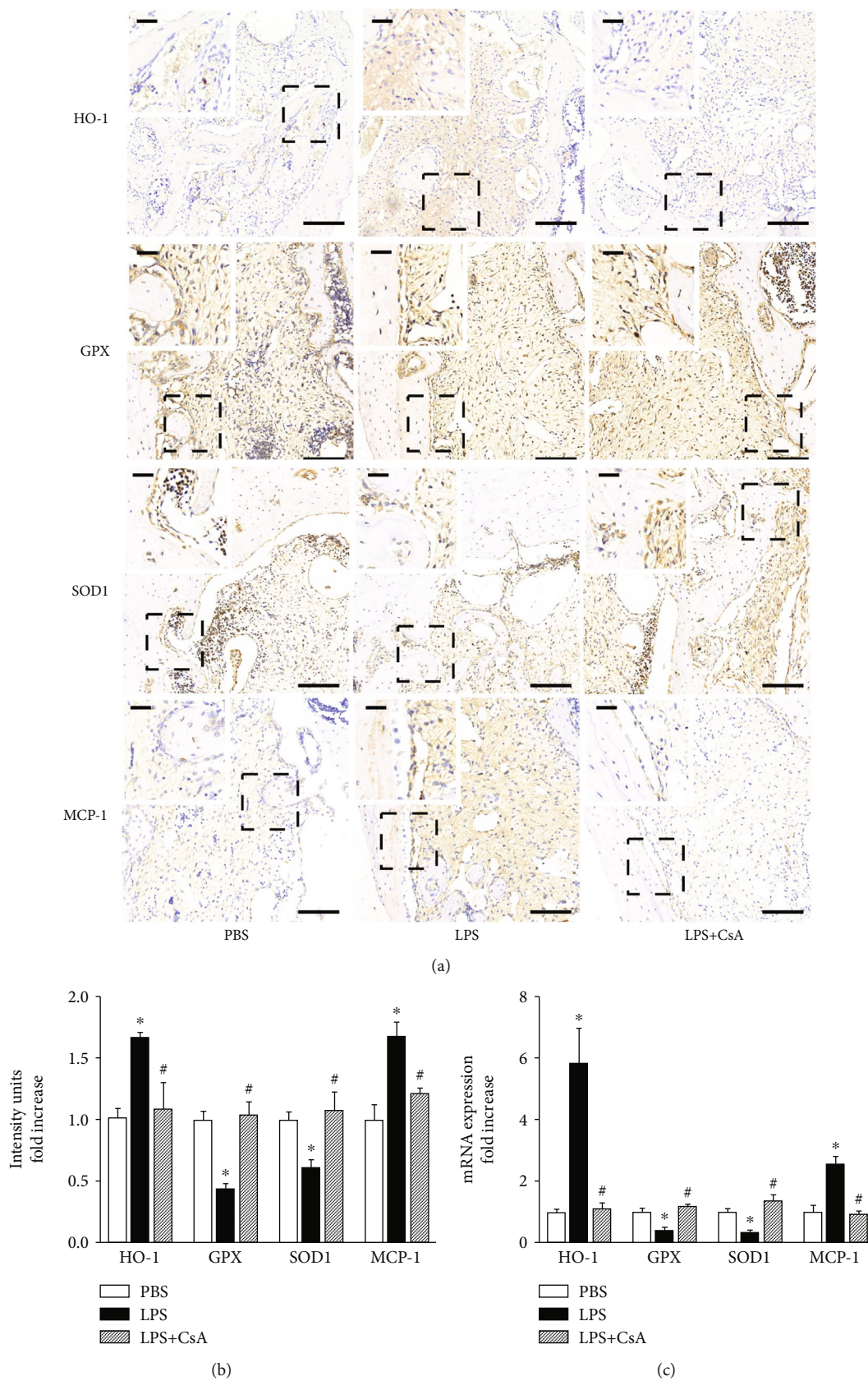
Figure 5: CsA suppressed oxidative stress under an LPS environment *in vivo*. (a) Immunohistochemistry staining of HO-1, GPX, SOD1, and MCP-1. (b) IOD values in each group, $n = 6$. (c) Relative expression of HO-1, GPX, SOD1, and MCP-1 in the alveolar bone defect area by RT-qPCR analysis, $n = 6$. The longer scale bar = 100 μm, and the shorter scale bar = 20 μm. $*P < 0.05$ vs. PBS group and $\#P < 0.05$ vs. LPS group.

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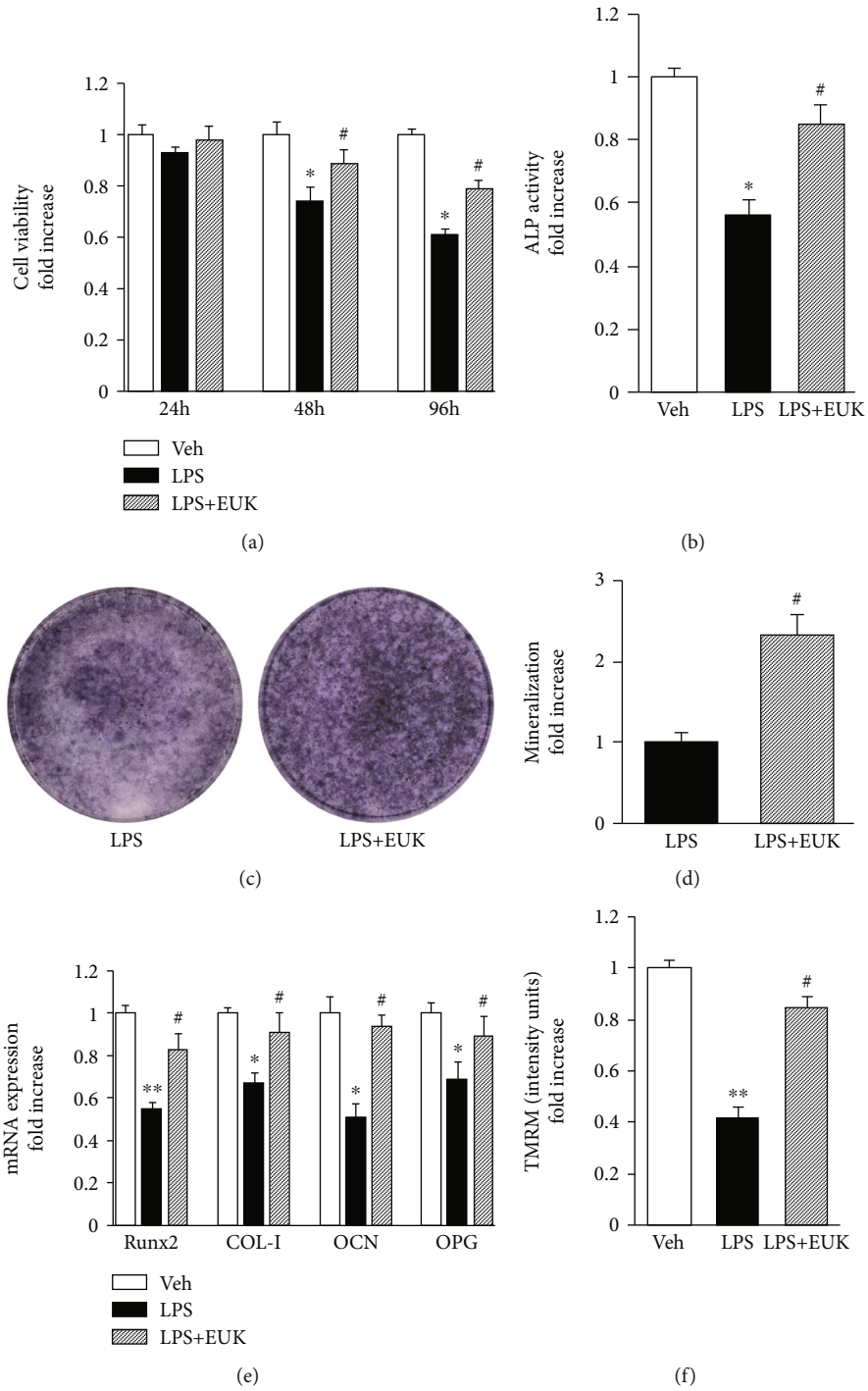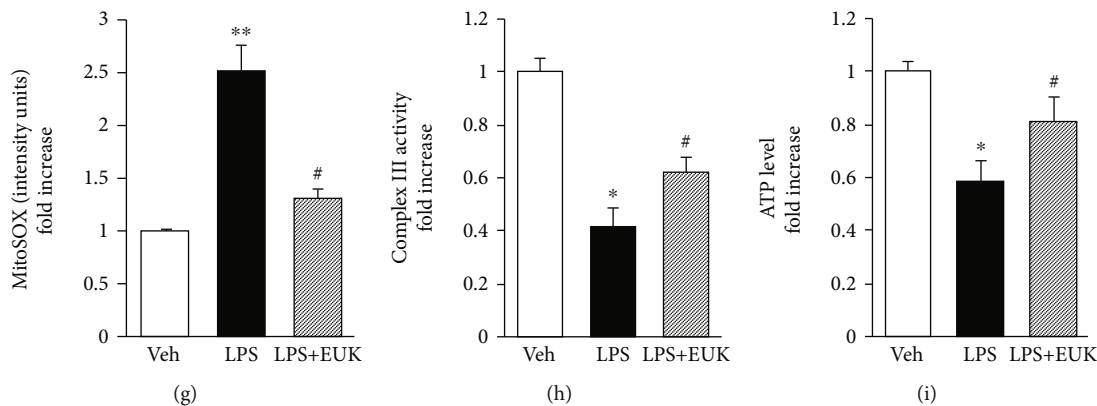
FIGURE 6: Antioxidant (EUK134) reversed LPS-induced OB dysfunction and mitochondrial dysfunction. (a) Cell viability evaluated by the CCK-8 assay at 24, 48, and 96 h, $n = 6$. (b) ALP activity was detected by the ALP assay, $n = 3$. (c) ALP staining after an osteogenic induction of 7 days, $n = 3$. (d) Quantification of mineralization nodules in different groups, $n = 3$. (e) Relative expression of Runx2, COL-I, OCN, and OPG detected at 24 h by RT-qPCR analysis, $n = 3$. (f) TMRM staining intensity, $n = 3$. (g) MitoSOX staining intensity, $n = 3$. (h) Complex III activity was detected by complex activity assay, $n = 3$. (i) ATP production was detected by an ATP assay kit, $n = 3$. $^{**}P < 0.01$ vs. PBS group; $^{*}P < 0.05$ vs. PBS group; $^{\#}P < 0.05$ vs. LPS group.

markers and ALP activity in murine osteoblasts. We also observed impaired cellular viability in osteoblasts, suggesting that osteogenic activity of osteoblasts was inhibited in an inflammatory environment, while CsA reversed these effects.

In vivo, the present study found that the injection of LPS could inhibit bone formation in the mandibular central incisor tooth extraction site. However, no significant inhibition was observed in the LPS and CsA cotreated groups, and surprisingly, it was even better than that in the PBS group. The BMD and BV/TV values of the alveolar bone defects significantly reduced after LPS treatment. However, when the mice were treated with CsA, the osteogenic damage performance in the alveolar bone defects significantly improved. The results of HE staining showed that bone fragments increased, the number of inflammatory cells increased, and no obvious granulation tissue was seen in the LPS-treated group, indicating that LPS had a strong inhibitory effect on bone healing in the bone defect area. After treatment with CsA, granular tissues similar to those in the PBS group were seen in the alveolar bone defects, indicating that CsA might be involved in bone formation disorders caused by LPS. In published papers, CsA was reported to have acute anti-inflammatory effects in immunocompetent animals [47]. Moreover, CsA has become one of the immunosuppressive drugs widely used for the treatment of ocular inflammation [48]. The aforementioned results indicated that the changes in the expression levels of bone formation-related transcription factors in the alveolar bone defects of mice and in osteoblasts were consistent with the results of the morphological examination, revealing the important regulatory effect of CsA on the remodeling process of alveolar bone defects in inflammation as well as on the osteoblast function.

Considering the relationship among CsA, inflammation, and oxidative stress, we focused on the role of ROS. ROS induced oxidative stress is the main cause of cell damage in many inflammatory diseases. Since excessive ROS production may be an important factor in bone defects under

inflammation, it will be of great significance to study the molecular characteristics of these effects. Mitochondria not only produce ROS but are also the principal target of ROS attacks. Impaired mitochondrial function can lead to increased ROS generation and result in oxidative injury in the cells and tissues. Komarova et al. suggested that mitochondrial activity and the principal pathways of energy metabolism were vital in the osteoblast function. The respiration, ATP production, and transmembrane potential in mitochondria significantly increased during osteoblast differentiation [49]. All these findings indicated that the maintenance of normal mitochondrial function contributed a lot to the normal functioning of osteoblasts. In the present study, LPS resulted in mPTP opening (TMRM decrease). Also, complex III activity and ATP levels decreased, while these adverse effects on mitochondrial function were reversed by CsA treatment. When the ROS levels exceeded the capacity of the cell in general and the mitochondria in particular to scavenge and render themselves harmless, the resulting oxidative stress initiated mitochondrial permeability transition [50], which then in turn potentiated the oxidative stress. In the present study, LPS was found to increase ROS production in mitochondria, as shown by MitoSOX staining. *In vivo*, results from immunohistochemistry showed that the expression of HO-1, an oxidative stress regulator, was increased. And we observed an increased activator of monocytes (MCP-1) and decreased free radical scavenger (GSH and SOD1) in inflammatory mice. CsA can effectively suppressed oxidative stress status and mitochondrial dysfunction induced by LPS.

Mitochondrial dysfunction is a consequence of oxidative damage caused by increased oxidant levels. Therefore, decreasing oxidant generation and oxidative damage should be an effective way to inhibit mitochondrial impairment [51]. In addition, the present study showed that EUK134 supplementation, an antioxidant drug to suppress mitochondrial ROS generation, not only blunted oxidative stress but

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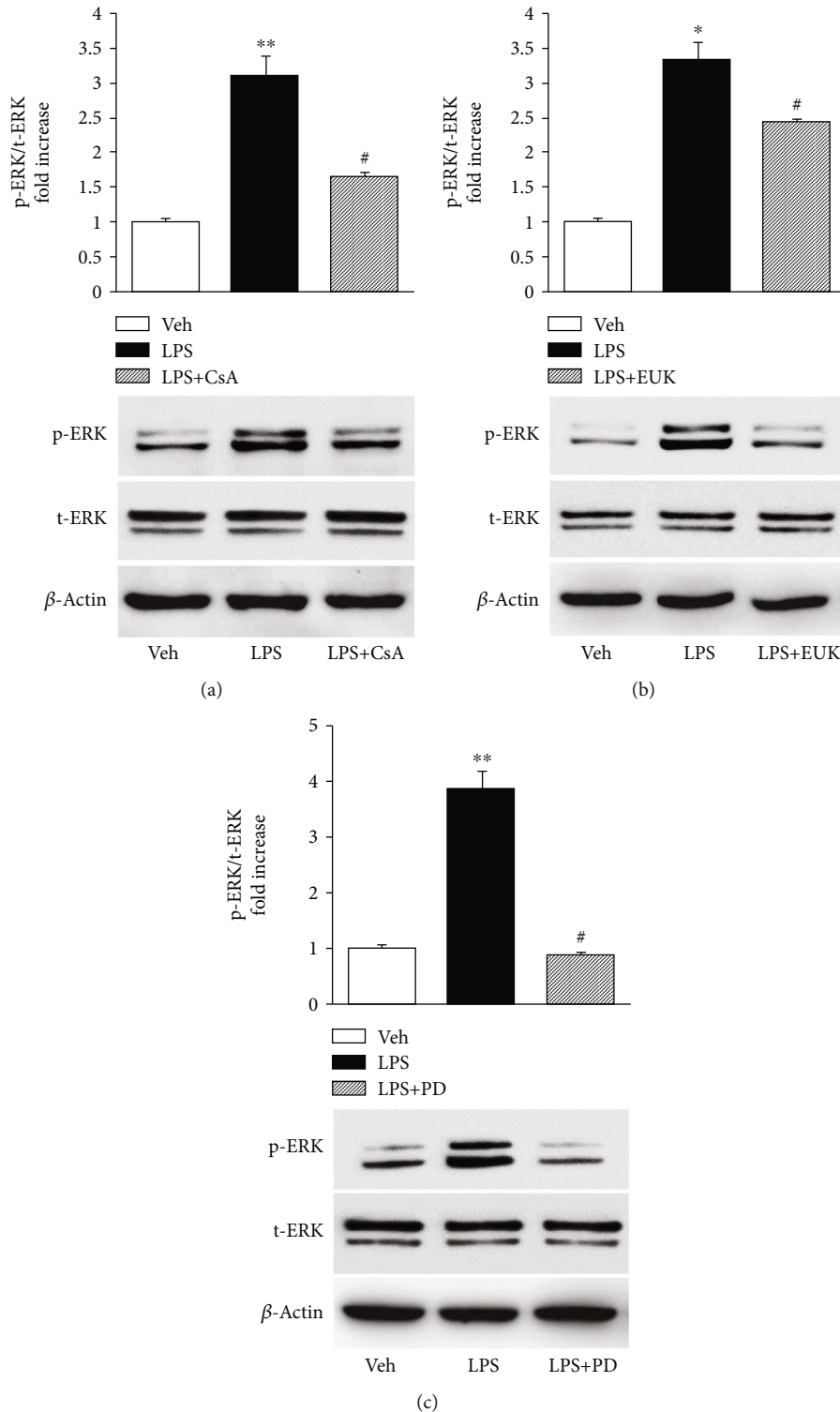
FIGURE 7: Effect of CsA and ERK1/2–MAPK was involved in the LPS-induced osteoblast dysfunction. (a–c) Ratio of phosphorylated ERK to total ERK levels. p-ERK: phosphorylated ERK (1/2); t-ERK: total ERK (1/2); t-ERK remains mostly unchanged. Representative immunoblots are shown in the lower panel. $^{**}P < 0.01$ vs. PBS group; $^{*}P < 0.05$ vs. PBS group; $^{#}P < 0.05$ vs. LPS group.

also significantly rescued mitochondrial dysfunction against LPS toxicity. Furthermore, the oxidative stress in LPS-induced inhibition of osteoblast differentiation was also recovered by EUK134 administration. All these results further confirmed that LPS-induced oxidative stress was cru-

cial in the osteoblast dysfunction, including mitochondrial function and osteogenic effect. However, the mitochondrial ROS level was significantly lower in cells treated with CsA. These data confirmed that CsA could promote ROS clearance or reduce intracellular ROS formation in the cells,

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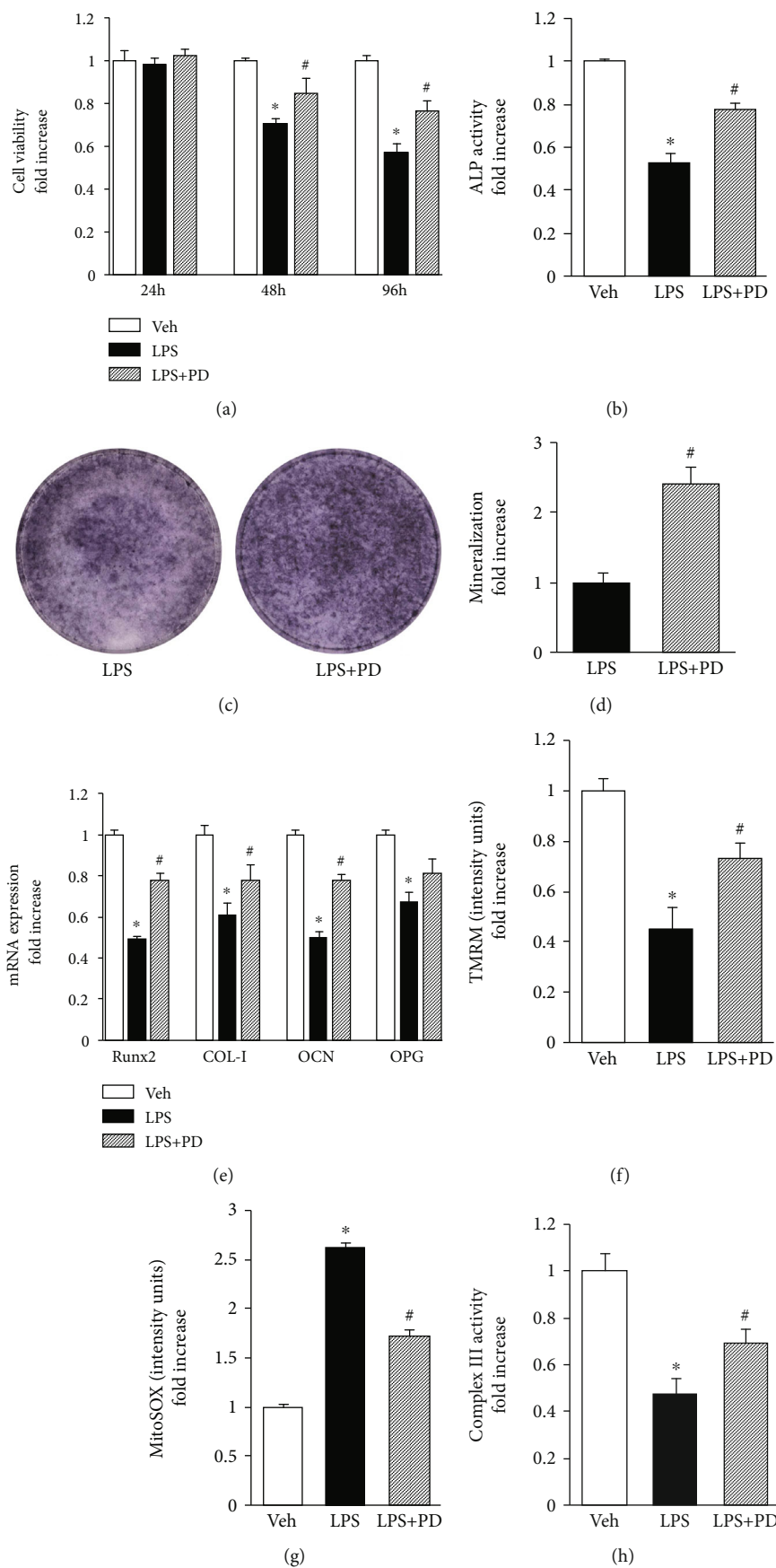
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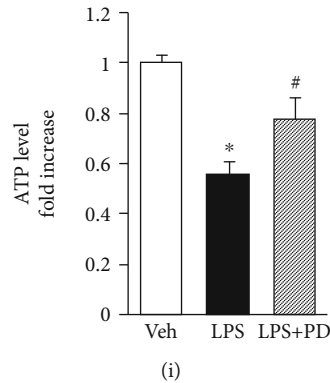
FIGURE 8: ERK1/2 inhibitor attenuated LPS-induced osteoblast dysfunction and mito dysfunction. (a) Cell viability evaluated by the CCK-8 assay at 24, 48, and 96 h, $n = 6$. (b) ALP activity was detected by the ALP assay, $n = 3$. (c) ALP staining after an osteogenic induction of 7 days, $n = 3$. (d) Quantification of mineralization nodules in different groups, $n = 3$. (e) Relative expression of Runx2, COL-I, OCN, and OPG detected at 24 h by RT-qPCR analysis, $n = 3$. (f) TMRM staining intensity, $n = 3$. (g) MitoSOX staining intensity, $n = 3$. (h) Complex III activity was detected by complex activity assay, $n = 3$. (i) ATP production was detected by an ATP assay kit, $n = 3$. $^{*}P < 0.05$ vs. PBS group and $^{#}P < 0.05$ vs. LPS group.
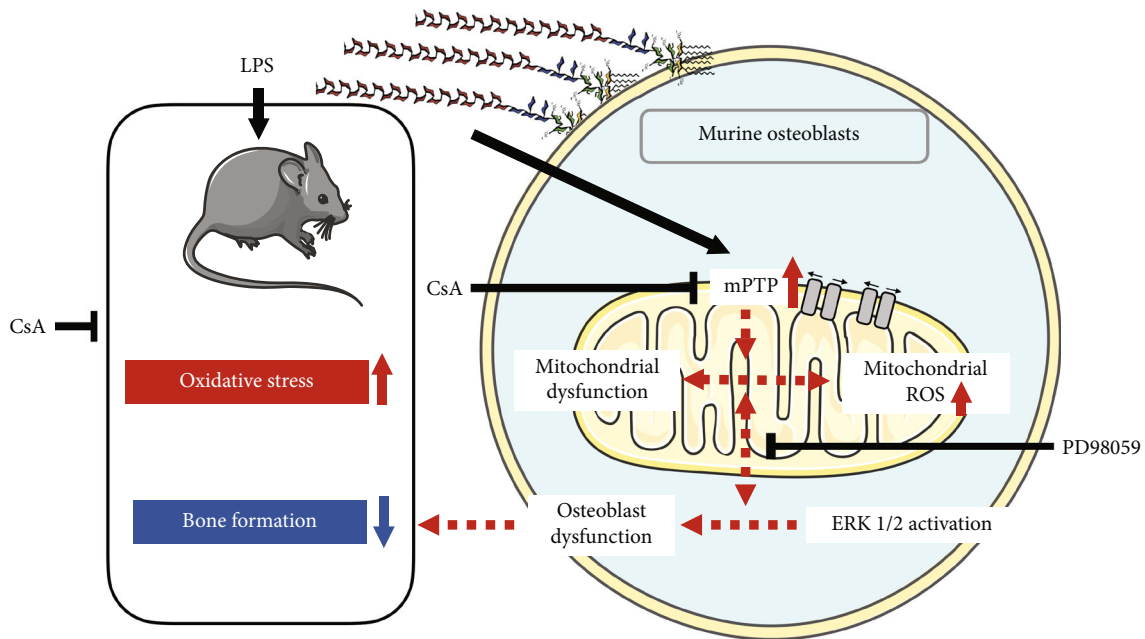
FIGURE 9: Diagram of CsA mechanism of action on bone remodeling in LPS-related inflammation.

suggesting that CsA treatment protected osteoblast cells from LPS-induced inhibition of osteogenic differentiation.

MAPK signaling pathways are known to be involved in both oxidative stress and osteoblast differentiation. The present study demonstrated that the levels of ERK1/2 phosphorylation significantly increased in LPS-treated osteoblasts. In agreement with the present findings, other studies showed that LPS suppressed osteoblastic differentiation of bone marrow progenitors and promoted osteoblast apoptosis or inflammatory osteolysis in an ERK-dependent manner *in vitro* and *in vivo* [52–54]. Moreover, antioxidant EUK134 blocked LPS-induced ERK1/2 activation, indicating a role of LPS-induced oxidative stress in the disruption of signal transduction such as ERK1/2 MAP kinase. Notably, LPS-induced ERK1/2 phosphorylation was blunted in osteoblasts treated with CsA. The addition of the specific ERK1/2 inhibitor (PD98059) resulted in pronounced preservation of mitochondrial function even in the face of LPS insults. Moreover, PD98059 even recovered cellular ALP activity, mineralization, and expression of osteogenic differentiation-related genes inhibited by LPS, suggesting that ERK1/2 was required for oxidative stress-induced inhibition of osteoblastic differentiation in osteoblasts, which was completely coincident with previous findings [38]. All these results indicated the involvement of ROS/LPS-associated ERK1/2 MAPK signaling in the disruption of osteoblast dysfunction. The findings suggested that ERK1/2 was a downstream target of LPS. Thus, it was proposed that LPS-induced inflammation and

impaired ROS production/accumulation in mitochondria are responsible for ERK1/2-MAPK activation, leading to osteoblast dysfunction.

5. Conclusions

In summary, the data provided new insights into the mechanism of inflammation-induced osteoblast dysfunction in the pathogenesis of inflammatory diseases in bones, especially the role of CsA in this model of alveolar bone defect inflammatory cell model. Oxidative stress is an important cause of reduced bone formation in inflammatory bone defects and mitochondrial dysfunction. CsA can effectively enhance bone formation in alveolar bone defects. Oxidative stress caused by inflammation promoted the opening of mPTP and consequently enhanced ROS production/accumulation, thus activating the ERK signal transduction pathway. All these events disrupt the cell viability and osteogenic effect of osteoblasts, ultimately causing cell injury and dysfunction (Figure 9).

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

All animal experiments in this study were conducted in strict accordance with the ARRIVE guidelines for animal experiments and approved by the Research Ethics Committee of West China Hospital of Stomatology.

Conflicts of Interest

The authors declare no competing interests associated with the manuscript.

Authors' Contributions

Yuwei Zhao and Ging Gao contributed equally to this study.

Acknowledgments

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Research Article

The Degree of *Helicobacter pylori* Infection Affects the State of Macrophage Polarization through Crosstalk between ROS and HIF-1α

Ying Lu ⓘ,[1] **Jianfang Rong** ⓘ,[1] **Yongkang Lai** ⓘ,[1] **Li Tao** ⓘ,[2] **Xiaogang Yuan** ⓘ,[3] **and Xu Shu** ⓘ[1]

[1]*Department of Gastroenterology, The First Affiliated Hospital of Nanchang University, No. 17 Yongwaizheng Street, Nanchang, 330006 Jiangxi Province, China*
[2]*Department of Gastroenterology, The Second Affiliated Hospital of Nanchang University, No. 1 Minde Road, Nanchang, 330006 Jiangxi Province, China*
[3]*Department of Gastroenterology, Longgang District People's Hospital of Shenzhen, Shenzhen, China*

Correspondence should be addressed to Xiaogang Yuan; xiaogangyuan@hotmail.com and Xu Shu; jxmushx@126.com

Background and Objective. Helicobacter pylori (*H. pylori*) is involved in macrophage polarization, but the specific mechanism is not well understood. Therefore, this study is aimed at investigating the effects of the degree of *H. pylori* infection on the macrophage polarization state and the crosstalk between reactive oxygen species (ROS) and hypoxia-inducible factor 1 α (HIF-1α) in this process. *Methods*. The expression of CD86, CD206, and HIF-1α in the gastric mucosa was evaluated through immunohistochemistry. RAW 264.7 cells were cocultured with *H. pylori* at various multiplicities of infection (MOIs), and iNOS, CD86, Arg-1, CD206, and HIF-1α expression was detected by Western blot, PCR, and ELISA analyses. ROS expression was detected with the fluorescent probe DCFH-DA. Macrophages were also treated with the ROS inhibitor NAC or HIF-1α inhibitor YC-1. *Results*. Immunohistochemical staining revealed that the macrophage polarization state was associated with the progression of gastric lesions and state of *H. pylori* infection. The MOI of *H. pylori* affected macrophage polarization, and *H. pylori* enhanced the expression of ROS and HIF-1α in macrophages. A low MOI of *H. pylori* promoted both the M1 and M2 phenotypes, while a high MOI suppressed the M2 phenotype. Furthermore, ROS inhibition attenuated HIF-1α expression and switched macrophage polarization from M1 to M2. However, HIF-1α inhibition suppressed ROS expression and inhibited both the M1 phenotype and the M2 phenotype. Inhibition of ROS or HIF-1α also suppressed the activation of the Akt/mTOR pathway, which was implicated in *H. pylori*-induced macrophage polarization. *Conclusions*. Macrophage polarization is associated with the progression of gastric lesions and state of *H. pylori* infection. The MOI of *H. pylori* influences the macrophage polarization state. Crosstalk between ROS and HIF-1α regulates *H. pylori*-induced macrophage polarization via the Akt/mTOR pathway.

1. Introduction

Macrophages play a central role in host defense and the inflammatory response and are significant components of the body's innate and adaptive immune systems [1, 2]. Macrophage polarization is a process by which macrophages respond to microenvironmental signals and stimuli in spe-

cific tissues and acquire specific phenotypes. Polarized macrophages can be functionally divided into two major categories: classically activated macrophages (M1) and alternatively activated macrophages (M2) [3]. M1 macrophages are characterized by the secretion of tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), interleukin- (IL-) 6, IL-1 beta (IL-β), and other chemokines and play

essential roles in the inflammatory response, antitumor response, and promotion of host immunity [4, 5]. M2 macrophages can secrete a great number of anti-inflammatory cytokines: transforming growth factor-β (TGF-β), IL-10, CD206, and arginase-I (Arg-1) [6]. It is generally believed that M1 macrophages perform proinflammatory, bactericidal, and cancer-suppressing functions, while M2 macrophages participate in parasite containment, tumor progression, and tissue remodeling promotion and have immunomodulatory functions [3]. Macrophages maintain a dynamic balance between the M1 and M2 phenotypes in healthy subjects. Once macrophages are extremely skewed toward either the M1 phenotype or the M2 phenotype over time, they can cause the progression of many diseases, such as rheumatoid arthritis, obesity, and cancer [7].

Helicobacter pylori (*H. pylori*), a gram-negative microaerophilic bacterium, infects approximately half of the population worldwide [8]. Long-term infection with *H. pylori* can result in chronic gastritis, peptic ulcers, and gastric adenocarcinoma [9]. *H. pylori* recruits macrophages to the gastric mucosa and induces them to secrete proinflammatory cytokines and chemokines, causing inflammation and damage to the gastric mucosa [10–12]. Several studies have reported the influence of *H. pylori* infection on macrophage polarization. *H. pylori* not only protects against chronic colitis by promoting M2 polarization [13] but also enhances M1 polarization in human and mouse gastric macrophages, leading to the occurrence of *H. pylori*-associated atrophic gastritis [14]. However, there have been no studies further investigating the role of the multiplicity of infection (MOI) of *H. pylori* in macrophage polarization, as illustrated in our study, although a previous study reported that high and low MOIs of *H. pylori* played converse roles in B lymphocyte apoptosis [15].

Reactive oxygen species (ROS), which are mainly derived from superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-), are cooperative or independent regulators of cellular signaling in response to different environmental stimuli rather than simply a harmful byproduct of cell metabolism [16]. It has been reported that ROS are involved in DNA repair, cell cycling, cell differentiation, chromatin remodeling, self-renewal, and other cell processes [17]. Furthermore, ROS play an essential role in the regulation of macrophage polarization. A reduced ROS level suppresses the M1 phenotype and promotes macrophage polarization into the M2 phenotype [18–21]. However, the link between ROS and *H. pylori*-induced macrophage polarization has not been well clarified. Hypoxia-inducible factor 1 α (HIF-1α) is involved in cell proliferation, tumor angiogenesis, apoptosis [22], infection [23], inflammatory diseases [24], and innate immune responses. Under normoxia, HIF-1α undergoes rapid degradation, but it has been reported that HIF-1α expression can be enhanced and maintained by either endogenous or *H. pylori*-induced elevated ROS level in the gastric mucosa under normoxia [25]. Matak et al. demonstrated that HIF-1α contributed to M1 macrophage polarization in *H. pylori*-mediated gastritis; interestingly, HIF-1α also had an anti-inflammatory function at the same time [26], which indicated that HIF-1α may play dual roles in

macrophage polarization. Nevertheless, the role of HIF-1α in macrophage polarization, especially in the context of *H. pylori*-associated polarization, has not been well explored.

H. pylori infection, ROS, and HIF-1α are involved in the process of regulating macrophage polarization. However, the role and specific mechanisms of *H. pylori* in macrophage polarization, especially the effect of the MOI of *H. pylori*, remain poorly understood. The roles of ROS and HIF-1α in *H. pylori*-induced macrophage polarization remain to be further explored. Hence, this study, for the first time, investigated the MOI of *H. pylori*, the interaction between ROS and HIF-1α, and their roles in regulating *H. pylori*-induced macrophage polarization. In addition, we explored the effects of ROS and HIF-1α on the Akt/mTOR signaling pathway in this process, as studies have reported a central role for the Akt/mTOR pathway in macrophage polarization [27].

2. Materials and Methods

2.1. Cell Culture.
RAW 264.7 cells from mice were cultured in Dulbecco's modified Eagle's medium (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (Gibco, CA, USA) and 1% penicillin/streptomycin (Solarbio Biotechnology, Beijing, China) at 37°C in a 5% CO_2 atmosphere. After digestion, cells were inoculated into six-well plates and cocultured with the *H. pylori* standard strain 43504 when the cells reached a logarithmic growth phase. The *H. pylori* strain 43504 was grown on solid brucella agar plates supplemented with 5% fresh sheep blood and 1% penicillin/streptomycin at 37°C under microaerophilic conditions. After coculture with *H. pylori* at different MOIs for 9 h, cells were collected for RT-PCR, Western blot, and ELISA analyses. Cells were also treated with the ROS inhibitor N-acetylcysteine (NAC; 10 mM, Sigma-Aldrich, MO, USA) or HIF-1α inhibitor [3-(5$'$-hydroxymethyl-2$'$-furyl)-1-benzylindazole] (YC-1; 10 μM, Sigma). Macrophages were further incubated with the Akt inhibitor LY294002 (20 μmol/L) (Selleck, USA) or the mTOR inhibitor rapamycin (20 nmol/L) (Selleck).

2.2. Western Blotting.
After washing twice with ice-cold phosphate-buffered saline (PBS), total protein was extracted from treated cells with a mixture of RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China), benzenesulfonyl fluoride (PMSF) (Beyotime Biotechnology), and a protein phosphatase inhibitor (Sigma). The protein concentration was determined via the BCA method after centrifugation at 12,000 rpm for 10 min. Protein was separated on a 10% SDS polyacrylamide gel, transferred to nitrocellulose membranes, and blocked in a blocking solution at room temperature for 1 h. The primary antibodies used were as follows: anti-HIF-1α (#36169), anti-Akt (#2920), anti-p-Akt (Ser473) (#4060), anti-mTOR (#2983), anti-p-mTOR (Ser2448) (#5536), and anti-β-actin (#4970S). All the primary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). After incubation with primary antibodies at 4°C overnight, the membranes were washed three times with Tris-buffered saline-Tween (TBST) (Solarbio Biotechnology) and then incubated with a horseradish peroxidase-conjugated secondary antibody (Zhongshan Golden Bridge

Biotech; dilution 1 : 5,000) for 1 h at room temperature. Protein bands were visualized with an enhanced chemiluminescence kit (Thermo Fisher Scientific, Suwanee, GA, USA). The band intensity of target proteins was normalized to that of β-actin.

2.3. Real-Time Quantitative PCR Analysis. Total RNA was extracted from RAW 264.7 cells utilizing the RNA simple Total RNA Kit (TIANGEN Biotech, Beijing, China) and converted to cDNA with the Fast Quant RT Kit (TIANGEN Biotech) according to the manufacturer's instructions. NanoDrop 2000 (Thermo Fisher Scientific) was used to measure the concentration and purity of the isolated RNA. Real-time quantitative PCR was conducted to detect the transcriptional levels of CD86, CD206, Arg-1, iNOS, and HIF-1α by using the Step-One™ Real-Time PCR System (Applied Biosystems, CA, USA). The transcriptional levels of target genes were normalized to that of GAPDH.

2.4. ELISA. The levels of iNOS and Arg-1, markers related to macrophage polarization, were measured with the Mouse iNOS ELISA Kit (Elabscience Biotechnology Co., Ltd., Wuhan, China) and Mouse Arg-1 ELISA Kit (Elabscience Biotechnology Co.), respectively, according to the manufacturer's instructions.

2.5. Gastric Specimens and Immunohistochemistry. We collected 240 endoscopic biopsy specimens from the Yudu County People's Hospital of Ganzhou and the Digestive Endoscopy Center of the First Affiliated Hospital of Nanchang University and obtained patient consent before collecting the specimens. We also recorded relevant patient information, which showed that there were no significant differences in the age and sex distributions among the patients (Supplemental Table S1). These specimens included equal numbers (60 cases) of chronic nonatrophic gastritis (CNAG), intestinal metaplasia (IM), dysplasia (Dys), and gastric cancer (GC) samples. Each group contained *H. pylori* negative (*H. pylori* (-), 20 cases), *H. pylori* moderately positive (*H. pylori* (+), 20 cases), and *H. pylori* strongly positive (*H. pylori* (+++), 20 cases), with the quantities of *H. pylori* based on a previous study [28]. Four slices of each subject were used to analyze the expression of target proteins by immunohistochemical staining, and the results were evaluated by two pathologists who were blinded to the identity of the samples and scored for intensity (level 0-3) and frequency (level 0-4) (a total of 100 cells were counted in five random fields). For statistical analysis, using the formula intensity × frequency, the levels of each target protein were reported according to an expression score with a range of 0 to 12.

2.6. ROS Detection. The level of ROS in RAW 264.7 cells was examined with Molecular Probes™ CM-H2DCFDA (Thermo Fisher Scientific). Cells were incubated with a CM-H2DCFDA staining solution at 37°C in the dark for 30 min and washed 3 times with sterile PBS. Then, 200 μL of cell suspension was added to the 24-well plate and imaged using a high-content fluorescence microscope.

2.7. Statistical Analysis. All statistical analyses were conducted with SPSS 20.0. The results included in this study were obtained from at least three independent experiments and are represented as the mean ± SEM. The statistical significance of variables was analyzed by ANOVA. For multiple comparisons, post hoc analyses were evaluated with LSD correction. A p value < 0.05 was considered statistically significant.

3. Results

3.1. The State of Macrophage Polarization Was Associated with Progression from CNAG to GC and Correlated with the State and Quantity of H. pylori Infection. Studies have reported that macrophage polarization is associated with gastritis [14] and GC [29]. To further explore the correlation between macrophage polarization and clinical progression from CNAG to GC, we detected the expression of macrophage polarization markers, including CD86 (indicative of M1 polarization) and CD206 (indicative of M2 polarization), and the macrophage marker CD68 in 240 human gastric tissue samples diagnosed with CNAG, IM, Dys, or GC through immunohistochemistry. As shown in Figures 1(a)–1(c), with the progression of gastric lesions, the expression of CD68 and CD206 increased gradually, whereas the level of CD86 was higher in CNAG but lower in IM, Dys, and GC, which indicated that there was macrophage infiltration in gastric lesions and the macrophage polarization state was implicated in the progression of gastric lesions. We found that macrophages appeared to exhibit the M1 phenotype in the early stage of gastric lesions, such as CNAG, while they tended to display the M2 phenotype in advanced pathological stages, such as GC. To determine the role of *H. pylori* in gastric macrophage polarization, we further divided these 240 human gastric tissue samples with different stages of pathological changes into three groups: *H. pylori* (-), *H. pylori* (+), and *H. pylori* (+++). Interestingly, as suggested in Figures 1(d)–1(f), the expression of CD68, CD86, and CD206 was correlated with the *H. pylori* infection status in gastric tissues. We discovered that in all stages of gastric lesions, the levels of CD206 and CD86 in the *H. pylori*-positive groups were higher than those in the *H. pylori*-negative group. CD68 expression in gastric carcinomas was higher in the *H. pylori*-positive groups than in the *H. pylori*-negative group. Additionally, the expression levels of CD86 and CD206 in the *H. pylori* (+) and *H. pylori* (+++) groups were different, which indicated that the quantity of *H. pylori* might affect macrophage polarization. Overall, we discovered that the state of macrophage polarization was implicated in the progression of gastric lesions and associated with the state and quantity of *H. pylori* infection.

3.2. HIF-1α Expression Was Positively Related to Markers of Macrophage Polarization in Gastric Tissues and Associated with the State and Quantity of H. pylori Infection. As HIF-1α is related to M2 macrophage polarization in cancer [30], we speculated whether HIF-1α can influence the progression of gastric lesions by regulating gastric macrophage polarization. Thus, we tested the expression of HIF-1α during the progression from CNAG to GC. As shown in Figure 2(a),

(a)

(b)

(c)

(d)

(e)
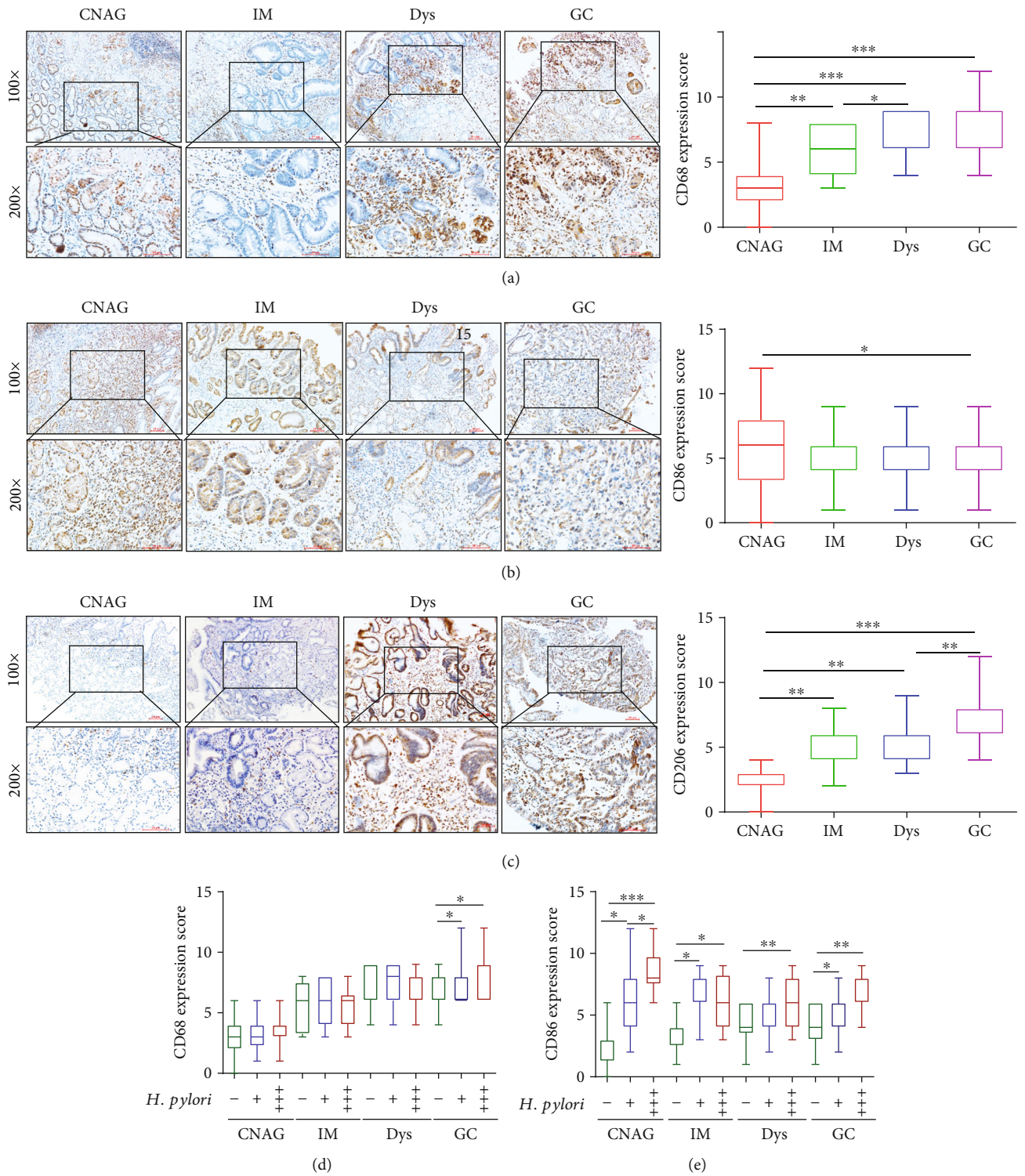
FIGURE 1: Continued.

(f)
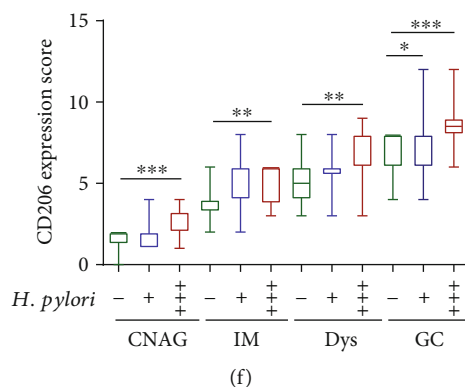
FIGURE 1: The state of macrophage polarization was associated with the progression from chronic nonatrophic gastritis to gastric cancer and correlated with the state of *H. pylori* infection. Representative images of immunohistochemical staining for CD68 (a), CD86 (b), and CD206 (c) in human gastric mucosa samples with CNAG, IM, Dys, or GC (magnification 200x, scale bars = 50 μm). Scores were assessed, and statistical comparisons were conducted to evaluate the results for the expression of CD68, CD86, and CD206, as shown on the right side of the representative images. Levels of CD68 (d), CD86 (e), and CD206 (f) compared between *H. pylori* (-), *H. pylori* (+), and *H. pylori* (+++) gastric tissue samples at different stages. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

HIF-1α expression increased gradually with the progression of gastric lesions. We further investigated the relationship between HIF-α and gastric macrophage polarization and found that HIF-1α levels were positively associated with the expression of CD68, CD86, and CD206 via Pearson correlation analysis (Figures 2(b)–2(d)). Additionally, the level of HIF-1α was correlated with the state and quantity of *H. pylori* (Figure 2(e)). All these results suggested that HIF-1α was correlated with gastric macrophage polarization and associated with the state and quantity of *H. pylori* infection during the progression of gastric lesions.

3.3. The MOI of H. pylori Affected the State of Macrophage Polarization and Expression of HIF-1α and ROS. Our previous study revealed that the ROS level was elevated by *H. pylori* infection in an MOI-dependent manner in GC [31], and in the present study, we found that the state and quantity of *H. pylori* infection affected macrophage polarization and HIF-1α expression. We speculated whether the MOI of *H. pylori* can influence the state of macrophage polarization and the expression of HIF-1α and ROS in macrophages; thus, we conducted the following experiments. We cocultured the macrophage cell line RAW 264.7 with *H. pylori* strain 43504 at various MOIs (0, 25, 50, 100, and 200) for 9 h, and the expression of iNOS, CD86, CD206, Arg-1, HIF-1α, and ROS was detected. As shown in Figures 3(a)–3(c), the levels of CD86 and iNOS were found to be much higher in the *H. pylori* infection groups than in the uninfected control group ($p < 0.05$). Moreover, the levels of CD86 and iNOS were positively associated with the MOI of *H. pylori*, which suggested that *H. pylori* could promote macrophage polarization toward the M1 phenotype in an MOI-dependent manner. Likewise, the levels of CD206 and Arg-1 were higher in the *H. pylori*-infected groups than in the uninfected control group. However, interestingly, although the increases in the CD206 and Arg-1 levels were dependent on the MOI at low MOIs (25, 50, and 100), the expression of CD206 and Arg-1 was suppressed slightly in the MOI 200 group (the MOI 200 group compared to the MOI 100 group, $p < 0.01$)

(Figures 3(d)–3(f)), which suggested that M2 macrophage polarization was inhibited by a high MOI of *H. pylori* compared with a low MOI. Similarly, the mRNA and protein expression of HIF-1α showed trends similar to those of CD206 and Arg-1 (Figures 3(g) and 3(h)). As expected, the level of ROS in macrophages was also significantly increased by *H. pylori* in an MOI-dependent manner (Figure 3(i)). All these results indicated that the MOI of *H. pylori* influenced the macrophage polarization status and expression of HIF-1α and ROS in macrophages.

3.4. ROS and HIF-1α Influenced the Macrophage Polarization Induced by H. pylori. Given that *H. pylori* infection affected macrophage polarization and upregulated the levels of ROS and HIF-1α in macrophages, we further evaluated whether ROS and HIF-1α expression influence the macrophage polarization induced by *H. pylori*. We treated RAW 264.7 cells with the ROS inhibitor NAC (10 mM), and decreases in iNOS and CD86 mRNA expression and increases in Arg-1 and CD206 mRNA levels were observed in the cells treated with the combination of NAC and *H. pylori* compared to the cells treated with *H. pylori* alone by using RT-PCR (Figures 4(a), 4(b), 4(d), and 4(e)). We also detected iNOS and Arg-1 levels by ELISA and further determined the upregulation of Arg-1 expression and downregulation of iNOS expression in groups treated with the combination of NAC and *H. pylori* compared with groups treated with *H. pylori* alone (Figures 4(c) and 4(f)). These results demonstrated that ROS inhibition suppressed macrophage polarization toward the M1 phenotype and promoted macrophage polarization toward the M2 phenotype. In contrast, we discovered that the HIF-1α inhibitor YC-1 (10 μM) decreased the elevated expression of iNOS, CD86, Arg-1, and CD206 induced by *H. pylori* utilizing RT-PCR (Figures 4(g), 4(h), 4(j), and 4(k)) and ELISA (Figures 4(i) and 4(l)), which indicated that inhibition of HIF-1α restrained M1 and M2 macrophages. In summary, our results indicated that ROS and HIF-1α could regulate *H. pylori*-mediated macrophage polarization.

(a)

$r = 0.567$
$p = 0.0001$

(b)

$r = 0.130$
$p = 0.039$

(c)

$r = 0.581$
$p = 0.0001$

(d)

(e)
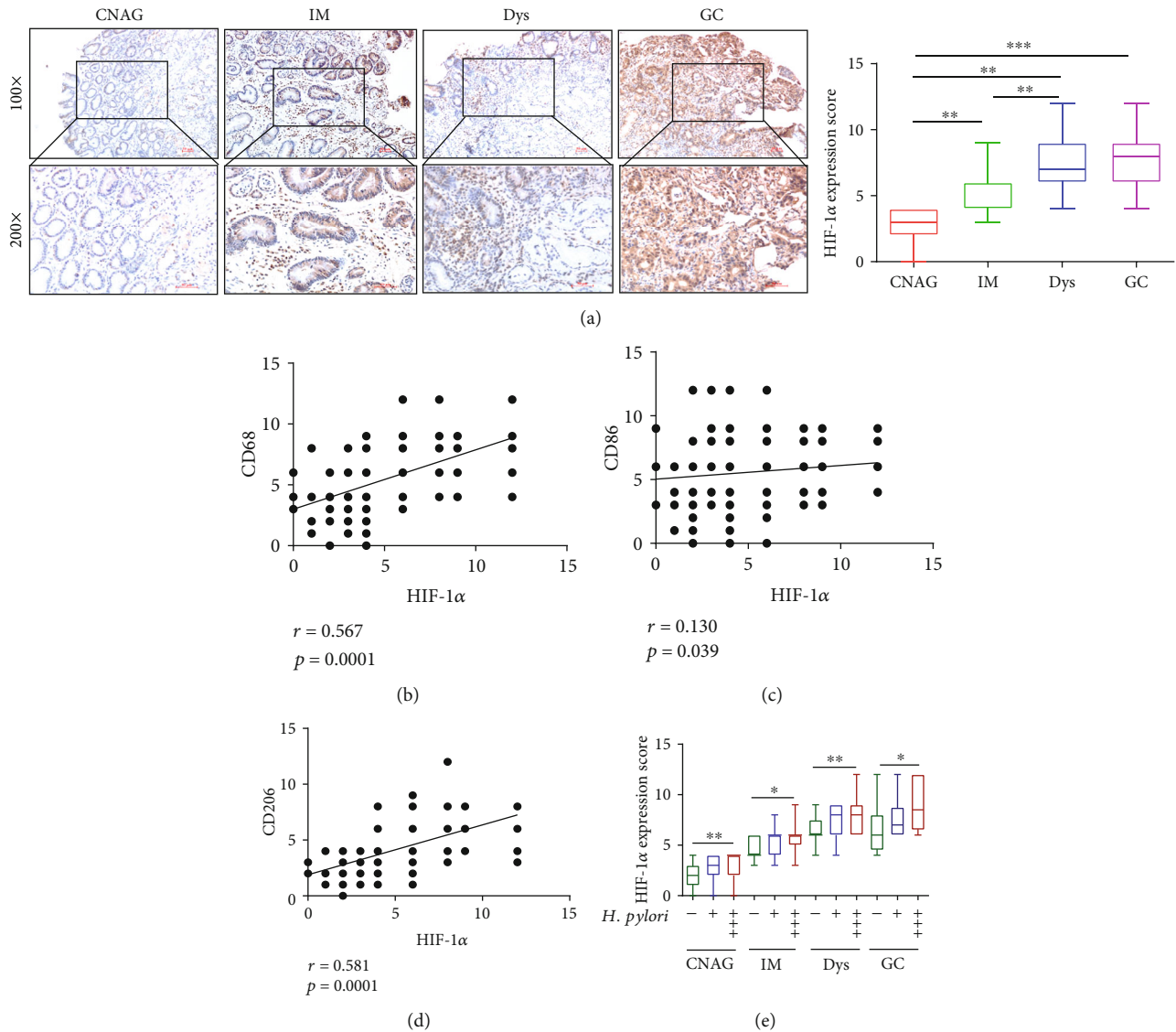
FIGURE 2: HIF-1α expression was positively related to markers of macrophage polarization in gastric tissues and associated with the state and quantities of *H. pylori* infection. Representative images of immunohistochemical staining for HIF-1α (a) in human gastric mucosa samples with CNAG, IM, Dys, or GC (magnification 200x, scale bars = 50 μm). Scores were assessed, and statistical comparisons were conducted to evaluate the results for the expression of HIF-1α. Pearson correlation analyses showing that the expression score of HIF-1α was positively associated with the scores of CD68 (b), CD86 (c), and CD206 (d). HIF-1α expression (e) between *H. pylori* (-), *H. pylori* (+), and *H. pylori* (+++) gastric tissue samples at different stages. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3.5. Crosstalk between ROS and HIF-1α in H. pylori-Infected Macrophages. Based on the results above and our previous research showing that ROS inhibition decreased the *H. pylori*-induced enhancement of HIF-α expression in BALB/c mice (Supplemental Figures 1(a)-1(c)), we suspected whether there was an interaction between ROS and HIF-1α in macrophages during *H. pylori* infection. Thus, we treated RAW 264.7 cells with 10 mM NAC (a ROS inhibitor) alone or in combination with the *H. pylori* strain. The data shown in Figure 5(a) suggested that NAC treatment attenuated both the ROS expression and the augmented ROS level induced by *H. pylori* infection. Moreover, the mRNA and protein levels of HIF-1α were suppressed in cells treated with NAC alone, and the *H. pylori*-induced elevations in HIF-1α mRNA and protein expression levels were also

sharply decreased by NAC treatment (Figures 5(b) and 5(c)), which indicated that ROS inhibition could inhibit HIF-1α expression in macrophages. Interestingly, when we treated RAW 264.7 cells with 10 μM YC-1 (a HIF-1α inhibitor), similar results were observed. YC-1 attenuated the expression of HIF-1α and ROS compared with the control group. Furthermore, YC-1 obviously decreased the augmented *H. pylori*-induced levels of HIF-1α (Figure 5(d)) and ROS (Figure 5(e)) in *H. pylori*-infected RAW 264.7 cells. Taken together, these results revealed that there was crosstalk between ROS and HIF-1α in *H. pylori*-infected macrophages.

3.6. ROS and HIF-1α Regulated H. pylori-Induced Macrophage Polarization via the Akt/mTOR Pathway.

(a)

(b)

(c)

(d)

(e)

(f)

(g)

(h)

FIGURE 3: Continued.

H. pylori (MOI)   0   25   50   100   200

(i)
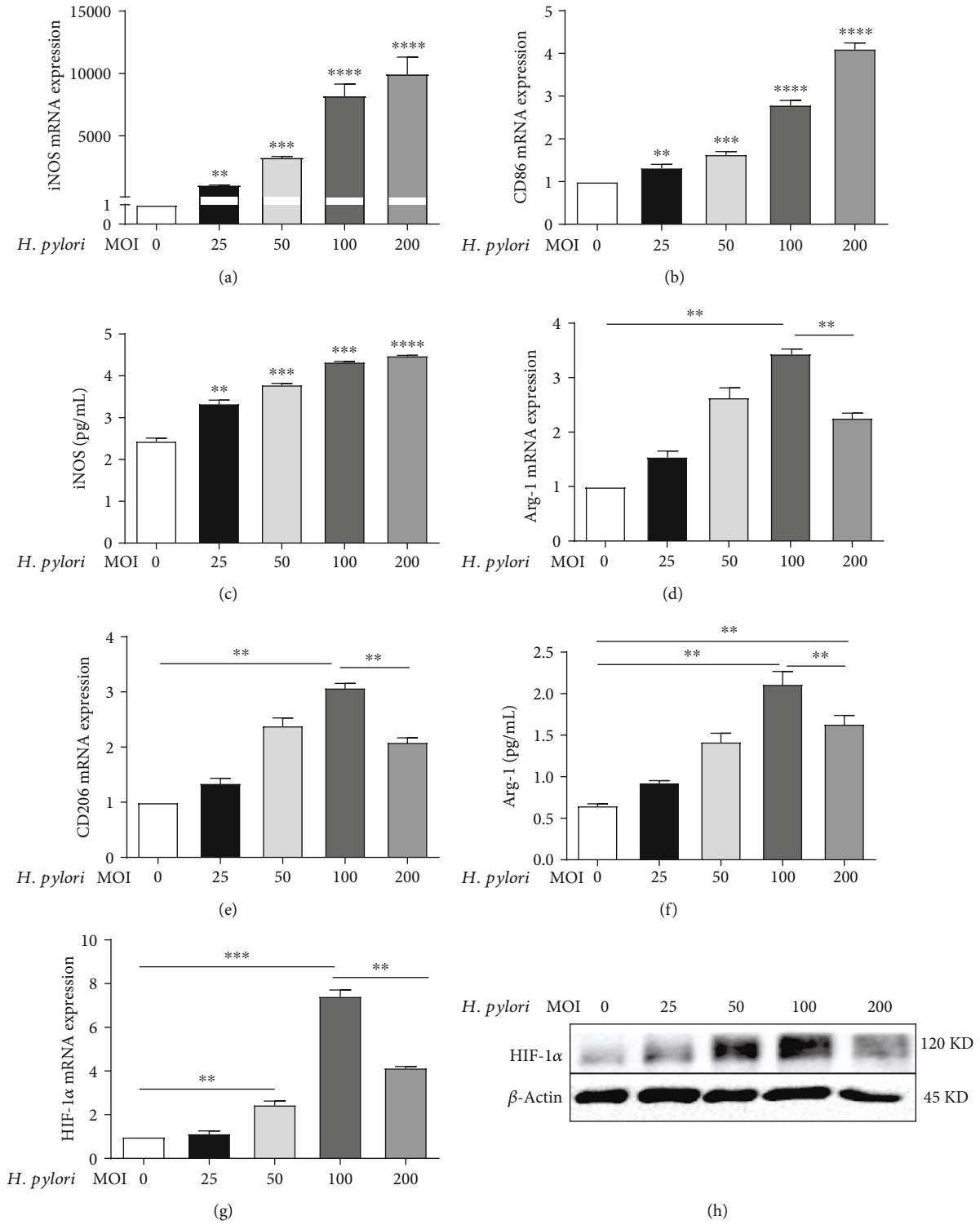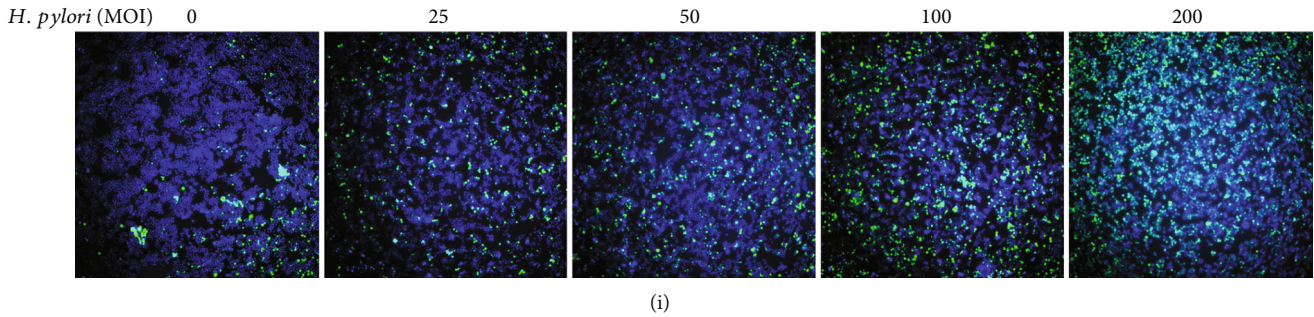
FIGURE 3: The MOI of *H. pylori* affected the state of macrophage polarization and expression of HIF-1α and ROS. Macrophages (RAW 264.7 cells) were incubated with *H. pylori* at various MOIs for 9 h. The mRNA expression levels of iNOS (a), CD86 (b), Arg-1 (d), and CD206 (e) measured by real-time PCR. ELISA detection of iNOS (c) and Arg-1 (f) levels. HIF-1α expression analyzed by real-time PCR (g) and immunoblotting (h). ROS expression evaluated with the fluorescent probe DCFH-DA (i), green: ROS, blue: nucleus. All experiments were independently repeated three times. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Several studies have demonstrated a crucial role for the Akt/mTOR pathway in the M1 and M2 polarization of macrophages [32–34]. However, there have been no studies on the function of the Akt/mTOR pathway in *H. pylori*-induced macrophage polarization. As shown in Figure 6(a), the protein levels of p-Akt (Ser473) and p-mTOR (Ser2448) were much higher in *H. pylori*-infected macrophages than in uninfected controls, while the total protein expression of Akt and mTOR was not different between the *H. pylori*-infected and control groups. Moreover, as the MOI of *H. pylori* increased, the levels of p-Akt (Ser473) and p-mTOR (Ser2448) showed trends toward increasing gradually at low MOIs and decreasing slightly at high MOIs (MOI = 200), which was similar to the results observed for HIF-1α, Arg-1, and CD206. We further treated cells with an inhibitor of Akt (LY294002, 20 μmol/L) or mTOR (rapamycin, 20 nmol/L) and observed sharp attenuation of the enhanced expression of p-Akt (Ser473) or p-mTOR (Ser2448) induced by *H. pylori* infection (Figures 6(b) and 6(c)). Moreover, as shown in (Figures 6(d)–6(k)), the augmented levels of iNOS, CD86, Arg-1, and CD206 in *H. pylori*-infected macrophages were reduced by LY294002 and rapamycin, which suggested that the Akt/mTOR pathway plays an important role in *H. pylori*-mediated macrophage polarization. Since we found that the crosstalk between ROS and HIF-1α regulated the polarization of macrophages induced by *H. pylori* infection, we further explored the roles of ROS and HIF-1α in this pathway. Western blot analysis showed that both NAC (10 mM) and YC-1 (10 μM) extremely inhibited the levels of p-Akt (Ser473) and p-mTOR (Ser2448) in macrophages and attenuated the elevated p-Akt (Ser473) and p-mTOR (Ser2448) expression induced by *H. pylori* infection (Figures 6(l) and 6(m)). All these data indicated that ROS and HIF-1α might regulate *H. pylori*-induced macrophage polarization via the Akt/mTOR pathway.

4. Discussion

In this study, we found that *H. pylori* infection was related to macrophage polarization, as reported in several previous studies [13, 14]. Moreover, clinical specimens revealed that different quantities of *H. pylori* infection had different effects

on macrophage polarization. Thus, we speculated whether the MOI of *H. pylori* is associated with macrophage polarization, as reported in a previous study showing that a low MOI of *H. pylori* suppressed B lymphocyte apoptosis, while a high MOI promoted B lymphocyte apoptosis [15]. The present study is the first to demonstrate that the MOI of *H. pylori* is associated with the state of macrophage polarization. We found that a low MOI of *H. pylori* promoted the M1 and M2 phenotypes of macrophages, while a high MOI partially inhibited the M2 phenotype compared with low MOIs. This indicated that macrophages were in a mixed state of M1 and M2 cells in the context of a low MOI, but with an increased MOI, M1 macrophages were enhanced, while M2 macrophages were suppressed. Whereas in studies of clinical specimens, compared to that in the *H. pylori* (+) group, the expression of M2-related markers in the *H. pylori* (+++) group did not show a decreasing trend. These contradictory results might be explained by the following two factors: one is that the MOI of in vitro experiments does not exactly match the positive grade of *H. pylori* in clinical specimens, and the concentration of *H. pylori* in the *H. pylori* (+++) group may not have reached the level of the high MOI (MOI = 200); the other is the insufficient number of clinical specimens. However, clinical specimens still revealed certain differences between the *H. pylori* (+) and *H. pylori* (+++) groups.

In our study, we found that *H. pylori* infection enhanced the ROS level in an MOI-dependent manner, which was consistent with our previous study [31]. We also discovered that HIF-1α expression in macrophages treated with different MOIs of *H. pylori* showed a trend similar to those of markers of the M2 phenotype, which we suspected would be explained by the dual role of ROS. A previous study revealed that there is a positive feedback loop between ROS and HIF-α: ROS upregulate and stabilize HIF-1α expression; in turn, elevated HIF-1α expression can increase the ROS level. However, when the ROS amount increases to a certain level, it may activate some molecules that in turn downregulate the expression of ROS and HIF-1α [35]. Thus, we surmised that the extremely high ROS level induced by the high MOI might slightly suppress the expression of HIF-1α through potential molecular mechanisms.

(a)

(b)

(c)

(d)

(e)

(f)

Figure 4: Continued.

(g)

(h)

(i)

(j)

(k)

(l)
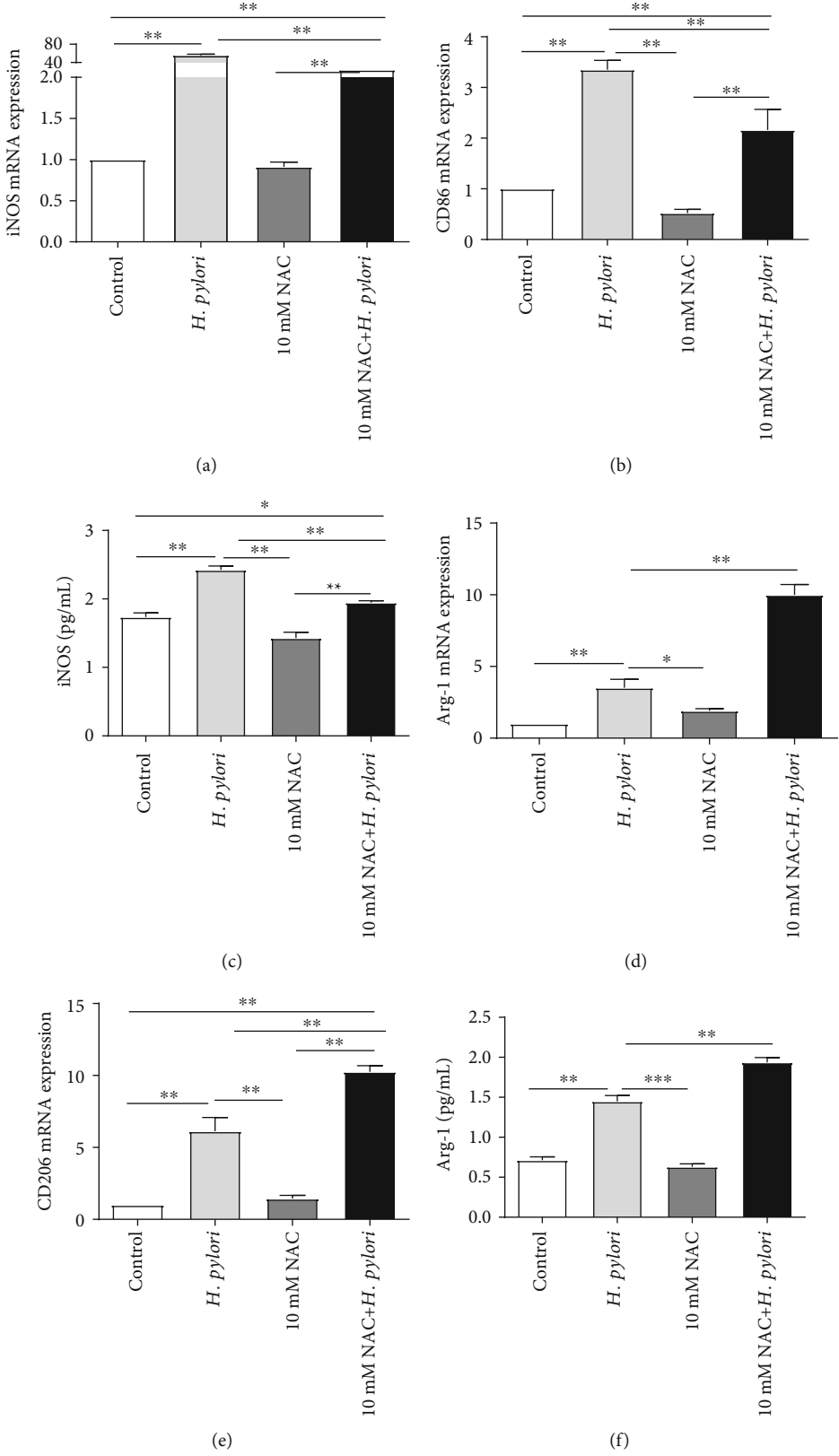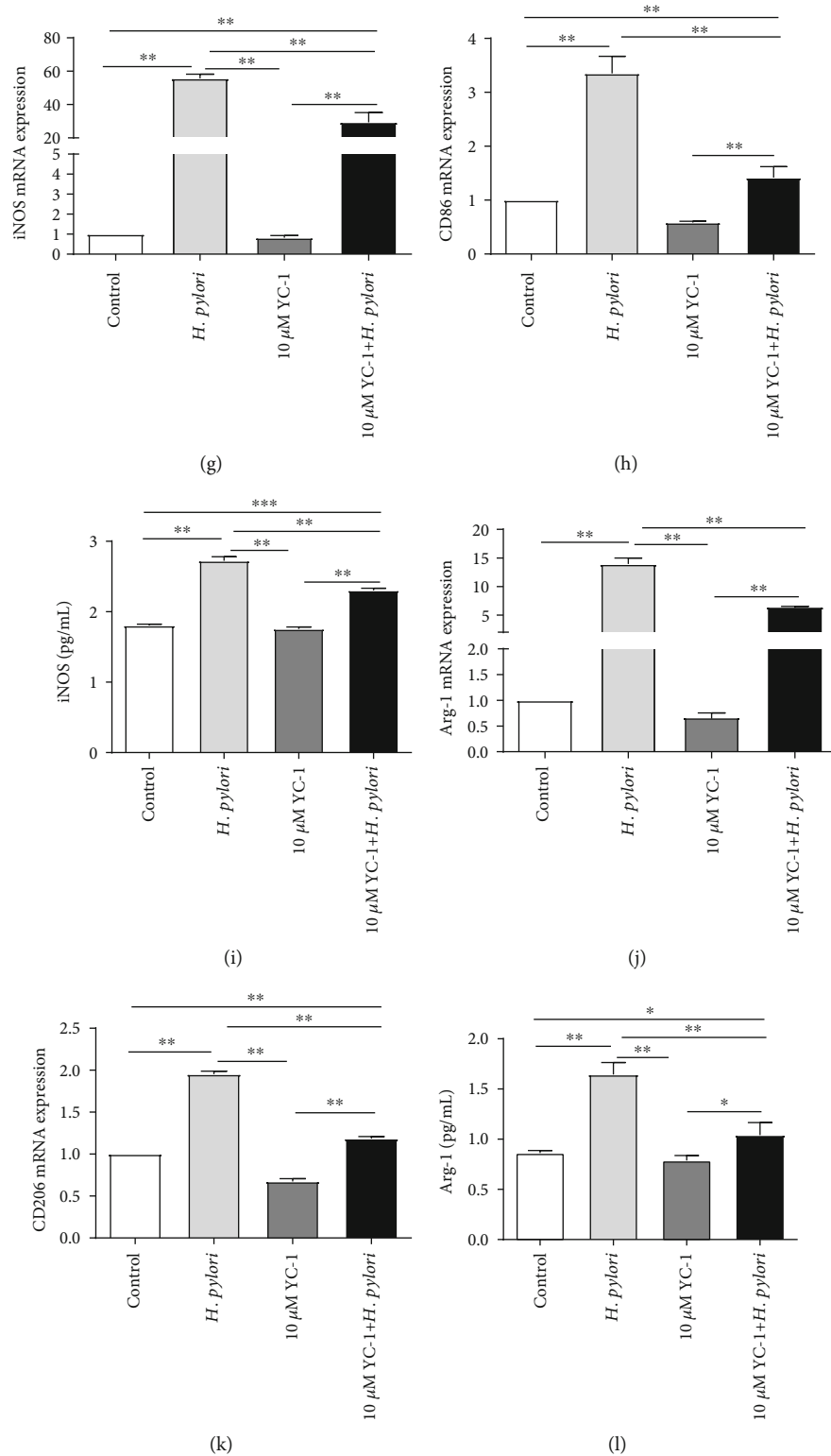
Figure 4: ROS and HIF-1α influenced the macrophage polarization induced by *H. pylori*. (a–f) RAW 264.7 cells were incubated with *H. pylori* (MOI = 100) alone or in combination with NAC (10 mM). NAC treatment inhibited the M1 phenotype but promoted the M2 phenotype. (g–l) RAW 264.7 cells were incubated with *H. pylori* (MOI = 100) alone or in combination with YC-1 (10 μM). YC-1 treatment inhibited both M1 and M2 macrophage polarization. The expression of iNOS, CD86, CD206, and Arg-1 was detected by real-time PCR ((a–d), (g), (h), (j), and (k)). The expression of iNOS (c, i) and Arg-1 (f, l) was also analyzed by ELISA. All experiments were independently repeated three times. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

(a)

(b)

(c)

(d)

(e)
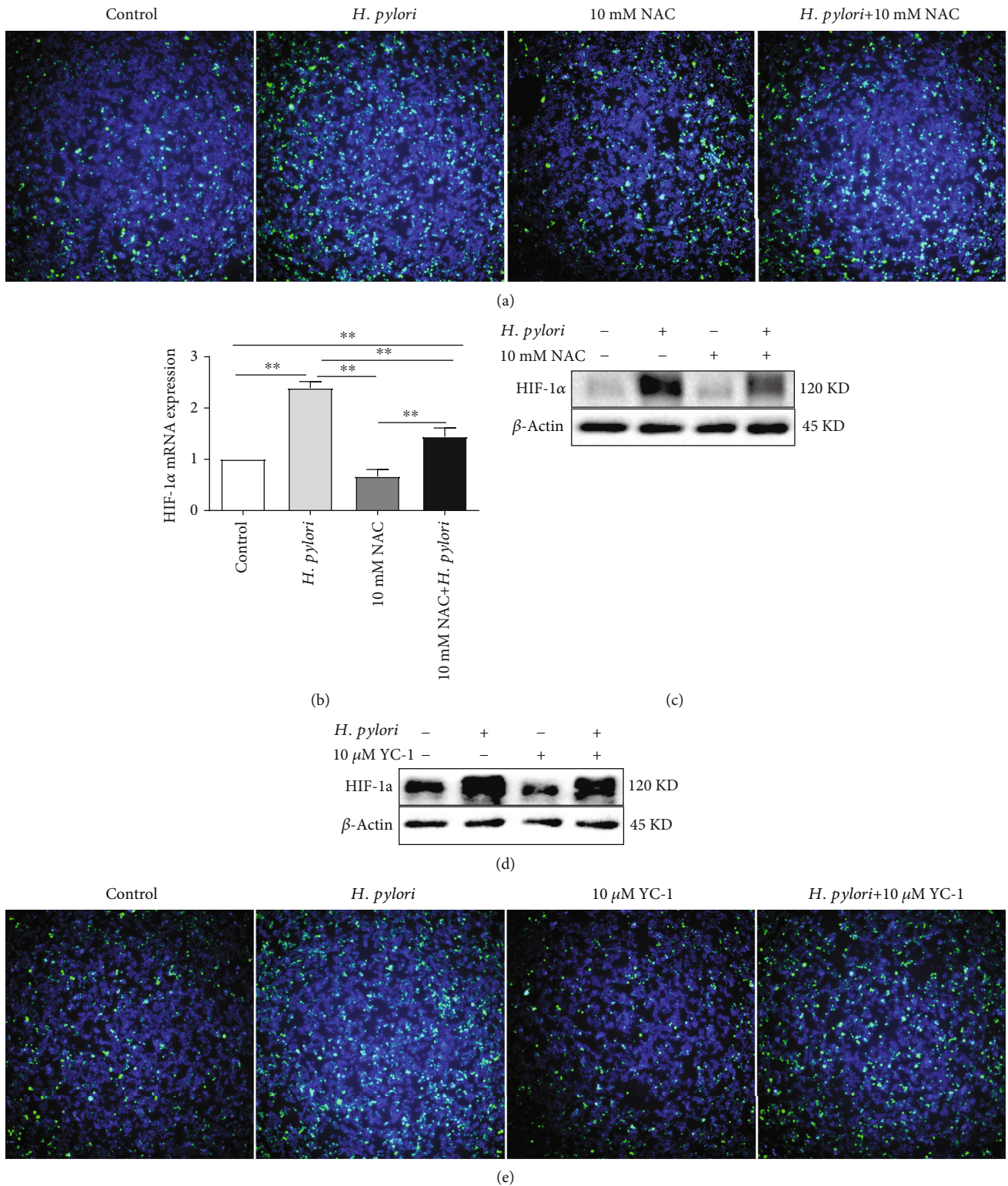
FIGURE 5: The crosstalk between ROS and HIF-1α in *H. pylori*-infected macrophages. (a–c) RAW 264.7 cells were incubated with *H. pylori* (MOI = 100) alone or in combination with NAC (10 mM). ROS inhibition decreased the enhanced expression of HIF-1α induced by *H. pylori*. (d, e) RAW 264.7 cells were incubated with *H. pylori* (MOI = 100) alone or in combination with YC-1 (10 μM). HIF-1α inhibition downregulated the augmented expression of ROS induced by *H. pylori*. ROS expression was detected with the fluorescent probe DCFH-DA (green: ROS, blue: nucleus). HIF-1α expression was tested by real-time PCR and Western blotting. Representative images are shown. All experiments were independently repeated three times. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

(a)

(b)

(c)

(d)

(e)

(f)

(g)

(h)

Figure 6: Continued.

(i)

(j)

(k)

(l)

(m)
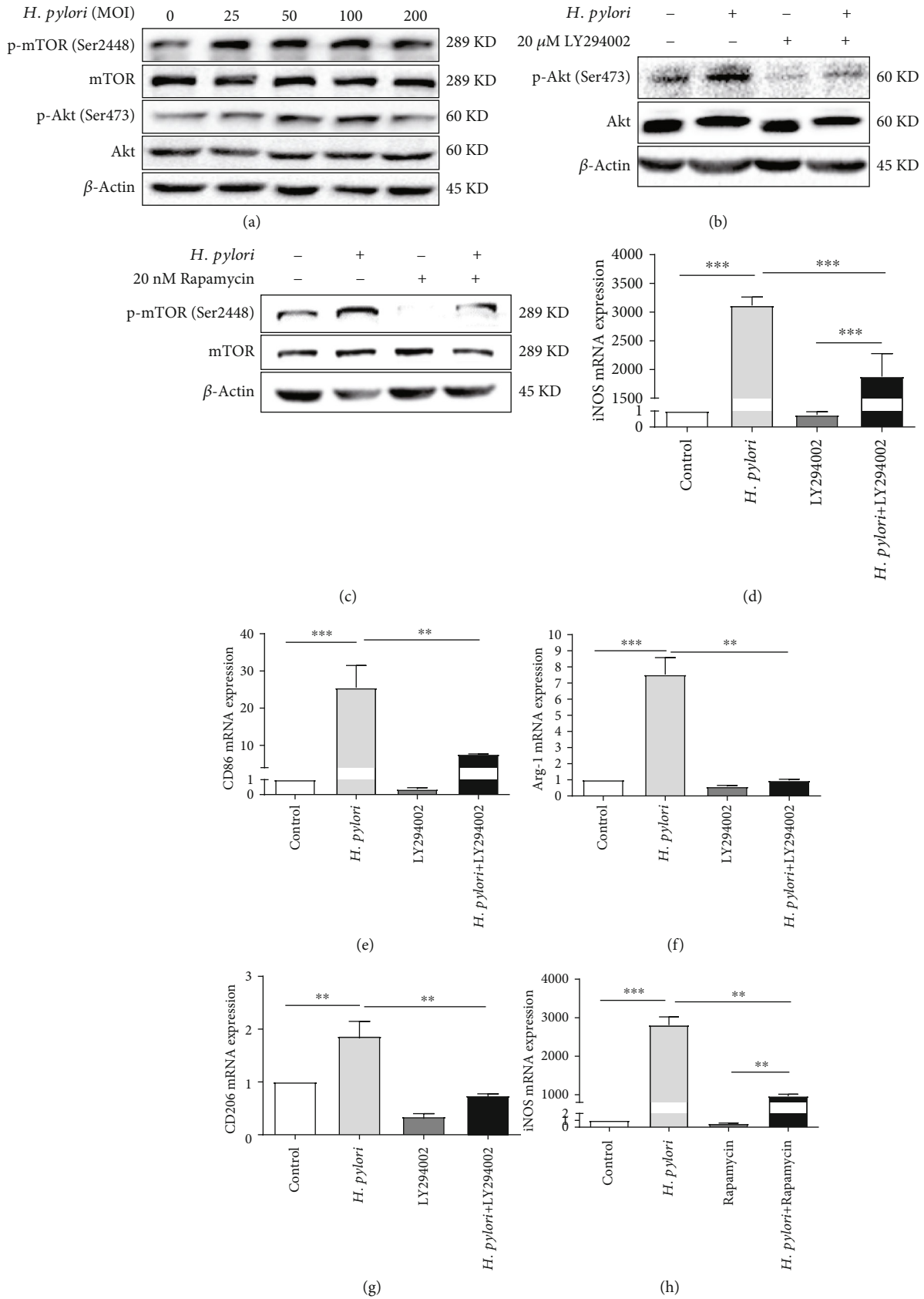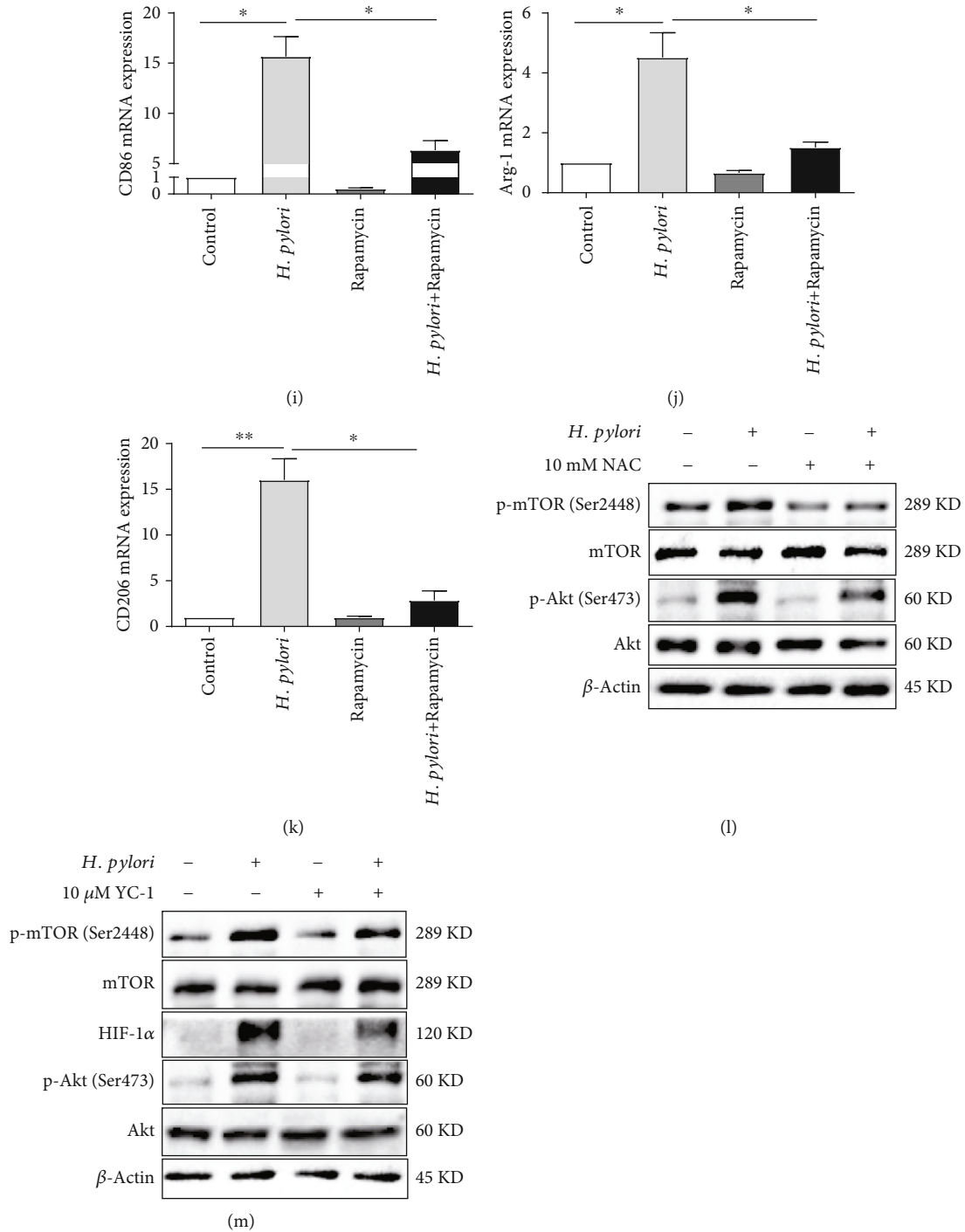
FIGURE 6: ROS and HIF-1α regulated *H. pylori*-induced macrophage polarization via the Akt/mTOR pathway. Increased expression of p-mTOR (Ser2448) and p-Akt (Ser473) was observed in RAW 264.7 cells treated with *H. pylori* at different MOIs for 9 h (a). RAW 264.7 cells were treated with *H. pylori* (MOI = 100), LY294002 (20 μmol/L), rapamycin (20 nmol/L), the combination of *H. pylori* and LY294002 (20 μmol/L), or the combination of *H. pylori* and rapamycin (20 nmol/L). LY294002 and rapamycin significantly attenuated the levels of p-Akt (Ser473) (b) and p-mTOR (Ser2448) (c), as well as the M1 (d–g) and M2 (h–k) phenotypes induced by *H. pylori*. RAW 264.7 cells were incubated with *H. pylori* (MOI = 100) alone or in combination with NAC (10 mM) (l) or YC-1 (10 μM) (m). Both NAC and YC-1 treatment reduced the augmented p-mTOR (Ser2448) and p-Akt (Ser473) levels induced by *H. pylori*. All experiments were independently repeated three times.

As previously reported, ROS play a critical role in macrophage polarization [36, 37], but the link between ROS and *H. pylori*-induced macrophage polarization has not been identified. In this study, we discovered that inhibiting ROS with NAC inhibited M1 polarization and contributed to M2 polarization mediated by *H. pylori* infection and that ROS inhibition downregulated the expression of HIF-1α, which was consistent with a previous study in which ROS production induced by *H. pylori* infection led to constant expression and stabilization of HIF-1α [25]. For HIF-1α, a high HIF-1α level is involved in increases in M2 polarization and accelerates hepatocellular carcinoma progression [30]. Moreover, recruitment of M1 macrophages is dependent on the presence of HIF-1α [38, 39]. Our study, for the first time, demonstrated that HIF-1α contributed to both M1 and M2 polarization induced by *H. pylori* infection. Moreover, *H. pylori*-mediated M1 and M2 polarization was attenuated by HIF-1α inhibition with YC-1, and a reduction in the ROS level was also observed with this treatment. Hence, we concluded that ROS combined with HIF-1α promoted M1 polarization and that HIF-1α enhanced M2 polarization when cells were treated with a low MOI of *H. pylori*. In contrast, upon treatment with a high MOI of *H. pylori*, M1 polarization was maintained by ROS, while M2 polarization was partially suppressed, which might be due to the decrease in the HIF-α level induced by extremely high ROS expression. However, we did not identify the specific molecular mechanism underlying the mutual regulation between ROS and HIF-1α in this study, and this mechanism needs to be further elucidated in our future studies. Although many studies have illustrated that ROS and HIF-1α are involved in macrophage polarization, we are the first to discover the crosstalk between ROS and HIF-1α in *H. pylori*-induced macrophage polarization, and the expression of ROS and HIF-1α was associated with the MOI of *H. pylori*.

Activation of the Akt/mTOR pathway has been determined to play a central role in the regulation of M1 and M2 macrophage polarization in various diseases [27, 40–43]. M2 macrophage polarization induces tamoxifen resistance through the activation of the PI3K/Akt/mTOR pathway in breast cancer [44]. In the present study, elevated levels of Akt and mTOR phosphorylation were observed in macrophages treated with *H. pylori*. Inhibition of the Akt/mTOR pathway greatly inhibited the levels of p-Akt and p-mTOR, resulting in reductions in both M1 and M2 macrophage polarization induced by *H. pylori*. More importantly, both inhibition of ROS and inhibition of HIF-1α significantly attenuated the elevated levels of p-Akt and p-mTOR induced by *H. pylori* in macrophages. Hence, we concluded that ROS and HIF-1α could regulate *H. pylori*-mediated macrophage polarization via the Akt/mTOR pathway.

Long-term sustained *H. pylori* infection can cause chronic gastritis, peptic ulcers, and gastric adenocarcinoma [9]. During the histopathological Correa cascade of gastric tumorigenesis (from CNAG to GC), M2 macrophage numbers increased gradually, while M1 macrophage numbers showed a trend toward a slight decrease, which was consistent with previous studies that reported that M1 macrophages are mainly involved in proinflammatory processes,

while M2 macrophages are associated with tumor transformation [45–47]. Tumor-recruited M2 macrophages contribute to GC metastasis [48] and enhance the resistance of gastric cells to cisplatin treatment [49]. Accordingly, the M2 phenotype of macrophages might be involved in the progression of *H. pylori*-associated gastric carcinoma, which needs to be clarified in our future studies.

In conclusion, our study, for the first time, shows that the MOI of *H. pylori* affects the state of macrophage polarization and the expression of HIF-1α and ROS in macrophages. Additionally, ROS and HIF-1α regulate *H. pylori*-mediated macrophage polarization via the Akt/mTOR pathway, and there is crosstalk between ROS and HIF-1α during macrophage polarization induced by *H. pylori*. Our study describes a new mechanism of *H. pylori*-induced macrophage polarization. Further investigations into the correlation among ROS, HIF-1α, *H. pylori* infection, and gastric carcinoma could lead to the development of novel strategies for the therapy of *H. pylori*-associated GC.

Data Availability

The datasets supporting the conclusions of this article are included within the article and supplemental files.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Xu Shu and Xiaogang Yuan designed the study and supervised the structure and quality of the manuscript. Ying Lu and Jianfang Rong collected and analyzed the data and drafted the manuscript. Yongkang Lai and Li Tao analyzed immunohistochemical data and revised the paper. Ying Lu and Jianfang Rong contributed equally to this research. All authors confirmed the final paper.

Acknowledgments

Supplementary Materials

Supplemental materials contain one table, one Excel spreadsheet, and figures which include statistical graphs of the results of multiple Western blot experiments. *(Supplementary Materials)*

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Review Article

Dual and Opposite Roles of Reactive Oxygen Species (ROS) in Chagas Disease: Beneficial on the Pathogen and Harmful on the Host

Edio Maldonado [iD],[1] **Diego A. Rojas** [iD],[2] **Sebastian Morales** [iD],[1] **Vicente Miralles** [iD],[3] **and Aldo Solari** [iD][1]

[1]*Programa Biología Celular y Molecular, Facultad de Medicina, Universidad de Chile, Santiago, Chile*
[2]*Instituto de Ciencias Biomédicas, Facultad de Ciencias de la Salud, Universidad Autónoma de Chile, Santiago, Chile*
[3]*Departamento de Bioquímica y Biología Molecular, Universidad de Valencia, Valencia, Spain*

Correspondence should be addressed to Edio Maldonado; emaldona@med.uchile.cl and Aldo Solari; asolari@med.uchile.cl

Chagas disease is a neglected tropical disease, which affects an estimate of 6-7 million people worldwide. Chagas disease is caused by *Trypanosoma cruzi*, which is a eukaryotic flagellate unicellular organism. At the primary infection sites, these parasites are phagocytized by macrophages, which produce reactive oxygen species (ROS) in response to the infection with *T. cruzi*. The ROS produce damage to the host tissues; however, macrophage-produced ROS is also used as a signal for *T. cruzi* proliferation. At the later stages of infection, mitochondrial ROS is produced by the infected cardiomyocytes that contribute to the oxidative damage, which persists at the chronic stage of the disease. The oxidative damage leads to a functional impairment of the heart. In this review article, we will discuss the mechanisms by which *T. cruzi* is able to deal with the oxidative stress and how this helps the parasite growth at the acute phase of infection and how the oxidative stress affects the cardiomyopathy at the chronic stage of the Chagas disease. We will describe the mechanisms used by the parasite to deal with ROS and reactive nitrogen species (RNS) through the trypanothione and the mechanisms used to repair the damaged DNA. Also, a description of the events produced by ROS at the acute and chronic stages of the disease is presented. Lastly, we discuss the benefits of ROS for *T. cruzi* growth and proliferation and the possible mechanisms involved in this phenomenon. Hypothesis is put forward to explain the molecular mechanisms by which ROS triggers parasite growth and proliferation and how ROS is able to produce a long persisting damage on cardiomyocytes even in the absence of the parasite.

1. Introduction

Protozoan parasites from the *Trypanosomatidae* family are unicellular and diploid organisms, which cause neglected diseases in many regions of the world, mainly in developing countries with tropical and subtropical climates. Chagas disease is a neglected tropical disease, which affects over seven million people around the world. It is caused by the unicellular flagellate protozoan *Trypanosoma cruzi* and affects over 8 million people worldwide, causing approximately 50,000 deaths each year [1, 2]. Another

70-100 million people living in endemic areas are at risk of infection [1, 2]. For most of the Latin American countries, it is one of the main public health problems. Many other factors such as migration events are increasing the number of diagnosed cases in nonendemic regions such as Europe, North America, and Western Pacific areas [3].

Chagas disease is transmitted by blood-sucking bugs of the subfamily *Triatominae*. The disease has two successive phases. The first phase is an acute one characterized by high parasitemia and a second chronic phase is usually with chronic cardiomyopathy. Most of the infected individuals

in the second phase (60-70%) never develop chronic symptoms or signs associated with the disease; however, the rest of the patients (30-40%) will show signs of heart impairment. Chagas disease displays a variety of clinical forms, and the possible outcomes of this disease involve interplay between environmental and genetic factors associated with both the host and the infecting parasite [4, 5]. During the initial acute phase, the infecting parasites (blood trypomastigotes) can invade tissues and they mainly multiply inside the macrophages leading to high parasitemia and inflammation. Most individuals then circumvent this acute phase of infection and enter in a new phase, which is characterized by a low parasite numbers in the blood and no apparent pathological features. However, for 30-40% of patients, a chronic phase appears 20-30 years later and is characterized by low parasitemia but an increased tissue injury, which results in severe digestive and/or cardiac damage that can be lethal if patients are not treated [6]. Two drugs have been commonly used to treat *T. cruzi* infections for the last 40 years, which are benznidazole and nifurtimox [6, 7]. They are believed to exert their trypanocidal activity through the bioreduction of the nitro group by trypanosomal type I reductase and followed by covalent linkage of the reduced drug to internal thiols which can form toxic adducts on the parasite DNA.

The *Trypanosomatidae* family belongs to the *Kinetoplastida* order, which contains a single and unusual mitochondrial DNA or kinetoplast DNA containing several dozens of maxicircle copies, which code for proper mitochondrial functions and several thousand of minicircles copies coding for guide RNAs, which edit mitochondrial mRNA; all of them are concatenated in a single net that occupies the only one mitochondrion in this flagellate [8, 9].

T. cruzi goes through extensive morphological and biochemical changes during its life cycle, which alternates between the vertebrate and the invertebrate hosts (*Triatomine* bugs). Noninfective epimastigotes proliferate in the hindgut of the insect vector, which are haematophagous bugs. Once in the rectum of the insects, parasites differentiate into the nondividing infectious metacyclic trypomastigotes [10, 11]. These parasites are excreted with the insect excrements after they feed on the mammalian host and can infect this host by passing through different mucous membranes, such as the stomach or skin lesions produced during or after the insect blood meal. These infective metacyclic forms invade host cells, mainly macrophages, where they transform into the replicating intracellular amastigote stage [10, 11]. After multiplying by binary division in the cytoplasm, amastigotes differentiate into infective nonreplicating trypomastigotes. Both forms are released into the bloodstream of the mammalian host upon host cell lysis. Subsequently, trypomastigotes can penetrate other nucleated cell types, including skeletal and cardiac muscle cells (cardiomyocytes), which depend on the immune competent state of the host, or they can be taken up again by the insect during a blood meal, starting a new infecting cycle [10, 11].

In 1968, the reactive oxygen species (ROS) produced by phagocytes during respiratory burst was proposed as the killing mechanism of pathogens. By the late 1970 and early 1980, it was proposed and assumed that ROS from the macrophages was the responsible of the killing of *T. cruzi*; however, a few years ago (2012), the old paradigm has shifted. *T. cruzi* can live inside the macrophages under oxidative stress conditions; moreover, antioxidants are detrimental for its growth. In this review, we will summarize the mechanisms by which *T. cruzi* can deal with the host-oxidative assault, and also we will discuss the most important work demonstrating that ROS produced by the host cells in response to the infection can promote *T. cruzi* cell growth and differentiation and how ROS produced by the infected cell contributes to the progressive oxidative damage produced in Chagas heart disease at the chronic stage.

1.1. Trypanosoma cruzi and Oxidative Damage.

T. cruzi is a well-equipped organism to deal with endogenous and/or exogenously produced oxidants. As an obligatory intracellular parasite, *T. cruzi* must be able to withstand its own endogenous toxic metabolites, produced during its aerobic mitochondrial metabolism, and also must cope with the oxidative burst from the host immune system, which includes several reactive nitrogen and oxygen species (RNS and ROS, respectively). Moreover, they can use ROS as a signal for cellular growth and proliferation. In this way, *T. cruzi* has evolved mechanisms to deal and use oxidative signals in their own benefit. This fact is reflected by the efficient and well-compartmentalized antioxidant network that the parasite uses in the detoxification of ROS and RNS species produced during parasite-host cell interactions and the efficient use of ROS to grow and proliferate.

In *T. cruzi*, the antioxidant defenses are composed for several enzymes and nonenzymatic redox active molecules, which are located mainly in the mitochondria, endoplasmic reticulum, glycosomes, and the cytosol [12–14]. The final electron donor for the enzymatic reactions is NADPH derived from the pentose pathway. In such a way, the reducing equivalents from NADPH are delivered to a variety of enzymatic detoxification systems of the parasite, mainly through the dithiol trypanothione [$T(SH)_2$, N1, N8-bisglutathionylspermidine] and the thioredoxin homologue named tryparedoxin (TcTXN) which are low-molecular-weight dithiol proteins belonging to the thioredoxin oxide reductase family or glutathione (GSH). Thus, despite the lack in the parasite of an enzyme-mediated pathway to detoxify H_2O_2, they rely mainly on trypanothione (TR) [12–14]. Trypanothione is synthetized by the trypanothione synthetase (TcTS) in an ATP-dependent reaction in which two molecules of GSH are covalently linked to spermidine. The reduced state of trypanothione is maintained by the NADPH-dependent flavoenzyme trypanothione reductase (TcTR) [15]. Trypanothione also provides reducing equivalents to the ribonucleotide reductase to generate deoxyribonucleotides for DNA synthesis [12–14]. In addition, *T. cruzi* has the complete biosynthetic pathway for ascorbate that can act both as a nonenzymatic antioxidant and as the reducing substrate for ascorbate-dependent hemoperoxidase (TcAPX) [12]. Other low-molecular-weight thiols such ovothiol A, which is found in the replicative stages but not in the blood forms of *T. cruzi*, might also possess antioxidant roles.

T. cruzi possesses five different peroxidases, which can differ in their specificity and subcellular location [16]. Two of these, the mitochondrial and cytosolic *T. cruzi* tryparedoxin peroxidases TcMPX and TcCPX, respectively, belong to the peroxiredoxin family of proteins, which are able to detoxify H_2O_2 short-chain organic hydroperoxides and peroxynitrite, which are as efficient as catalase and selenium-dependent glutathione peroxidase in H_2O_2 detoxification [16]. Another peroxidase, the ascorbate-dependent heme-peroxidase (TcAPX), is located in the endoplasmic reticulum and is able to confer resistance against H_2O_2 using ascorbate as reducing substrate. Other two peroxidases, which show similarity to glutathione-dependent peroxidases, are glutathione-dependent peroxidase I (GPX-I), which is located in the cytosol and glycosome, and the glutathione-dependent peroxidase II (GPX-II), which is present in the endoplasmic reticulum; however, they are unable to act against H_2O_2. Interestingly, a catalase enzyme is absent in *T. cruzi*; however, this enzyme is present in other kinetoplastides, which parasitize insects, and is also present in almost all aerobic organisms [17]. Efforts to answer this puzzling observation have been done with the generation of a *T. cruzi* cell line expressing a heterologous catalase (from *E. coli*), since it might be possible that $O_2^{\cdot-}$ and/or H_2O_2 can act as intermediates in cellular signaling pathways. The artificial expression of catalase increases the resistance to H_2O_2 compared to wild-type cells but reduces the parasite ability to adapt under a low H_2O_2 dose environment, suggesting that catalase has an effect in the parasite adaptation ability, which is promoted by pretreatment of the parasites with low doses of the oxidant [16]. Moreover, the expression of the heterologous catalase is able to reduce the levels of trypanothione reductase, but increases the levels of superoxide dismutase, which results in higher levels of residual H_2O_2 after the treatment with this oxidant [17].

In the antioxidant network dependent on trypanothione, this molecule is reduced by the enzyme TcTR which transfers the reducing equivalents from NADPH via trypanothione to tryparedoxin TcXNI, which is the source of reducing equivalents for cytosolic tryparedoxin peroxidase TcCPX [12–14]. The rate-limiting step in the antioxidant trypanothione-dependent system is the interaction between trypanothione and TcXN. The TcTXN possesses an important role to protect the parasites against fluctuating levels of ROS which is generated during the physiological process, since it can be modulated by the exposure of the parasites to H_2O_2 and has a higher expression in culture-derived trypomastigotes treated with H_2O_2, and its expression levels are higher in strains more resistant to the oxidative damage caused by this compound [18–21]. This enzyme is also released into the culture medium; however, the mitochondrial TcMPX is not, but the reason for this observation is not known. TcMPX is located inside the mitochondrion, toward the cell periphery in close contact with the kinetoplast DNA, and therefore might protect mitochondrial DNA from peroxide-mediated damage [20, 21]. It has been hypothesized that TcMPX acts in a similar way, as TcCPX, since both can act on the same substrates; however, trypanothione TcTR and TcXN have not been found inside the *T. cruzi* mitochondrion. So far,

the mechanism by the mitochondrial NADPH which is regenerated inside the mitochondrion remains unclear. It seems to be that *T. cruzi* relies only on TcCPX to detoxify the H_2O_2 generated inside the mitochondrion [14, 18, 19].

Two thiol-dependent tryparedoxin peroxidases (TcTXNI and TcTXNII) exist in *T. cruzi*. Tryparedoxins are dithiol proteins of low molecular weight, which belong to the thioredoxin family of proteins. TcTXNI can interact with several cytosolic endogenous proteins involved in the antioxidant system, energy metabolism, and protein translation machinery [14, 18–20]. One of the interacting partners, as expected, is TcCPx. On the other hand, TcTXNII is a mitochondrial transmembrane protein and is anchored to the surface of this organelle and also on the surface of the endoplasmic reticulum with the redox active center exposed towards the cytosol. TcTXNII is able to interact with protein members of the antioxidant system, cytoskeleton, energy metabolism, and protein synthesis machinery. Also, both TcCPx and TcMPX were found to be able to interact with TcTXNII [18, 19]. However, it is believed that tryparedoxins are not required for mitochondrion redox metabolism and TcMPX might use a reduction system that does not require tryparedoxins. However, it has been found that TcTXNI and TcXNII peroxidases can efficiently reduce H_2O_2 and peroxynitrite *in vitro* [22, 23] and this could contribute to their role as virulence factors reported *in vivo*. It is also likely that thiol-dependent tryparedoxin peroxidases could play a minor nonessential role in the parasite antioxidant defense system, and perhaps they play an important role in other biological processes. An overview of the enzymes involved in the oxidative stress and their cellular location is displayed in Figure 1.

The mitochondrion is the principal site for oxidant formation during the aerobic metabolism. Those oxidants can be able to exert damage and/or participate in signal transduction processes. Superoxide radical, generated by electron leakage from the respiratory chain (mainly at the complex III), is metabolized by an iron-dependent superoxide dismutase (FeSOD-A) [24] to produce H_2O_2, an oxidant that must be further catabolized. Meanwhile, the nitric oxide (NO) which reaches the mitochondria can inhibit parasite mitochondrial respiration (at the complex IV) enhancing the generation of $O_2^{\cdot-}$ (by complex III) which produces intramitochondrial peroxynitrite formation ($ONOO^-$). This process can outcompete the dismutation of $O_2^{\cdot-}$ by the enzyme FeSOD-A [24]. To overcome this, there exists a typical two-cysteine peroxiredoxin located at the mitochondrial matrix (TcMPX) which efficiently reduces and detoxifies the $ONOO^-$ and H_2O_2 to NO_2 and H_2O, respectively [25]. On the other hand, in the endoplasmic reticulum, two distinct peroxidases, which are named ascorbate-dependent heme-peroxidase (TcAPX) [26] and glutathione-dependent peroxidase II (GPX-II), can metabolize H_2O_2 and lipid hydroperoxides, respectively [13, 14]. Meanwhile, the glycosomes, which are specific trypanosomal organelles and the places where the first set reactions of the glycolysis pathway occur, contain Fe-SOD, glutathione-dependent peroxidase I (GPX-I), and GPX-II [13, 14]. The enzyme GPX-I can metabolize H_2O_2; however, the enzyme GPX-II has a more restricted specificity directed towards lipid hydroperoxides. Finally,
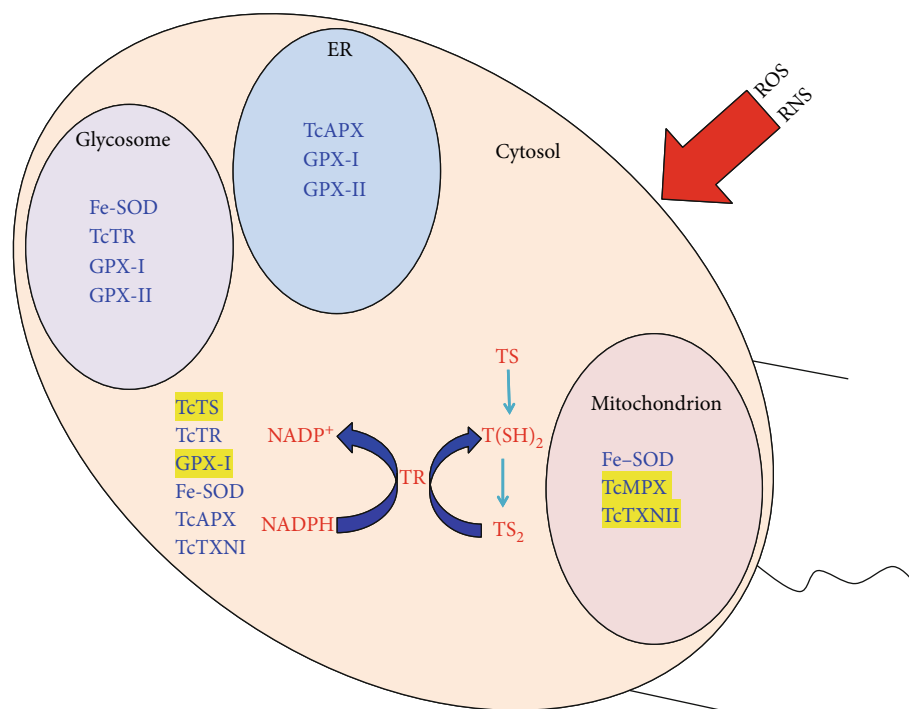
FIGURE 1: Antioxidant defenses location in *T. cruzi*. The dithiol trypanothione [T(SH)$_2$] and the *T. cruzi* antioxidant defense enzymes are distributed into the ER, glycosome, mitochondrion, and cytosol. ROS: reactive oxygen species; RNS: reactive nitrogen species; TR: trypanothione; TcTS: trypanothione synthetase; TcTXN: tryparedoxin; TcTR: trypanothione reductase; TcAPX: ascorbate-dependent hemoperoxidase; TcMPX/TcCPX: tryparedoxin peroxidase; GPX-I: glutathione-dependent peroxidase I; GPX-II: glutathione-dependent peroxidase II; Fe-SOD: iron-dependent superoxide dismutase.

the cytosolic antioxidant defenses are supplied by the presence of GPX-I, Fe-SOD, and also by the cytosolic peroxiredoxin TcCPX. The relevance of each of those components of the parasite antioxidant defenses has been demonstrated by the increased resistance against H$_2$O$_2$/ONOO$^-$ treatment, which is afforded when those enzymes are overexpressed in *T. cruzi* epimastigote cells.

In spite of the antioxidant defenses of the parasites that might look undefeatable, the macrophage-produced peroxynitrite can damage and control *T. cruzi* proliferation [27, 28]. Cytotoxic actions of peroxynitrite against parasites include alteration of Ca^{2+} homeostasis which compromises cellular energetic charge and mitochondrial physiology and can mediate either necrotic or apoptotic death pathways, depending on the severity of the oxidative insult [27, 28]. The ultimate success of the parasite to infect the host will depend on the macrophages' ability to destroy the infecting parasite, the host immune system, and the virulence factors of the parasite.

1.2. Oxidative Stress in the Acute Phase of Chagas Disease. In order to get a successful infection, the metacyclic trypomastigotes are required to invade the host cells. In the vertebrate host, resident macrophages, present at the parasite invading site, are among the first professional phagocyte cells to be invaded *by T. cruzi*; therefore, they are key players in the infection control [28–31]. The naive macrophages (noncytokine primed) internalize infecting metacyclic trypomastigotes into the phagosome vacuole and

this event activates the membrane-associated NADPHox [31]. The phagocytic stimuli event makes that cytosolic subunits of the NADPHox can associate with the other plasma membrane counterparts and can form an active complex, a flavoenzyme which is responsible for the generation of large amounts of O$_2^{\cdot-}$ directed towards the internalized invading parasite [31]. Since the O$_2^{\cdot-}$ has an anionic nature that does not diffuse through membranes, its action spectrum is reduced only at the production site. The O$_2$ radical can spontaneously produce H$_2$O$_2$ or can suffer an enzyme-mediated dismutation to H$_2$O$_2$ by the action of superoxide dismutase, which produces an oxidant with higher diffusional capacity. Some metal transition ions in the presence of H$_2$O$_2$ are able to generate hydroxyl radical (OH$^{\cdot}$), an oxidant with high reactivity, but displays poor selectivity against cellular targets. Although resident naïve macrophages can produce ROS at the infection site, this by itself is not sufficient to clear the parasite inside the macrophage phagosome vacuole. Macrophages produce proinflammatory cytokines such as IL-12, IFNγ, and TNF-α, and this results in the priming of macrophages. This event can lead to the induction of the inducible nitric oxide synthase (iNOS) system, which is able to generate high amounts of NO that can be maintained during 24 h, releasing from the macrophages all the whole oxidant cytotoxic power [29, 30]. The NO produced in the cytosol is a hydrophobic radical that diffuses to the phagosome vacuole where it reacts with O$_2$ to produce peroxynitrite (ONOO$^-$), which is a powerful oxidant and

cytotoxic effector molecule, which can kill a variety of pathogens including trypanosomes [32, 33]. However, ONOO⁻ is a short-lived molecule but can cause damage, by direct reactions via one or two electron oxidation mechanisms to several molecules such as thiols and metal centers. Therefore, the ONOO⁻ production by the macrophages during the first hours of infection is fundamental to control the intraphagosomal parasite survival, before the replicative amastigotes can reach the safe cytoplasmic environment [33].

During the first week of infection, several proinflammatory cytokines are produced, preferentially IFNγ by the host natural killer cells. This event is important, since the cytokine production can prime naive macrophages to induce iNOS, which will generate trypanocidal oxidative mediators [16]. Weeks after, the inflammatory cytokines are produced by the host T lymphocytes and in the mean, while the adaptive immune response against the parasite is mounting. The IL-12/IFNγ/iNOS axis has a pivotal role in the control of $T.\ cruzi$ infection and also has important implications for the outcome in Chagas disease [34–36]. These observations have been well documented in conclusive experiments where it was used knockout mice for IFNγ, IL-12, and iNOS [35, 36].

In a very recent study to investigate the role of NOX2-derived ROS, it was found that in $T.\ cruzi$-infected mice, which lack a functional NOX2 oxidase, the cardiomyocytes displayed a proarrhythmic phenotype in the acute phase of experimental Chagas disease [37]. Moreover, it was found that an imbalance between the production of ROS and NO can increase the cellular electromechanical dysfunction which can lead to severe arrhythmias. However, it is possible to inhibit NO production, by a treatment with a nonspecific NOS inhibitor, and attenuate the imbalance between ROS and NO, which leads to a recovery of the cardiomyocyte electrical function and to a decrease of in vivo arrhythmia [37]. The effect of lack of NOX2 oxidase on the heart cells at the chronic phase of Chagas disease has not been described yet.

1.3. ROS as a Promoter of T. cruzi Infection. Upon infection by $T.\ cruzi$, host cells can respond in producing several factors, such as NOS, ROS, and cytokines, among others. As we have discussed, NO and ROS are combined to produce ONOO, which is a powerful killing agent against phagocyted parasites. For several decades, it was believed that ROS was used to eliminate the invading parasites. However, recent evidences have suggested that the parasite proliferation is stimulated in oxidative environments [38–41]. Whether or not an oxidative environment provides the ideal conditions for $T.\ cruzi$ growth is still a matter of discussion. However, Goes et al. [39] have proposed that ROS might play a dual role in the $T.\ cruzi$ life cycle, in which high levels of ROS (oxidative environment) are necessary for proliferation, while a low level of ROS (reduced environment) is obligatory to promote metacyclogenesis.

The recent evidence suggests that ROS are able to produce signals to stimulate $T.\ cruzi$ growth [39, 40]. However, it must be noted that counterevidence for the role of ROS as promoter of cell growth also exists. It has been demonstrated that oxidative stress generated by $T.\ cruzi$ infection

can lead to an increase in the replication rate of the parasite [38–40]. Also, it has been proposed that the reason for this finding is the availability of iron, from heme, to be used by the parasite, since $T.\ cruzi$ is auxotrophic for heme, which is an important cofactor for several enzymes involved in key biological processes and also a source of iron [38]. Heme-induced ROS is able to stimulate epimastigote cell proliferation [38] and recently has been shown that heme regulates the activity of $T.\ cruzi$ TcK2 kinase [42]. TcK2 is an eIF2A kinase, which phosphorylates the alpha subunit of initiation factor 2 involved in protein synthesis. The phosphorylation of this factor by TcK2 decreases translation and in the absence of heme TcK2 is active and promotes cell growth arrest, which leads to the differentiation of proliferative amastigote cells into infective nonproliferative trypomastigote cells [42].

It has been largely documented that an increased ROS production can produce oxidative damage to the host during both the acute and chronic phases of Chagas disease [40, 43, 44]. The increased ROS production has been ascribed to NADPHox2 activation in the unstimulated infected host macrophages [40, 44]. Thus, it might be possible that reduction of the oxidative stress could reduce tissue damage in Chagas disease. Indeed, NADPHox2 enzyme inhibition can ameliorate $T.\ cruzi$-induced myocarditis during Chagas disease [40]. Even though it is expected that antioxidants can increase parasite burden, however, they are unable to do so. Moreover, the treatment of mice, at the beginning of the infection, with antioxidant cobalt protoporphyrin (CoPP), reduces parasite burden. CoPP activates the nuclear factor erythroid-derived 2-like 2 (NRF2), which in turn increases the expression of heme oxygenase (HO-1) [39, 45]. NRF2 is a transcriptional activator that controls gene expression of antioxidant enzymes, when cells are under oxidative stress conditions [39]. The antioxidant was able to reduce parasitemia, tissue parasitism, and reduced macrophage parasitism. Prooxidants were able to promote macrophage parasite burden, indicating that the redox state of the cell is important for $T.\ cruzi$ infection [45, 46]. The effects of CoPP were not directly on the parasites, instead were dependent on the induction of the NRF2/HO-1 pathway. Moreover, either the overexpression of NRF2 or HO-1 can reduce macrophage parasite burden [40]. One of the first studies is launched to systematically assess the effects of oxidative stress on the parasite burden during acute $T.\ cruzi$ infection, contradicted the paradigm of ROS as trypanocidal agent [40, 45]. In this study, nrf2 and ho-1-knockout mice were produced to assess the parasite burden in macrophages derived from those knockout mice. The treatment with CoPP reduced the parasite burden in infected macrophages from ho-1-knockout mice; however, those derived from nrf2-knockout mice did not [40]. However, transfected THP-1 human macrophages with either ho-1 or nrf2 genes, before infection, have reduced parasite burden [40]. Those observations indicate that the effects of CoPP are dependent on the redundant action of the NRF2-controlled genes, since this transcription factor controls the expression of several genes involved in the antioxidant defense. Incubation of infected macrophages with several antioxidants or with the enzyme polyethylene glycol-

conjugated superoxide dismutase (PEG-SOD) reduces parasite burden while respiratory burst-inducer phorbol 12-myristate 13-acetate (PMA), H_2O_2, and prooxidant paraquat can increase the parasite burden [44]. It has been observed that the treatment of $T.$ $cruzi$ cells with low concentrations of H_2O_2 can improve the proliferation and they increase the resistance against sublethal doses of H_2O_2 which can cause oxidative stress [46]. In those studies, it was also observed that H_2O_2 induce the increasing levels of TcCPX and this may be an initial cell attempt to promote detoxification [47]. These observations suggest that adaptation to oxidative stress is assured when cells are first exposed to low concentrations of H_2O_2 and are afterwards exposed to higher concentrations at H_2O_2. Another observation is that $T.$ $cruzi$-infected macrophages, which lack NADPHox, have reduced parasite burden than their wild-type counterparts; however, when those infected macrophages are incubated with H_2O_2, the infection index increases [40]. That indicates that H_2O_2 supplies an additional proliferation stimulus to $T.$ $cruzi$, which is lacking in macrophages that do not have the NADPHox enzyme. From those studies, we can have a general picture that emerges and shows that ROS is able to fuel $T.$ $cruzi$ infection in macrophages during the early acute phase. Whether or not ROS could reach high concentrations inside the invaded macrophages, capable of killing or restraining amastigote growth instead of stimulating their proliferation, is still an unknown matter and it must be studied further on. When infected macrophages are incubated with sublethal doses of up to 100 μM H_2O_2, this promotes a more intensive amastigote proliferation [47]; however, it is unknown the actual concentration of H_2O_2 that can be reached inside the cytosol after this treatment. It is known that the cytosolic concentration of H_2O_2 during macrophage respiratory burst is within the 1-4 μM range [47].

The role played by ROS-producing macrophages at the chronic stage of infection has not been evaluated yet, but it seems likely that at this stage of the disease, most of them are activated by IFNγ to induce the iNOS pathway to produce NO and ONOO with trypanocidal activity. On the other hand, the idea that ROS promotes $T.$ $cruzi$ parasitism might help to explain several results reported earlier in the literature. Cruzipain is the major cysteine protease from $T.$ $cruzi$, and the expression of this an enzyme increases the susceptibility of macrophages to $T.$ $cruzi$ infection [48] and it is the major inducer of NADPHox2 activation, which can produce ROS during macrophage infection and in such a way induces parasite cell growth. Cruzipain is able to cleave chemokines and this activity might be in part responsible for the increased susceptibility of macrophages to infection, due to the lack of macrophage activation. On the other hand, the lack of the gene of signaling lymphocytic activation molecule family member (Slamf1) is a condition that reduces NADPHox2 activation in myeloid cells and was found that it increases resistance to $T.$ $cruzi$ infection [49]. Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that plays important roles in the immune response controlling the expression of genes involved in immune responses, including the activation and differentiation of specific T cell subsets and antigen-presenting cells, and also regulates ROS

production in response to infections. AhR is a suppressor of cytokine signaling pathway in the spleen and heart and as consequence influences NO and ROS production. Indeed, infected AhR-knockout mice displayed significantly reduced parasitemia, inflammation, and fibrosis of the myocardium [50]. This was associated with an anticipated increased immune response characterized by increased levels of inflammatory cytokines and low ROS production. In $vitro$, AhR deficiency caused impairment in parasite replication and decreased levels of ROS production [50]. Also, the treatment of infected mice with curcumin [51] or melatonin [52], which both are able to activate the host antioxidant defenses, reduced parasite burden in blood and heart tissue. Taken altogether, those results are in complete agreement with the general idea that ROS can promote $T.$ $cruzi$ infection.

However, the molecular mechanisms by which ROS can promote $T.$ $cruzi$ infection have to be still fully understood. It has been shown that antioxidants upregulated the expression of H-ferritin (H-Ft), a cytosolic protein that can bind iron and ferroportin-l (Fpn-1), a channel that allows iron efflux [53]. The increased expression of those proteins decreases the labile iron pool (LIP) in macrophages [53, 54]. The decreasing of LIP also decreases the parasite burden, while the increase of the LIP has the opposite effect. Iron also increased the parasite burden of infected $gp9l$ macrophages (which lack NADPHox) to an extent similar to that found in wild-type macrophages. These results can be interpreted that regulation of LIP is the main mechanism underlying the increase in macrophage parasitism produced by oxidative stress [55].

As mentioned before, several research groups have explored the idea that the cellular oxidative environment by itself is a direct growth stimulus for $T.$ $cruzi$. It is most likely that $T.$ $cruzi$ profits from the stress generated from ROS, and this might provide an adaptive evolutionary advantage for the parasite. Many evidences point towards that direction: parasites can proliferate in response to H_2O_2 [47] and the increased growth of epimastigotes in response to H_2O_2 involves a calmodulin-dependent protein kinase II CAMKII-like enzyme in the proliferation pathway and a cascade of events including AMP kinase in the host [38, 56, 57] and also the mitochondrial ROS produced into the mitochondria, which results from heme metabolism and enhances the parasite growth [49]. As mentioned earlier, heme also controls the activity of TcK2 and in turn the process of translation in the parasite. Parasites lacking TcK2 lose this differentiation capacity, and heme is not stored in reserve organelles, remaining in the cytosol. On the other side, TcK2 null cells display growth deficiencies, accumulating H_2O_2 driving the generation of ROS [42]. The augmented level of H_2O_2 occurs as a consequence of increased superoxide dismutase activity and decreased peroxide activity. These observed phenotypes can be reverted by the reexpression of the wild-type TcK2 but not for a dead mutant version of the kinase [42]. These findings indicate that heme is a key factor for the growth control and differentiation through regulation of an unusual type of eIF2α kinase in $T.$ $cruzi$ [42, 58].

The effects of antioxidants, oxidants, and prooxidants have been evaluated on epimastigotes, and while antioxidants

were able to reduce proliferation and increased metacyclogenesis, the oxidants and prooxidants had the opposite effect [44]. Those findings indicate that the redox state of the *T. cruzi* cell is important for cell growth and there must be a cell signaling pathway responsible of transducing the signal. Taken altogether, those results indicate that ROS has a direct effect on the parasite growth through a still unknown pathway of cell signaling.

1.4. Oxidative Stress in Chronic Phase Cardiomyopathy. Researchers disagree about the reasons for Chagas heart disease during the chronic phase, which develops in almost 30-40% of the patients and many years after the vector infects the host, but a strong connection between disease and the presence of the infective agent is still elusive. Chagas heart disease can occur after a cumulative and irreversible tissue destruction damage [30]; however, the reasons for the tissue damage are still not clear enough. More recently, the BENE-FIT clinical trial treatment with benznidazole [59, 60], which was used at the chronic stage of Chagas disease, has revealed that once the damage is established, the progression of heart disease cannot be prevented even if a parasitological cure is achieved, as determined by the parasite clearance in the blood [61], indicating that the parasite presence is not responsible for disease progression. Those results suggest that other factors are involved in the heart disease progression produced by *T. cruzi* infection. However, those factors have not been fully identified yet.

Several works have succeeded to prevent [62, 63] and even reverse [57] the established functional heart disease by using an antioxidant therapy in mice and in an independent way of the therapy's effects on heart parasite burden. Those results suggest that Chagas cardiomyopathy is a ROS-dependent pathology, or at least ROS can contribute in a great extension to it [64]. In *T. cruzi*-infected cardiomyocyte cells, ROS is able to activate the NF-κB activation pathway which induces the production of proinflammatory cytokines such as tumor necrosis factor (TNF) and IL-lβ [65] that might lead to inflammatory responses able to affect heart function [66–68]. It is also possible that ROS might function as a funnel, in which several pathways of injury-causing factors, such as autoantibodies with adrenergic activities or others, can converge to disturb the myocardium physiology, most likely through the Calcium/Calmodulin Protein Kinase II-dependent phosphorylation pathway (CAMKII-like) and AMPK pathway of the host cells [69–71]. A successful work in the treatment of experimental Chagas disease at the chronic stage was done by Vilar-Pereira and colleagues using resveratrol [72]. This is a multitarget drug with cardioprotective properties exerted through NRF2, AMPK, SIRT1, SIRT3, and PGC1α activation [73, 74]. They infected mice with a lineage I *T. cruzi* strain and performed individual electrocardiography (ECG) and echocardiography studies before starting the treatment with the antioxidant resveratrol (at 60 days postinfection) and after another treatment was given at 90 days postinfection. At 90 days postinfection, those infected mice which were treated with resveratrol presented faster heart rate and shorter P wave duration, PR, and QT intervals when compared to infected mice treated without treatment [72]. Also, the treatment with resveratrol shortened P wave duration, PR, and QTc intervals, and increased individual heart rates during 60-90 days postinfection, and those benefits were not seen in infected mice without treatment. The resveratrol treatment activates the AMPK pathway and reduces parasite burden as well as the oxidative stress. Those results indicate that despite all tissue damage, there is a relevant physiological dysfunction in chronic Chagas heart disease which can be eventually reversed and improve cardiac function. This observation is important for the treatment of Chagas disease at the chronic stage.

Also, activation of the AMPK pathway or reducing ROS had similar beneficial effects as resveratrol on heart function during chronic Chagas disease. Metformin is an AMPK activator and has cardioprotective effects and also increases the expression of antioxidant enzymes such as mitochondrial SOD2, while tempol is a drug that neutralizes ROS, since it is a SOD-mimetic drug. The results of the treatments were similar to those with resveratrol, since the treated infected mice showed decreased PR and QTc intervals and also increased heart rates compared to infected mice without treatment [72]. However, metformin or tempol does not reduce parasite burden and these results indicate they do not act in a similar way as resveratrol. Taken altogether, these results suggest that reducing ROS and activating the AMPK pathway are sufficient to improve heart function in chronic Chagas disease even in the presence of the parasite.

In patients with Chagas disease, the activities of the mitochondrial respiratory complexes are depressed, a phenomenon associated with increased ROS production, which is correlated with a mitochondrial dysfunction [62]. Also, there is a high production of superoxide and H_2O_2 in the mitochondria of cardiomyocyte cells of mice chronically infected with *T. cruzi* [63]. This process is the result of electron leakage at the respiratory chain [64]. On the other hand, the treatment of the infected mice with the ROS-scavenger antioxidant PBN improves respiratory chain function and reduces electron leakage in the myocardium of those *T. cruzi*-infected mice [63]. Those results suggest that ROS production and mitochondrial dysfunction are intertwined in a positive feedback loop in this disease. Mice subjected to an acute *T. cruzi* infection produce a severe myocarditis which is characterized by inflammatory infiltrates containing macrophages, neutrophils, CD8 T cells, and tissue oxidative adducts. All of those effects can be prevented, or at least diminished, in the infected mice by the treatment with the NADPHox (NOS) inhibitor apocynin from day 0 of the infection [40]; however, the treatment increased blood and tissue parasite burden. The treatment decreases ROS produced by NADPHox, cytokine production, and T cell proliferation. When the treatment was extended to the chronic phase, no increase was observed in hypertrophy markers (increased cardiomyocyte cell size), fibrosis, or heart weight [40]. It was concluded from these studies that ROS produced by the NADPHox is a critical regulator of the splenic response such as cytokines, T cell, and phagocytic responses, which result in cardiac remodeling in mice with experimental Chagas disease at the chronic phase [40].

An imbalance between antioxidant and oxidant factors is considered the main cause of Chagas disease progression. As discussed earlier, immune cells produce ROS, RSN, proinflammatory cytokines, and peroxynitrite (ONOO-), which is a potent cytotoxic agent against parasites; however, it can also damage host cells. Cardiomyocytes also respond to _T. cruzi_ infection by producing ROS, and several studies have shown that mitochondrion is the main source of ROS (mitochondrial ROS) in those infected cells [67, 68]. Increased mitochondrial ROS production is the result of loss of the structural integrity of the mitochondrial membrane and an effect on the membrane potential [67]. Altered mitochondrial function has been shown in _T. cruzi_-infected cardiomyocytes and in the myocardium of chronically infected animals [69, 75]. Studies in mice have shown that mitochondrial alterations and increased mitochondrial ROS are present in the chronic stage of infection [76, 77]. On the other hand, the increased ROS production during Chagas disease might cause the loss of key antioxidant enzymes of the host. It has been demonstrated that increased production of mitochondrial ROS inhibits the expression and activity of mitochondrial antioxidant enzyme MnSOD and the cytosolic GPx activity and also decreases the content of GSH in the myocardium of chronically infected animals and in Chagas patients [70]. As mentioned earlier, NRF2 is a known transcription factor that controls the gene expression of key antioxidant enzymes in cells under oxidative stress conditions [71]. Activation of NRF2 activity by CoPP induces HO-1 heme oxygenase, which reduces _T. cruzi_ parasitemia and tissue parasitism; conversely, HO-1 inhibition increases _T. cruzi_ in the blood [71]. Investigations on the role of mitochondrial ROS produced by _T. cruzi_ infection show an improvement of the mitochondrial ROS scavenging capacity of infected mice by overexpressing the manganese superoxide dismutase (MnSOD) [78, 79]. In those studies, overexpression of MnSOD preserved the NRF2 transcriptional activity and binding to antioxidant response elements (ARE) on the promoter genes of antioxidant enzymes and also the expression of several enzymes of the antioxidant system, such as HO-1, gamma-glutamyl cysteine synthase (yGCS), and glutathione S transferase (GST). More importantly, the heart structure and function of those infected overexpressing transgenic mice was preserved [78]. Since _T. cruzi_ infection in wild-type mice results in a ROS-dependent decrease of the NRF2 transcription factor activity indicates that preserving the NRF2 pathway will arrest the mitochondrial and cardiac oxidative damage in Chagas disease [78, 79]. These authors also considered that activation of other pathways, which have cardioprotective capacities in ROS-dependent cardiomyopathies, such as diabetes, could potentially restore mitochondrial functions, oxidative phosphorylation (OXPHOS) capacity, mitochondrial biogenesis, and all processes which are impaired in the myocardium of chronically infected mice [80]. For example, the sirtuin 1 (SIRT1) pathway can be activated in response to fasting as stressor and is able to activate several cell functions by deacetylating key cellular proteins, and it is connected to the energy-sensing AMP protein kinase pathway (AMPK) [81]. These two pathways seem to be coordinated with each other and can activate key proteins such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) [80]. PGC1α is a transcriptional coactivator that regulates mitochondrial biogenesis, energy expenditure, OXPHOS, and antioxidant defenses [81]. The authors were able to treat _T. cruzi_-infected mice during the late acute stage with the SIRT1 agonist SRT1720 and assessed heart mechanical function during chronic infection [80]. The SIRT1 activity in heart cells is decreased during the infection; however, resveratrol or SIRT1720 agonist treatment improved its activity along with left ventricular function, although it had no effects on cardiac remodeling or interstitial fibrosis [80]. The coactivator PGC1α was highly acetylated, but the agonist treatment did not improve the compromised mitochondrial biogenesis or the expression of mitochondrial genes. The SRT1720 therapy resulted in a decrease in H_2O_2 production, proinflammatory cytokine production, and cell infiltrate in the myocardium with Chagas disease [80]. The authors associated those benefits to the inhibition of the NFκB transcriptional activity by a SIRT1-dependent mechanism [80]. Those results indicate that chronic cardiomyopathy in Chagas disease is a pathology that depends on ROS production. More importantly, it should be noted that the authors were able to intervene during the development of the disease and achieved long-term prevention, although they were not able to revert it. Taken altogether, those studies indicate that there is an exhaustion of the host antioxidant system during Chagas disease progression due to the increased mitochondrial ROS production and this can result in a persistent mitochondrial dysfunction and ROS production in cardiomyocytes [70, 76, 82]. The persisting mitochondrial ROS production can lead to the expression of fibrotic genes and this might contribute to the chronic cardiomyopathy. ROS also produces an effect on the antioxidant and immune response which could lead to a constant inflammation and the damage of proteins, lipids, and DNA which are characteristics of Chagas disease. It is also possible that mitochondrial ROS could signal long-lasting epigenetic modifications that keep an altered response in cardiomyocytes. All those observations are summarized in Figure 2.

The accumulated evidence suggests that oxidative stress is an important pathogenic factor in Chagas cardiomyopathy. The inhibition of oxidative stress seems to be critical for both improving and preventing heart dysfunction from developing in individuals with Chagas disease. One of the first antioxidants tested as a therapy for Chagas disease was vitamin E and it attenuates oxidative damage of the heart and in conjunction with benznidazole is able to prevent heart oxidative damage [83]. Selenium is already being tested as an antioxidant therapy in Chagas disease [84, 85]. It is expected that soon antioxidants acting through the NRF2 and/or SIRT1 pathways can be tested in clinical trials. All these results are pointing to a physiological heart dysfunction, caused mainly by ROS in individuals with Chagas disease, which can be treated and reversed with the appropriate antioxidants, AMPK activators, and SIRT agonists, despite the interference with parasite burden or cardiac hypertrophy.
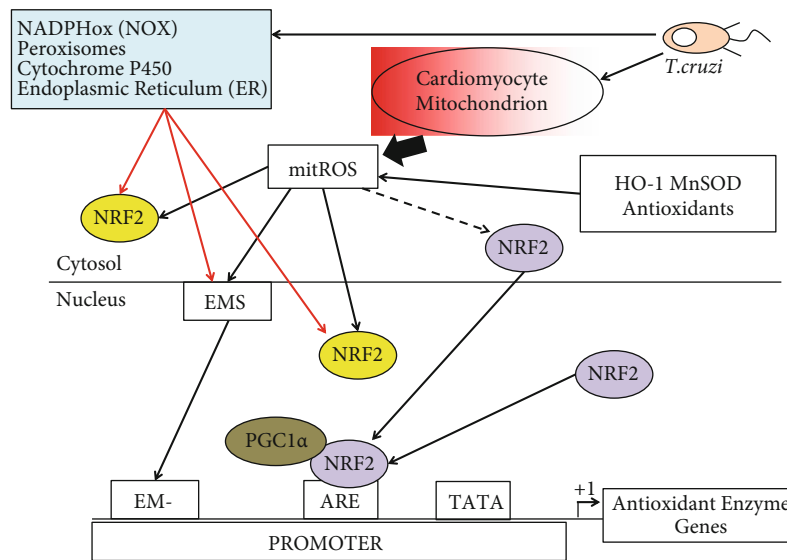
FIGURE 2: A model to explain the effect of increased mitochondrial ROS production on the antioxidant defenses of the cardiomyocytes. The main endogenous ROS sources are indicated. Also, exogenous ROS sources such as UV light, ionizing radiation, environmental agents, and drugs can contribute to ROS production. Mitochondrial ROS (mitROS) can impede the translocation to the nucleus of NRF2 and in addition can inactivate it both in the cytosol and nucleus (yellow NRF2) inhibiting the expression of the antioxidant enzyme genes, which can produce damage on the cell. On the other hand, antioxidant enzymes and antioxidants can neutralize the ROS effects on the NRF2 transcription factor (broken arrow) and this can translocate to the nucleus and activates the expression of antioxidant enzyme genes, leading to overcome the ROS effects. Additionally, ROS can activate an epigenetic marking system (EMS) in order to make a negative epigenetic mark (EM-) on the gene promoters of antioxidant enzymes, which leads to inhibition of the expression of those genes.

Some of those drugs are being tested experimentally in mice, and we hope soon those drugs can be tested in clinical trials to evaluate their use in patients with Chagas disease [86]. More importantly, those drugs and new research could open new frontiers in the treatment and in the development of specific drugs to treat and cure Chagas disease.

1.5. DNA Metabolism under Oxidative Stress in T. cruzi. When the production of ROS exceeds the antioxidant defense capacity, lesions on the DNA are formed, which can be lethal unless they are repaired before replication occurs. As we have discussed, *T. cruzi* is an intracellular parasite and the host-invaded cells can produce high amounts of H_2O_2 in response to the infection. H_2O_2 can trespass membranes and produce damage of parasite and host molecules. *T. cruzi* must to be able to prevent oxidant formation and repair an eventual damage of its own DNA, in order to survive inside the host cells. *T. cruzi* has evolved a series of evolutionary adaptation mechanisms to adapt to the harsh oxidative conditions inside the host cells. In this section, we will focus on the mechanisms by which *T. cruzi* can deal with DNA lesions produced by ROS. However, we must also remember that host-infected cells can be able to activate DNA repair pathways in response to the high ROS produced by the infection which elicits DNA damage.

The DNA is a key target of ROS into the cells, since it represents the template molecule for replication and transcription. Another ROS targets are proteins, membrane phospholipid, and RNAs; however, those are in many copies and in a constant turnover and might not be as important as the DNA. ROS attacks the nitrogenated bases and the sugar-phosphate backbone of DNA producing single- and double-strand breaks (SSB and DSB, respectively), base modifications, apurinic/apyrimidinic (AP) sites, and DNA-protein crosslinks [72, 81, 82]. While DBS are lethal if left unrepaired, oxidized bases might be mutagenic, cytotoxic, or even both of them. One of the most prevalent and mutagenic lesions is 8-oxoguanine (8-oxoG) [87–89]. In eukaryotic cells, different kinds of lesions can be produced in more than one repair DNA system that has evolved to deal with oxidative damage and they form an intricate network that many times can have a functional overlap between them. The DNA breaks are repaired by the recombination pathways, which can proceed through nonhomologous end joining (NHE) or homologous recombination (HR) [87–89]. On the other hand, modified bases are primarily removed by DNA glycosylases, which can produce abasic sites that are repaired by the base excision repair (BER) pathway [87–89]. It has been also suggested that the nucleotide incision repair (NIR) pathway could serve as a backup for BER in the removal of cytosine-derived lesions [87–89]. The nucleotide excision repair (NER) system has been also described as an alternative system for BER to deal with oxidative lesions such as thymine glycol and abasic sites, and the NER enzymes can be regulated by oxidative stress [72, 81, 82]. Moreover, the mismatch repair (MMR) system can control the levels of 8-oxoG in DNA and its role is in the recognition of oxidative lesions which is generally accepted by the researchers in the field [87–89]. The DNA repair process in trypanosomatids has not been extensively studied; however, its understanding has advanced by the publication of the genome sequences from several of them, including *T. cruzi* [90, 91]. The majority of the enzyme-

encoding genes involved in the aforementioned DNA repair processes are present in *T. cruzi* [90, 91].

Those DNA lesions in which the bases are modified by the addition or loss of a small chemical group are repaired by the BER system [87–89]. The classical BER repair pathway begins with the recognition of modified by the DNA glycosylases. Monofunctional DNA glycosylases remove the modified base producing an intact AP site, which is subsequently recognized and acted upon by AP endonucleases (such as APE1), which leaves a free $3'$-OH and a $5'$-deoxyribose phosphate terminus. However, most of the DNA glycosylases involved in the DNA repair of oxidative damage are bifunctional and have an associated AP lyase enzyme activity [87–89]. After the recognition of the lesion, the enzyme can remove the modified base and cleave the DNA at $3'$ side from the AP site produced. This event produces a baseless sugar retained on the $3'$ end, which is subsequently removed by another endonuclease, named APE1. No $5'$-deoxyribose phosphate flap is produced in this case [87–89]. After the base is removed, short patch (SP-BER, single-nucleotide replacement) or long patch (LP-BER, multinucleotide replacement) repair might proceed. All these DNA repair mechanisms end up with the actions of DNA polymerases and ligases to seal the gap. Both processes SP-BER and LP-BER involve distinct sets of enzymes and accessory proteins. In SP-BER for example, DNA pol β synthesizes the missing nucleotide extending from the free $3'$-OH terminal [87–89]. When a $5'$-deoxyribose phosphate flap is present, DNA pol β uses its lyase activity to remove it. The gap is sealed by DNA ligase III and stimulated by XRCC1 [87–89]. In LP-BER, on the other hand, replicative DNA polymerases, such as polδ and polε, are the enzymes in charge of the replication/elongation step and the sealing is carried out by DNA ligase I [87–89]. Sequences encoding essential DNA repair enzymes such as replication factor C, PCNA, APE1/APE2, FEN-1, ligase I, PARP1, DNA polβ, DNA polδ and DNA polε, and several other DNA polymerases have been identified in the *T. cruzi* genome [90, 91]. Also, a gene encoding a DNA glycosylase has been found in *T. cruzi* genome [92]. However, genes encoding DNA ligase III and XRCC1 have not been found, which implies that other enzymes are responsible for the ligation step in SP-BER. Evidence indicates that treatment of *T. cruzi* epimastigotes with high concentrations of ROS can damage the nuclear and kinetoplast DNA and this damage might be partially repaired by the BER pathway [93]. An inhibitor of the BER pathway repair system (methoxyamine) augments the negative effect of ROS on cell viability, suggesting the involvement of this pathway [93].

Interestingly, *T. cruzi* possesses two DNA pol β homologues: Tcpolβ and Tcpolβ-PAK [85]. Both enzymes are localized in the parasite kinetoplast and they present intrinsic $5'$-dexoyribose phosphate lyase activity *in vitro*, the same as the mammalian counterparts [94]. This implies that in *T. cruzi*, in the absence of a nuclear polβ to carry out SP-BER, LP-BER would be most likely the sole BER pathway available in the nucleus. It is noteworthy mentioning that recombinant Tcpolβ-PAK is able to perform DNA synthesis over 8-oxoG *in vitro* suggesting its involvement in a pathway named translesion synthesis (TLS) in mitochondrial DNA [94]. This pathway will be discussed in more detail later. On the other hand, Tcpolβ does not possess this ability; however, its overexpression enhances the survival of *T. cruzi* strains to H_2O_2 [94]. Moreover, recombinant Tcpolβ requires additional cellular proteins to be able to repair *in vitro* short gaps of DNA, suggesting that in vivo is part of a multiprotein repair system [95]. Whether or not Tcpolβ can perform mitochondrial TLS *in vivo* remains to be investigated. There is evidence for a role of Tcpolβ in dealing with oxidative lesions of the kinetoplast DNA, most likely through the BER mechanism of repair. Indeed, when epimastigote cells are treated with H_2O_2, a new focus for Tcpolβ localization is detected, besides the two Tcpolβ foci, which are localized on kinetoplast DNA antipodal sites in the absence of treatment, suggesting that it could correspond to a DNA repair site [94]. The parasites overexpressing Tcpolβ displayed reduced levels of 8-oxoG in the kinetoplast DNA when compared to normal cells [94]. Furthermore, we have demonstrated that *T. cruzi* epimastigote and trypomastigote cells can respond to H_2O_2 treatment expressing higher levels of Tcpolβ compared to untreated cells [87]. Tcpolβ can be found in two forms in *T. cruzi* cell extracts. The high form is heavily phosphorylated (H), while the low form is unphosphorylated (L). Interestingly, the high form is more active in *in vitro* DNA synthesis [96]. The levels of the phosphorylated active form of the enzyme are higher in H_2O_2-treated cells compared with untreated cells, suggesting that this activity is involved in repair of the kinetoplast DNA and perhaps is also responsible of the kinetoplast DNA replication [96]. A mechanistic model to explain the overexpression of Tcpolβ is outlined in Figure 3 and it can be observed the different protein families involved in the posttranscriptional expression of the enzyme. Tcpolβ can be crosslinked to kinetoplast DNA, but no to nuclear DNA, indicating that the enzyme is always in contact with kinetoplast DNA [96]. We believe that several protein kinases are involved in the phosphorylation of Tcpolβ and those kinases are involved in the signal transduction pathway triggered by the H_2O_2 treatment. Perhaps, a similar pathway operates to transduce the positive growth signal triggered by ROS on *T. cruzi*. Those protein kinases can act either by phosphorylating Tcpolβ and/or by modifying proteins that can be bound to the Tcpolβ mRNA in order to make it a better template for protein translation, thus increasing the activity and the amount of the enzyme. Most likely, other proteins and DNA polymerases involved in the process of DNA repair can be positively modulated by H_2O_2 treatment, but those experiments will wait until specific antibodies against them can be developed. Another mitochondrial located DNA polymerase which is able to perform TLS *in vitro* is Tcpolκ, which can bypass 8-oxoG lesions *in vitro*, and its overexpression together with Tcpolβ and Tcpolβ-PAK in *T. cruzi* cells augments parasite survival against benznidazole [97].

Since the trypanosomatids possess a single mitochondrion, it is possible that they need a powerful system for DNA repair, because the mitochondria are important source of oxidative stress and many mitochondrial-encoded proteins are important for parasite survival. Also, the efficient kinetoplast DNA replication is essential for parasite duplication, since any blocks in the replication fork could affect the
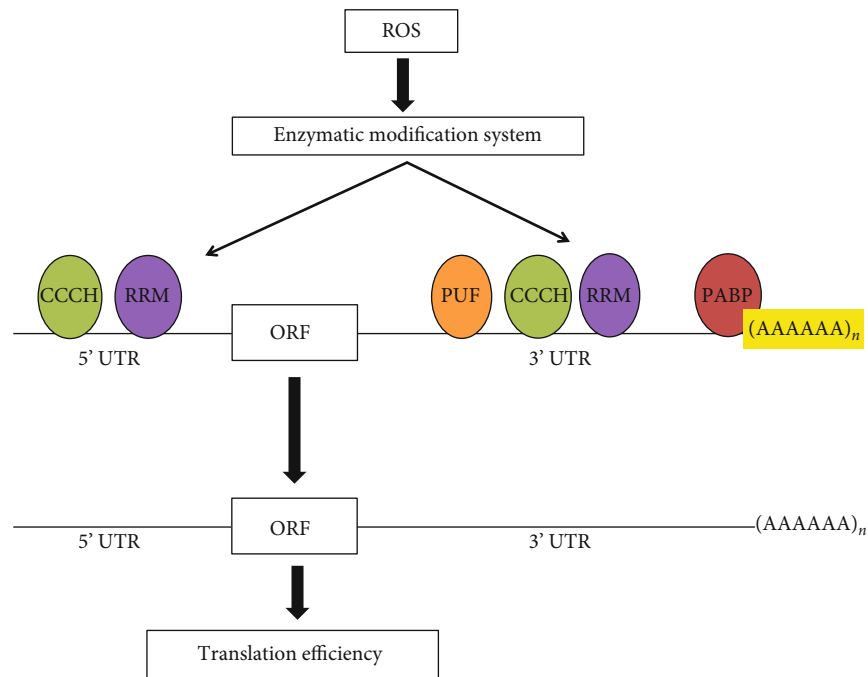
Figure 3: Regulation of Tcpolβ expression at the posttranscriptional level. The Tcpolβ mRNA is bound at the $5'$ UTR, $3'$ UTR, and poly A tail by different RNA binding proteins, which mainly contains the RNA recognition motif (RRM), Pumilio/Fem-3 mRNA domain (PUF), the zinc finger CCCH domain, and the poly A binding protein (PABP), which can act from splicing to RNA function and turnover. The Tcpolβ mRNA can be bound by any of those proteins and increase the splicing rate and/or decrease the translation efficiency of the mRNA. A signal provided by ROS could act on an enzymatic modifying system, which in turn acts on the bound factors, causing the release from the mRNA to increase translation efficiency. The PUF family binds to the $3'$ UTRs, while CCCH domain and the RRH domain binds to both $5'$ and $3'$ UTRs. Those proteins bind to specific sequences on the UTRs.

parasite viability. Therefore, the mitochondrial BER activity given by Tcpolβ and TLS activity of Tcpolβ-PAK and Tcpolκ are essential for the parasite and hence allow the progression of kinetoplast DNA replication even when oxidative lesions are present.

As mentioned earlier, in addition to BER and the repair systems mentioned earlier, a tolerance pathway, called TLS, has evolved from bacteria to humans. This system contains DNA polymerases able to replicate the DNA across several DNA lesions, such as 8-oxoG and thymine glycol. This TLS system is able to ensure genome integrity, but generates increased mutations, since the TLS polymerases have lower fidelity than those involved in DNA replication. Several genes encoding TLS DNA polymerases are present in the *T. cruzi* genome including polβ, polη, and polκ, indicating TLS can be operative in *T. cruzi* [87–89, 97, 98]. On the other hand, it has been shown that Tcpolη, a nuclear located DNA polymerase, has TLS activity *in vitro* and its overexpression in *T. cruzi* cells leads to higher parasite survival in the presence of H_2O_2 [98]. This DNA polymerase can bypass cyclobutene pyrimidine dimers and also can bypass 8-oxoG lesions. Its ability to confer resistance to oxidative lesions might be associated with the ability to bypass 8-oxoG lesions in the DNA. This suggests that Tcpolη has a role in TLS of nuclear DNA oxidative damage.

Despite the fact that BER is the main repair pathway to repair the oxidized base 8-oxoG, another repair pathway might also operate in *T. cruzi* to repair this lesion in the

nuclear DNA. In higher eukaryotes, it is involved in the repair of oxidative lesions in the nuclear DNA and it is named mismatch repair (MMR) system. The proteins able to recognize the mismatches in the DNA are a dimer of MSH2-MSH6, which is the *E. coli* MutS system counterpart. Both MSH2 and MSH6 genes are present in *T. cruzi*, and different isoforms of MSH2 exist [99, 100]. Moreover, *T. cruzi* strains, in which one of the chromosomal copies of MSH2 was deleted, are more sensitive to H_2O_2 treatment than the wild-type cells [100]. On the contrary, *T. cruzi* strains lacking both copies of MSH2 genes are more resistant to H_2O_2 treatment than the wild-type parasites [100]. Although MSH2 might have a role dealing with oxidative lesions of the DNA, it seems to be that the MMR is not directly involved in the repair of oxidative damage and perhaps the MSH proteins could be involved in other signaling pathways, such as the signaling per se of oxidative damage to other repair systems. The finding that basal 8-oxoG levels are higher in H_2O_2 *T. cruzi* hybrid strains (lineage II) compared to *T. cruzi* of the lineage I could corroborate the hypothesis that *T. cruzi* from the lineage I are more resistant to H_2O_2 than those hybrid strains. This means that higher 8-oxiG levels in *T. cruzi* hybrid strains would implicate in the recruitment of high numbers of MSH2 protein to deal with the lesions, thus affecting the MMR pathway efficiency under stressful conditions, such as treatment with genotoxic agents [101]. An overview of the *T. cruzi* DNA repair system location is given in Figure 4. BER repair system has not been found in the
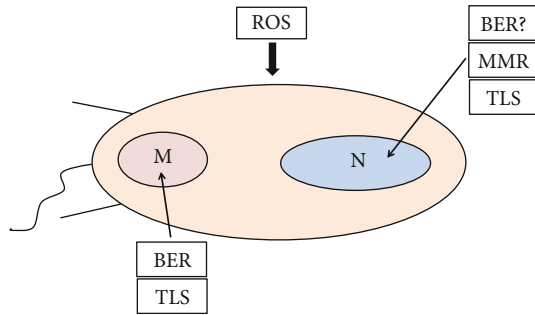
FIGURE 4: DNA repair mechanisms under oxidative stress. *T. cruzi* has several mechanisms to repair the damage caused by ROS. To repair, the nuclear DNA possesses the mismatch repair system (MMR), the translesion synthesis repair system (TLS), and likely a base excision-like repair system (BER-like). The kinetoplast DNA is repaired by a base excision repair system (BER, Tcpolβ, and Tcpolβ-PAK) and a TLS repair system. See text for details.

nucleus; however, a BER-like repair system operating at the nucleus cannot be ruled out yet.

In general, the role of the enzymes involved in the oxidative stress response can be demonstrated upon the overexpression of those enzymes and the increased tolerance that the parasites show upon ROS exposure. For example, the role of BER in the oxidative stress response can be observed in parasites overexpressing the APE1 endonuclease, which show an increased tolerance to persistent ROS exposure [102]. On the contrary, parasites treated with a BER pathway inhibitor methoxyamine have a diminished cell viability in the presence of high concentrations of ROS, which can cause nuclear and kinetoplast DNA damage [93].

2. Concluding Remarks and Future Directions

It has become clear that ROS produced by *T. cruzi*-infected cells have several functions on the host-pathogen interactions. ROS produces a positive signal on *T. cruzi*, which induces cell growth. On the other hand, ROS is able to activate a cell signal pathway in the host cell, leading to proinflammatory cytokine production. Moreover, ROS can produce a signal, which induces chronic heart tissue damage in the chagasic patient, even several years (decades) after the infection was produced. It can be concluded that *T. cruzi* possesses powerful antioxidant defense systems, which can deal with ROS produced by the host cell. The detoxification systems use thiol compounds and several enzymes to detoxify the ROS molecules produced mainly inside the mitochondria of the host-infected cell. To protect the DNA against ROS-produced oxidative damage, *T. cruzi* has several DNA repair systems including BER, TLS, MMR, and perhaps another unidentified yet DNA repair systems.

Seems to be that parasite burden is not necessarily responsible for chronic heart damage and classical antichagasic drugs no longer function to treat the disease at the chronic stage. Thus, there is a need to develop new drugs to treat the disease and antioxidants, drugs that can activate the expression of the host antioxidant enzymes or

that can inhibit cytokine, and ROS productions are the most promising to treat the Chagas disease at the chronic stage. Also, the possibility of using combined therapies must be considered.

Although there are plenty of information on the effects among host-pathogen interactions during *T. cruzi* infection, several issues on the molecular mechanisms by which ROS produces beneficial effects on the pathogen and harmful effects on the host must be explained. Although it is quite difficult to envision a mechanism to explain the persistent signal that ROS produces on the host leading to accumulative progressive heart tissue damage, even decades after the initial infection and in the absence of the pathogen. Also, it is necessary to explain whether or not ROS produced by the host acts directly on the parasite by producing a cell signal to enhance its growth.

We could put forward the following hypothesis to explain those observations:

(1) At the beginning of the infection, the ROS produced in response to *T. cruzi* infection can elicit a signal transduction pathway on the pathogen in order to be induced to proliferate. That signal could be sensed and transduced trough the MAPK pathway, since the *T. cruzi* genome contains several genes encoding potential MAPKs and in higher eukaryotes those are known to play important roles in cell signaling regulating key processes such as proliferation and stress response. We could image that a MAPK kinase senses and transduces the ROS signal phosphorylating and also by increasing the production of key enzymes and proteins, such as those involved cell division, transcription, DNA replication, DNA repair, and others. It must be noted that those MAPKs should act at the translational/modification level, since transcriptional regulation has not been described in *T. cruzi*

(2) At the chronic stage of the infection, *T. cruzi*-infected cardiomyocytes could produce a long-lasting mark on its own molecules, which persists for a long time. We could imagine that an epigenetic mark could be made on the genome DNA, specifically on those gene promoters, which encode proinflammatory cytokine genes in order to maintain the genes on. Alternatively, an epigenetic mark could be made on gene promoters encoding transcription factors, which control the expression of antioxidant enzymes, in order to keep these genes off. For example, that mark could be a *T. cruzi*-induced methylation on the gene promoters, which can regulate the activity of these gene promoters and therefore the expression of those genes. This hypothesis is outlined in Figure 5

The identification of MAPKs, transducing the ROS signal, might be good candidates for new drug development against *T. cruzi* in order to obtain a high arsenal of drugs to treat the Chagas disease, since there is a very limited number of drugs to treat the disease, and they are rather toxic. Assuming that a protein key kinase or phosphatase crucial
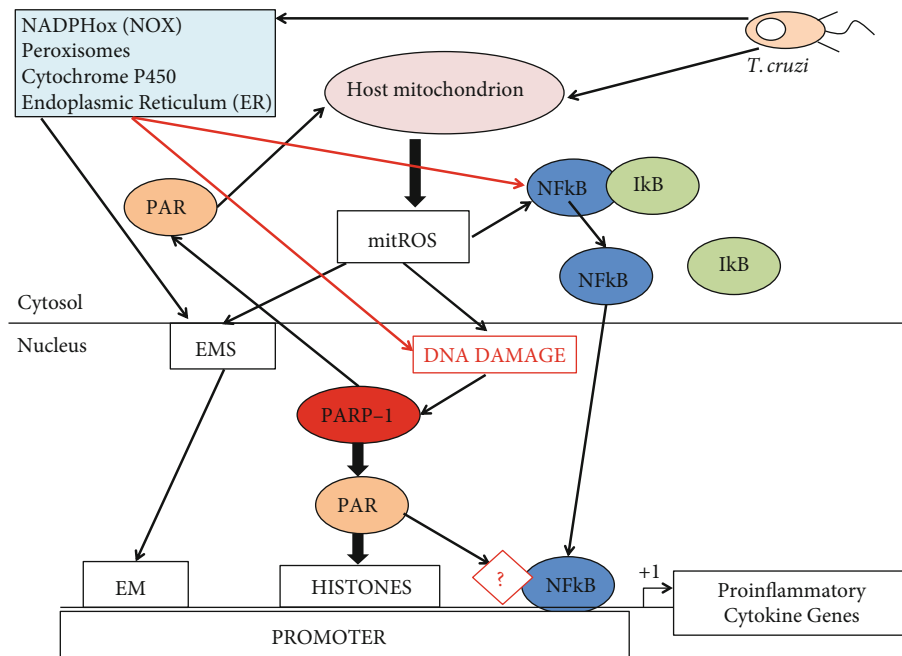
FIGURE 5: A model to explain the tissue damage in the host heart at the chronic stage of Chagas disease. The parasite inside the cells induces ROS production by the mitochondria of the host cell and also by the NADPHox. The ROS signal produces nuclear DNA damage, PARP activation, epigenetic marking system (EMS) activation, and NFκB translocation by dissociation of IκB. PARP-1 (poly ADP-ribose polymerase) produces poly ADP-ribose (PAR) polymers, which help the binding of NFκB on the gene promoter and produce epigenetic marks (EM) on the promoter histones, which together with the binding of NFκB can activate proinflammatory cytokine gene expression. Most likely another factor (?) is activated by PAR and helps NFκB to bind to the gene promoter. Also, an EM can be done on the gene promoter by the EMS keeping the proinflammatory cytokine genes active, leading to cardiac remodeling. On the other hand, PAR from the nucleus can travel to the cytosol and provides a signal to the mitochondrion to keep producing ROS. In addition, PARP-1 activity facilitates DNA repair. This model is complementary to that presented in Figure 2, since inactivation of the NRF2 axis can cause damage on the cell.

to regulate a biological process in *T. cruzi*, however, is different from a human counterpart, a specific inhibitor could be found or designed, thus affecting mainly the parasite protein without harming the host. With the availability of the *T. cruzi* genome, a detailed biochemical analysis of kinases and phosphatases could be done, to improve the knowledge of their characteristics and to be used to develop new therapeutic agents against Chagas disease. Studies can be designed to identify the long-lasting signal that persists in the cardiomyocytes, even in the absence of parasite burden, and additionally, the positive ROS-provided signal to the pathogens must be identified. It is possible that with the new tools of genomics, transcriptomics and proteomics those alterations and signals might be identified in a near future.

Abbreviations

ROS: Reactive oxygen species
RNS: Reactive nitrogen species
TR: Trypanothione
T(SH$_2$): Dithiol trypanothione
TcTXN: Tryparedoxin
TcTS: Trypanothione synthetase
TcTR: Trypanothione reductase
TcAPX: Ascorbate-dependent hemoperoxidase

TcMPX/TcCPX: Tryparedoxin peroxidase
GPX-I: Glutathione-dependent peroxidase I
FeSOD-A: Iron-dependent superoxide dismutase
MnSOD: Manganese-dependent superoxide dismutase
GPX-II: Glutathione-dependent peroxidase II
NADPHox: NADPH oxidase
IFN: Interferon
Tcpol: *T. cruzi* polymerase
BER: Base excision repair
MMR: Mismatch repair
TLS: Translesion synthesis
NFκB: Nuclear factor kappa-light-chain-enhancer of activated B cells.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Edio Maldonado and Diego A. Rojas contributed equally to this work.

Acknowledgments

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Research Article

Reactive Oxygen Species Induce Endothelial Differentiation of Liver Cancer Stem-Like Sphere Cells through the Activation of Akt/IKK Signaling Pathway

Zhengbin Zhao,[1] Jing Gao,[2] Caili Li,[3] Xiaoli Xu,[4] Yihuan Hu,[3] and Shuangsheng Huang [3]

[1]*Department of Infectious Diseases, The First Hospital of Lanzhou University, Lanzhou 730000, China*
[2]*Hospital of Northwest Minzu University, Lanzhou 730030, China*
[3]*Medical College of Northwest Minzu University, Lanzhou 730030, China*
[4]*School of Basic Medicine, Lanzhou University, Lanzhou 730000, China*

Correspondence should be addressed to Shuangsheng Huang; huangshsh02@qq.com

Cancer stem cells (CSCs) from various cancers are able to transdifferentiate into endothelial cells and further form functional blood vessels, indicating another possible resistance mechanism to antiangiogenic agents. However, it remains unclear whether CSCs from hepatocellular carcinoma have the ability to differentiate into endothelial cells, and thus resulting in resistance to antiangiogenic therapy targeting VEGF. Reactive oxygen species (ROS) are involved in the self-renewal and differentiation of CSCs, yet, their role in endothelial differentiation of CSCs has been poorly understood. In this study, we found that cancer stem-like sphere cells enriched from human hepatocellular carcinoma cell line Hep G2 could differentiate into endothelial cells morphologically and functionally, and this process could be blocked by Akt1/2 kinase inhibitor and IKK-β inhibitor BAY 11-7082 but not by Bevacizumab, a VEGFA-binding antibody, and DAPT, a γ-secretase inhibitor. Both hydrogen peroxide and BSO (an inhibitor of GSH biosynthesis) induce the differentiation of cancer stem-like sphere cells into endothelial cells, which can be canceled by the antioxidant N-Acetyl-L-cysteine (NAC). We also found that hydrogen peroxide or BSO induces the phosphorylation of Akt and IKK of endothelial differentiated sphere cells. Accordingly, both Akt1/2 kinase inhibitor and BAY 11-7082 inhibited hydrogen peroxide and BSO-mediated endothelial differentiation of cancer stem-like sphere cells. Collectively, the results of the present study demonstrate that cancer stem-like sphere cells from Hep G2 are able to differentiate into endothelial cells both morphologically and functionally, and this process is independent of VEGF and NOTCH signaling but dependent on the activation of Akt and IKK. ROS promote endothelial differentiation of cancer stem-like sphere cells through activation of Akt/IKK signaling pathway. Therefore, our study reveals a novel mechanism of resistance to conventional antiangiogenic therapy and may provide a potential therapeutic target for liver cancer treatment.

1. Introduction

Because vascular endothelial cells are genetically stable, it was once assumed that antiangiogenic therapy targeting endothelial cells would not become resistant. However, evidences from preclinical and clinical studies demonstrate that cancer cells can also acquire resistance to antiangiogenic agents. A variety of possible mechanisms, such as producing redundant angiogenic factors following anti-VEGF therapies, recruiting new vessels by vessel cooption or vasculogenic mimicry, and producing more invasive and metastatic tumor cells, have been elucidated [1–3]. Cancer stem cells (CSCs), also termed cancer initiating cells, are a small subgroup of cancer cells that have the ability to self-renew and differentiate to diverse cells that comprise the tumor. These cells are believed to be the primary cause of cancer recurrence and chemotherapy-resistance [4–6]. Recently, studies have shown that CSCs from some cancers, such as glioblastoma [7–10], breast cancer [11], and ovarian cancer [12], are able to transdifferentiate into endothelial cells and further form

functional blood vessels. Because the process of this new vessel formation is VEGF-independent, it is resistant to the anti-VEGF therapy, indicating another possible resistance mechanism to antiangiogenic agents.

Liver cancer is the sixth most commonly diagnosed cancer and the fourth leading cause of cancer death worldwide. It is estimated that there are 841,000 new liver cancer cases and 782,000 deaths during 2018 [13]. Despite significant advances in combined therapies, hepatocellular carcinoma (HCC) is still characterized by a poor prognosis and a low survival rate during the past decades, due to recurrence and metastasis, as well as the failure of chemo- and radiotherapy [14]. Because HCC is a highly vascularized tumor, anti-angiogenesis therapies have become the standard care for recurrent and unresectable HCC. Unfortunately, the efficacy is modest and limited. The recurrence rate and mortality have not been significantly improved [15, 16]. Studies have showed that endothelial cells from HCC possess increased angiogenic activity and resistance to chemotherapeutic drugs and angiogenesis inhibitors [17]. However, it is not clear whether there exist a proportion of CSCs-differentiated endothelial cells in HCC, which results in the resistance to antiangiogenic therapy.

Reactive oxygen species (ROS), acting as signaling molecules, can regulate the proliferation, differentiation, and apoptosis of various cancer cells [18]. Studies also demonstrate that ROS play important roles in stem cells. For example, it has been shown that hematopoietic stem cells contain lower levels of ROS than their more mature progeny and that these differences appear to be critical for maintaining stem cell function [19]. Accordingly, increasing the ROS level in hematopoietic stem cells by knockout the gene of Atm or FoxO may promote the differentiation of hematopoietic stem cells [20, 21]. These results indicate that ROS play an important role in regulating the differentiation of hematopoietic stem cells. Similar to hematopoietic stem cells, CSCs also contain lower levels of ROS than non-CSCs, which may be due to reduced ROS production and/or enhanced ROS scavenging systems in the CSCs [22]. However, whether ROS regulate endothelial differentiation of CSCs is not known yet. So we ask whether there exists a new angiogenic pattern, in which the endothelial cells of HCC are derived from the transdifferentiation of CSCs and whether this process is regulated by ROS. To test the hypothesis, we examined the ability of liver cancer stem-like cells to transdifferentiate into endothelial cells and the regulating role of ROS on this process.

2. Materials and Methods

2.1. Chemicals and Reagents.
Dulbecco's modified Eagle's medium (DMEM) and DMEM/F12 medium were obtained from Gibco (Thermo Fisher Scientific, Inc. Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from National HyClone Bio-Engineering Co., Ltd. (Lanzhou, China). Recombinant human vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) were purchased from Pepro-Tech (Rocky Hill, NJ, USA). B27 supplement without vita-min A and N2 supplement were purchased from Invitrogen (Carlsbad, CA, USA). Growth factor-reduced Matrigel was purchased from Becton Dickinson (Bedford, MA, USA). MCDB131 medium, hydrocortisone, sulforhodamine B (SRB), $2',7'$-dichlorodihydrofluorescein diacetate (DCFH-DA), N-Acetyl-L-cysteine (NAC), γ-secretase inhibitor (DAPT), Akt1/2 kinase inhibitor, and L-Buthionine-sulfoximine (BSO) were purchased from Sigma (St. Louis, MO, USA). Bevacizumab was purchased from Roche Pharma (Schweiz) Ltd. (South San Francisco, CA, USA). IκB phosphorylation inhibitor (BAY 11-7082) was obtained from Beyotime Biotechnology (Shanghai, China). Primary antibodies directed against IκB kinase (IKK; ab178870) and p-IKK (ab55341), rabbit polyclonal to CD31 (ab32457), and mouse monoclonal (4F9) to Von Willebrand Factor (vWF, ab20435) were purchased from Abcam (Cambridge, MA, USA). Primary antibodies directed against protein kinase B (Akt; sc-8312), p-Akt (sc-7985-R), and β-actin (sc-130656) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The secondary antibodies of Alexa Fluor®594-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) and Alexa Fluor®488-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG (ZB-2301) was purchased from ZSGB-BIO (Beijing, China).

2.2. Cell Culture.
Human hepatocellular carcinoma cell line Hep G2 cells were cultured in DMEM supplemented with 10% FBS. Human microvascular endothelial cell line HMEC-1 cells were cultured in MCDB131 medium supplemented with 1.18 mg/ml NaHCO$_3$, 20% inactivated FBS, 10 ng/ml EGF, and 1 μg/ml hydrocortisone. All cells were cultured in a highly humidified atmosphere of 5% CO$_2$ at 37°C.

2.3. Sphere Formation and Passage.
Sphere was cultured as previously described with some modification [23]. That is, Hep G2 cells were collected, washed to remove serum, and then suspended in serum-free DMEM/F12 medium supplemented with 10 ng/ml bFGF, 20 ng/ml EGF, 2% B27, and 1% N2 supplement. Subsequently, the cells were cultured in ultralow attachment 6-well plates at a density of 2,000 cells/ml in 2 ml culture medium. Sphere formation was then tracked on days 1, 3, 5, 7, 9, and 11 under an inverted microscope. To propagate spheres, the spheres were collected, dissociated with trypsin, and resuspended in serum-free DMEM/F12 medium. The dissociated single cells were then similarly transferred into ultralow attachment 6-well plates as mentioned above. The third passage of sphere cells was collected for the following experiments.

2.4. Endothelial Differentiation Assay.
Sphere cells dissociated with trypsin were cultured in MCDB131 complete endothelial medium as described in Section 2.2 with or without 10 ng/ml VEGF and passaged when they reached 95% confluence. After being passaged for two generations, cells were collected and regarded as endothelial differentiated sphere cells for the following experiments.

2.5. Immunofluorescence Assay. Cells were fixed with methyl and blocked with 1% BSA. The primary antibodies, including rabbit polyclonal to CD31 and mouse monoclonal to vWF, were added and incubated overnight at 4°C. After washing with PBS, the secondary antibodies labeled with fluorescent dyes were added and incubated at room temperature for 1 h. Then, the cells were counterstained with DAPI, and images were captured under a fluorescent microscope.

2.6. Tube Formation. Cells were seeded at a density of 7.5×10^4 per well in 100 μl MCDB 131 medium on 96-well plates precoated with Matrigel. After incubated for 24 hours, images were captured under an inverted microscope.

2.7. Cell Viability Assay. Cell viability was determined by SRB assay with some modifications [24]. Endothelial differentiated sphere cells were plated into 96-well plates at a density of 1×10^4 per well in 100 μl culture medium. After incubation overnight, cells were treated with different concentrations inhibitors for 24 h. The cultures were fixed with cold 10% trichloroacetic acid at 4°C for 1 h and then washed with water. After the plates were air-dried, the fixed cells were stained with 0.4% SRB for 10 min and washed repeatedly with 0.1% acetic acid to remove the unbound dye. The bound SRB was dissolved in 1% Tris base solution (pH 10.5). The optical density was measured at 510 nm on a microplate reader.

2.8. ROS Assay. To study intracellular ROS levels, cells were stained with 10 μM DCFH-DA at 37°C for 30 minutes. Subsequently, cells were rinsed with D-Hanks' solution to remove the excess dye. Then, the cells were resuspended in D-Hanks' solution and immediately analyzed by flow cytometry.

2.9. Western Blotting. After treatments, cells were lysed with RIPA buffer supplemented with proteinase inhibitors. Equal amounts of protein from each sample were resolved by 6%-10% SDS polyacrylamide gels and then transferred onto the polyvinylidene fluoride (PVDF) membrane. After blocking with 5% fat-free milk, the membrane was incubated with the primary antibody at 4°C overnight, followed by exposure to a horseradish peroxidase-conjugated secondary antibody for 2 h. Then, the protein bands were visualized using enhanced chemiluminescence reagents on X-ray film.

2.10. Statistical Analyses. Results were expressed as mean ± SD. Statistical analyses were performed by Student's t-test and ANOVA using the SPSS version 20 statistical analysis package (SPSS Inc., Chicago, IL, USA). P value less than 0.05 was considered significant.

3. Results

3.1. Hep G2 Cells Could Form Spheres. After being plated in sphere induction medium in ultra-low attachment 6-well plates, Hep G2 cells grew in an anchorage-independent fashion and formed spheres 7-9 days later. When the spheres were passaged, cells dissociated from spheres could form
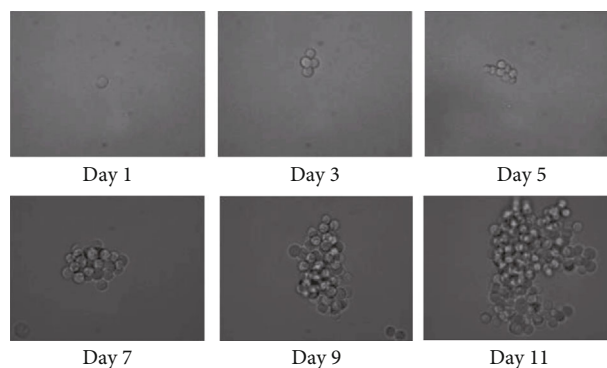
Figure 1: Hep G2 cells form nonadherent, self-renewing spheres in stem cell-conditioned medium. The sphere formation of a single cell was recorded at days 1, 3, 5, 7, 9, and 11 (200×).

new spheres again. Figure 1 recorded the process of single Hep G2 cell forming a sphere.

3.2. Sphere Cells Differentiate to Endothelial Cells. As shown in Figure 2, after cultured in MCDB131 endothelial medium for two generations, adherent Hep G2 cells did not express endothelial markers, including CD31 and vWF. In contrast, following the same culture conditions as Hep G2 cells, sphere cells, now regarded as endothelial differentiated sphere cells, expressed both endothelial markers. Moreover, addition of 10 ng/ml VEGF in MCDB131 endothelial medium did not affect the CD31 and vWF expression of sphere cells. Similar to sphere cells, HMEC-1 cells, which were selected as a positive control, also expressed both CD31 and vWF under the same culture conditions. Formation of capillary-like structures on Matrigel is one of the widely used functional tests for endothelial cells. We then examined the ability of tube formation with tube formation assay. After seeded on Matrigel for 24 h, adherent Hep G2 cells failed to form tube structures. However, both endothelial differentiated sphere cells and HMEC-1 cells developed tube structures. Addition of VEGF (10 ng/ml) did not affect the tube formation of endothelial differentiated sphere cells. All these results indicated that sphere cells from Hep G2 have the ability to differentiate into endothelial cells.

3.3. The Endothelial Differentiation of Sphere Cells Is Independent of VEGF and NOTCH Signaling but Dependent on the Activation of Akt and IKK. To further explore the underling mechanisms of endothelial differentiation of sphere cells, we investigated the impacts of Bevacizumab, a VEGFA-binding antibody currently in clinical use, DAPT, a γ-secretase inhibitor that effectively inhibits Notch signaling, Akt1/2 kinase inhibitor, and BAY 11-7082, an IKK-β inhibitor, on the tube formation of endothelial differentiated sphere cells. As shown in Figure 3, exposure to 1, 2 mg/ml Bevacizumab did not affect the tube formation of endothelial differentiated sphere cells, yet it blocked the tube formation of HMEC-1 cells. These results, combined with the above results that addition of VEGF did not affect the CD31 and vWF expression and tube formation of endothelial differentiated sphere cells, indicated that sphere cells differentiate into

(a)

(b)

(c)

(d)
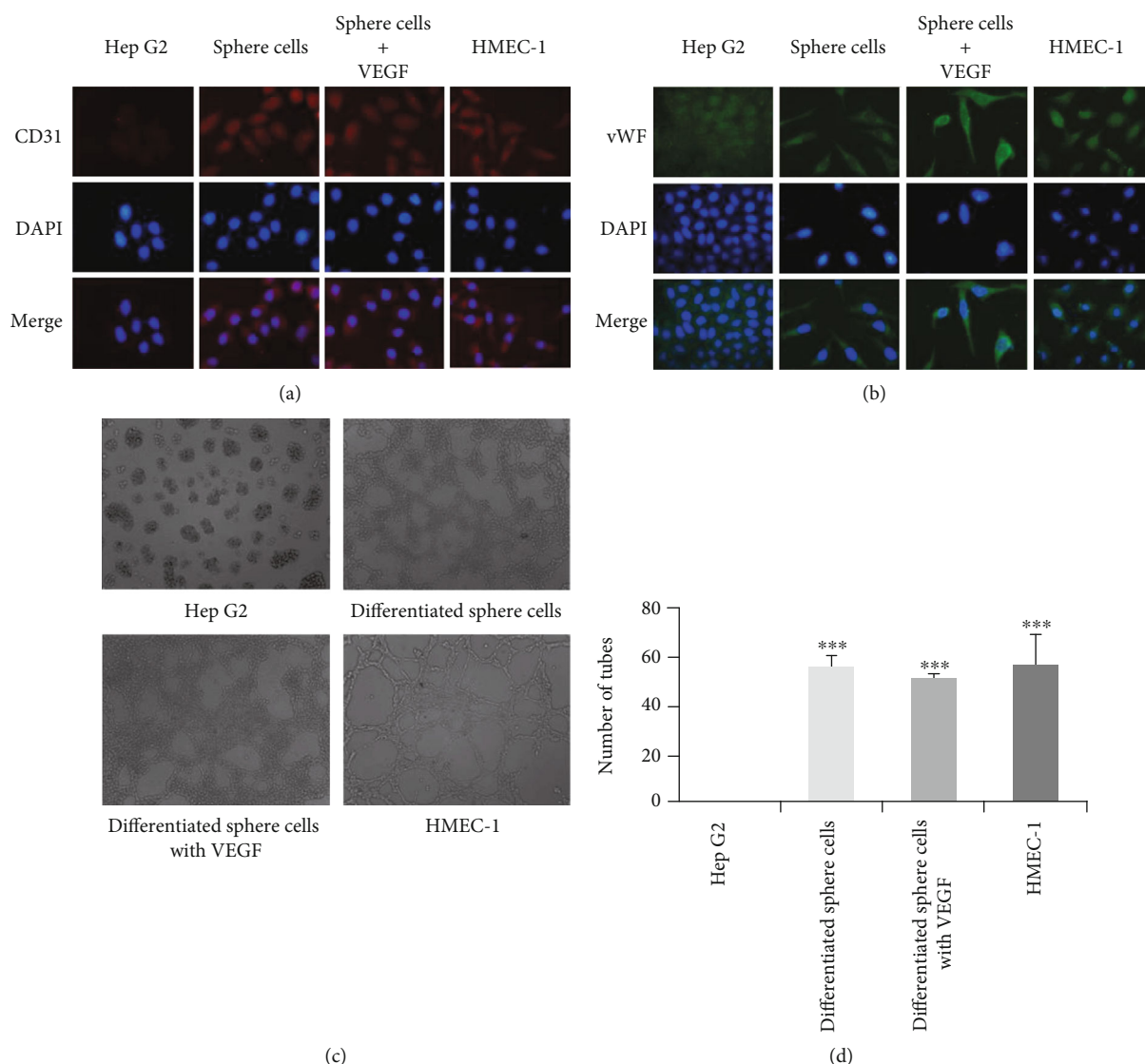
FIGURE 2: Cancer stem-like sphere cells differentiate to endothelial cells. Cells were cultured in MCDB131 endothelial medium with or without VEGF (10 ng/ml) for two generations. Expressions of endothelial markers CD31 (a) and vWF (b) were detected with immunofluorescence staining. The cell nuclei were counterstained with DAPI (200 ×). (c) Formation of capillary-like structure on Matrigel (100×). (d) A number of formed tubes were calculated. The data were presented as mean ± SD ($n = 3$). ***$P < 0.001$ vs. Hep G2 group.

endothelial cells in a VEGF-independent manner. Likewise, treatment with DAPT at the concentrations of 5, 10, and 20 μM did not affect the tube formation of endothelial differentiated sphere cells. In contrast, addition of Akt1/2 kinase inhibitor (2, 10, 50 μM) resulted in significant suppression of tube formation of endothelial differentiated sphere cells. Moreover, the same inhibition results were also observed when we treated endothelial differentiated sphere cells with different concentrations of BAY 11-7082. In addition, to determine whether the effects of Bevacizumab, DAPT, Akt1/2 kinase inhibitor, and BAY 11-7082 on the tube formation were related to their cytotoxicity, we then examined their effects on cell viability of endothelial differentiated sphere cells. As shown in Figure 4, treatment with Bevacizumab, DAPT, Akt1/2 kinase inhibitor at 2 and 10 μM, and BAY 11-7082 did not affect the viability of endothelial differentiated sphere cells, indicating that their effects on tube for-

mation were not due to the cytotoxicity. Akt1/2 kinase inhibitor at 50 μM inhibited the viability of endothelial differentiated sphere cells with an inhibition rate of 12.8%. Since this inhibitory effect on cell viability was much weaker than that of the tube formation, indicating that the effect of 50 μM Akt1/2 kinase inhibitor on the tube formation was also not the result of its cytotoxic effect. These results indicated that sphere cells are able to differentiate into endothelial cells in a VEGF- and NOTCH-independent, but an Akt- and IKK-dependent manner.

3.4. Hydrogen Peroxide Promotes the Differentiation of Sphere Cells into Endothelial Cells. It has been reported that liver cancer stem cells normally contain low levels intracellular ROS, and ROS also play an important role in the processes of self-renewal and differentiation of cancer stem cells [25, 26]. So we first measured the intracellular ROS levels in
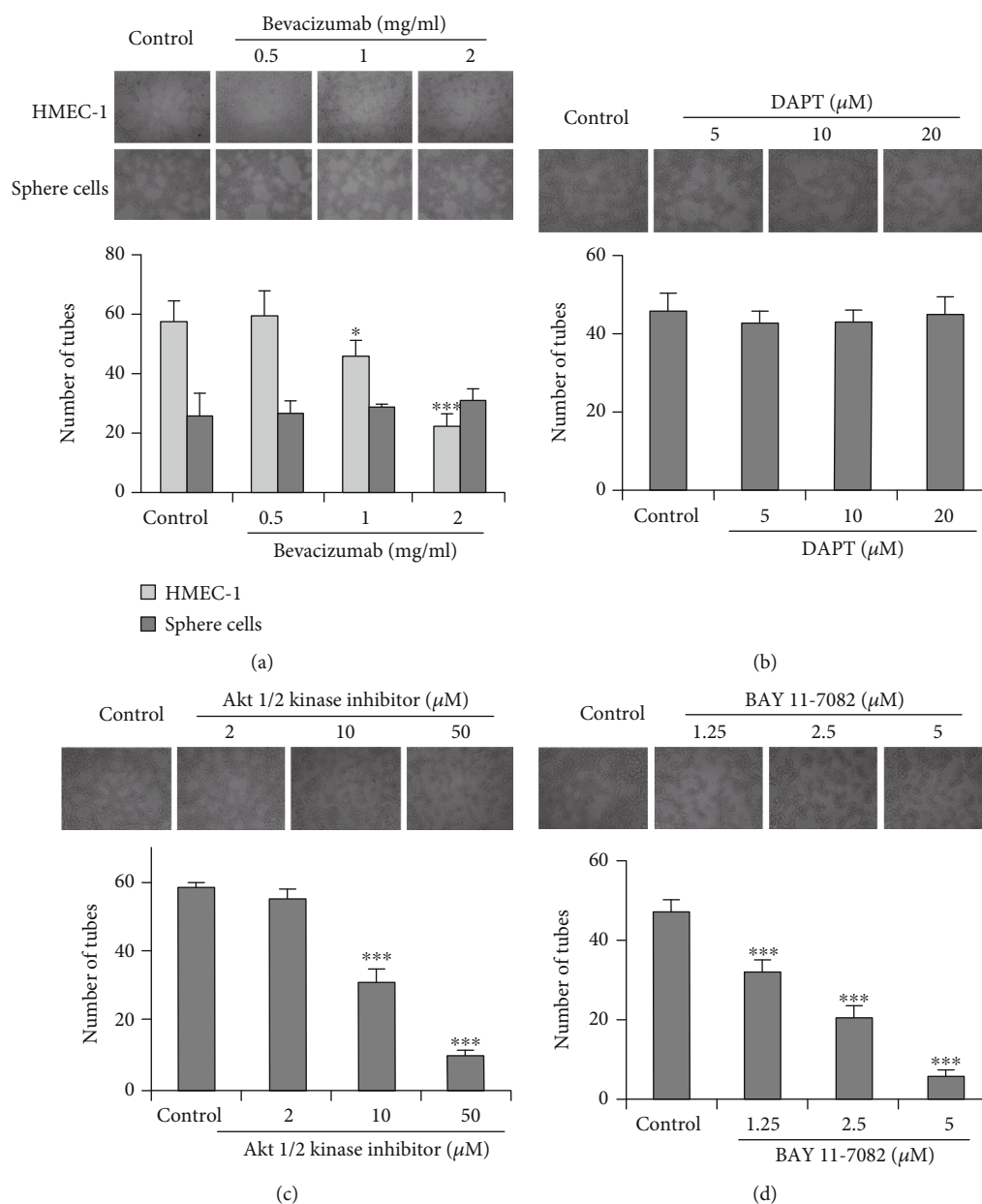
FIGURE 3: Effects of various signaling pathway inhibitors on the tube formation of HMEC-1 or endothelial differentiated sphere cells on Matrigel. HMEC-1 or endothelial differentiated sphere cells seeded on Matrigel were treated with or without indicated concentrations of Bevacizumab (a), DAPT (b), Akt1/2 kinase inhibitor (c), or BAY 11-7082 (d) for 24 h. The photographs of tube formation were taken (100×). The number of formed tubes was calculated. The data were presented as mean ± SD ($n = 3$). $^*P < 0.05$, $^{***}P < 0.001$ vs. control group.

sphere cells and their parental Hep G2 cells using DCFH-DA staining. As shown in Figure 5(a), sphere cells contained lower levels of intracellular ROS than their parental cells. Next, to determine whether ROS are involved in the process of endothelial differentiation of sphere cells, we treated the endothelial differentiated sphere cells with 100 μM hydrogen peroxide in the presence or absence of NAC (1 mM), which acts as a ROS scavenger by promoting intracellular biosynthesis of glutathione (GSH). As shown in Figures 5(b)–5(d), treatment with hydrogen peroxide for 24 h increased the CD31 and vWF expression of endothelial differentiated sphere cells. Under this treatment condition, hydrogen peroxide also increased the tube formation of endothelial differ-

entiated sphere cells. Importantly, pretreatment with NAC almost totally canceled the hydrogen peroxide-induce increase of the CD31 and vWF expression and tube formation. These results suggest that hydrogen peroxide could promote the endothelial differentiation of sphere cells.

3.5. L-Buthionine Sulfoximine (BSO) Promotes the Differentiation of Sphere Cells into Endothelial Cells. Studies have shown that lower ROS levels in cancer stem cells are associated with increased expression of free radical scavenging systems. Therefore, treatment of cancer stem cells with BSO, an inhibitor of GSH biosynthesis, results in the depletion of GSH levels, and thus the accumulation of intracellular

(a)

(b)

(c)

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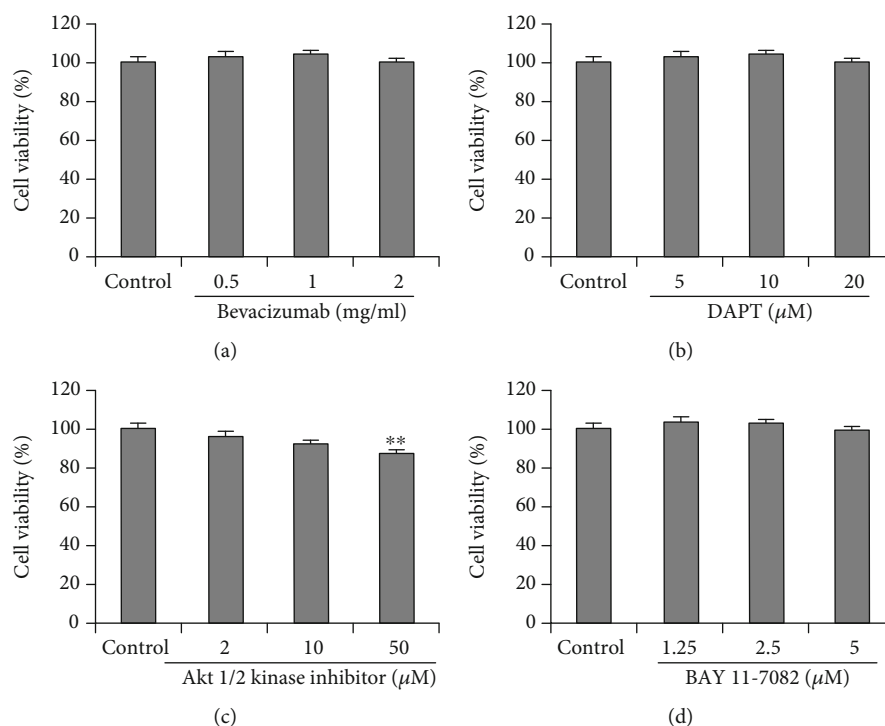
FIGURE 4: Effects of various signaling pathway inhibitors on the cell viability of endothelial differentiated sphere cells. Endothelial differentiated sphere cells were treated with or without indicated concentrations of Bevacizumab (a), DAPT (b), Akt1/2 kinase inhibitor (c), or BAY 11-7082 (d) for 24 h. Cell viability was measured with SRB assay. The data were presented as mean ± SD ($n = 6$). $^{**}P < 0.001$ vs. control group.

ROS [22]. To further explore the effect of endogenous ROS on the endothelial differentiation of sphere cells, we treated endothelial differentiated sphere cells with 1 mM BSO in the presence or absence of NAC (1 mM). As shown in Figure 6, exposure of endothelial differentiated sphere cells to BSO increased the CD31 and vWF expression. BSO treatment also induced the tube formation of endothelial differentiated sphere cells. Importantly, all these changes caused by BSO were abolished when the intracellular ROS were scavenged by NAC. Thus, the results suggest that elevating the intracellular ROS level by inactivating the antioxidant defense also induces the differentiation of sphere cells into endothelial cells.

3.6. Akt and IKK Activation Is Required for ROS-Mediated Endothelial Differentiation of Sphere Cells. As described above, the endothelial differentiation of sphere cells was Akt- and IKK-dependent. To clarify whether the promoting action of hydrogen peroxide and BSO on endothelial differentiation was related to the activation of both Akt and IKK, we first examined the effects of hydrogen peroxide or BSO on the activation of both Akt and IKK with western blotting. As shown in Figure 7(a), treatment with 100 μM hydrogen peroxide or 1 mM BSO did not affect the expression of total Akt but significantly increased the phosphorylation of Akt in endothelial differentiated sphere cells. The increase of Akt phosphorylation induced by hydrogen peroxide or BSO could be abolished by the pretreatment with 1 mM NAC. Likewise, treatment with 100 μM hydrogen peroxide or 1 mM BSO did not affect the level of total IKK but increased

the level of p-IKK. This increase of p-IKK was reversed by NAC (1 mM) (Figure 7(b)). Because IKK activation is one of the downstream events of Akt activation, these results indicated that ROS treatment activated the Akt/IKK signaling pathway. Next, to determine whether Akt/IKK signaling is indeed involved in ROS-induced endothelial differentiation of sphere cells, we explored the effect of Akt1/2 kinase inhibitor and BAY 11-7082 on the ROS-induced endothelial differentiation of sphere cells with tube formation assay. As shown in Figures 7(c) and 7(d), pretreatment with Akt1/2 kinase inhibitor (10 μM) or BAY 11-7082 (2.5 μM) inhibited hydrogen peroxide or BSO-induced tube formation of endothelial differentiated of sphere cells. These results suggest that Akt/IKK signaling pathway is involved in the ROS-induced differentiation of sphere cells into endothelial cells.

4. Discussion

Cancer stem cells are a small portion of cancer cells with the feature of stem cells. They have self-renewal and differential capacity and have been considered as the origin of tumor recurrence and metastasis. They are also responsible for the resistance to chemotherapy and radiotherapy in clinic. Currently, commonly used cell surface markers for isolating liver CSCs include CD133, CD90, CD44, epithelial cell-adhesion molecule (EpCAM), CD13, OV6, and ALDH [27]. However, none of these markers are specific for liver CSC. There is no generally accepted surface marker for liver CSC [28]. Sphere formation assay is a functional approach commonly used to identify CSCs and study their properties [29]. Studies have

(a)

(b)

(c)

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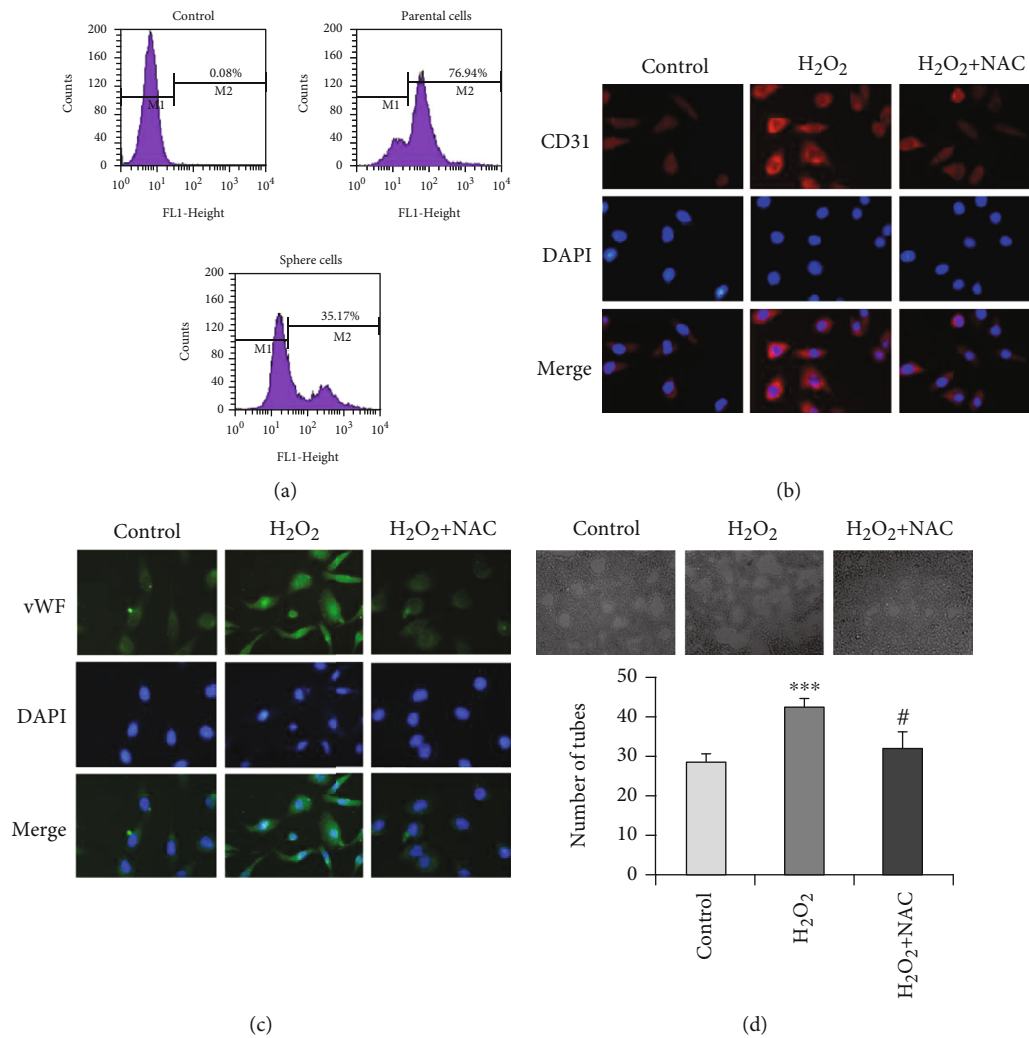
FIGURE 5: Hydrogen peroxide promotes the endothelial differentiation of sphere cells. (a) ROS levels in parental Hep G2 and sphere cells were examined with DCFH-DA staining and analyzed by flow cytometry. Endothelial differentiated sphere cells cultured in the presence or absence 1 mM NAC for 30 min were further treated with 100 μM H_2O_2 for 24 h. The cells were then subjected to immunofluorescence analysis for the expression of CD31 (b) and vWF (c) (200×). (d) Endothelial differentiated sphere cells were seeded on Matrigel in the presence or absence of NAC (1 mM) for 30 min and further treated with or without 100 μM H_2O_2 for 24 h. The photographs of tube formation were taken (100×). The number of formed tubes was calculated. The data were presented as mean ± SD ($n = 3$). ***$P < 0.001$ vs. control group and #$P < 0.05$ vs. H_2O_2 treatment alone.

shown that sphere cells enriched from HCC cell lines and primary tumor cells exhibited CSC properties, including proliferation, self-renewal, drug resistance, lower ROS levels, and high tumorigenicity [23, 26, 30]. More importantly, as Ma et al. reported, the cell surface marker selection only enriches one CSC subpopulation, while sphere-forming culture enriches different subpopulations of CSCs with certain HCC biomarkers, and thus the most complete CSC population from a bulk tumor [30]. Therefore, we finally choose sphere formation assay to isolate cancer stem-like cells in our study.

Recently, it is reported that glioblastoma [7–10], breast [11], and ovarian [12] CSCs have the ability to differentiate into endothelial cells both morphologically and functionally. Studies from Marfels et al. also demonstrated that chemoresistant hepatocellular carcinoma cells showed increased pluripotent capacities and the ability to transdifferentiate into

functional endothelial-like cells both *in vitro* and *in vivo* [31]. However, another report from Ghanekar et al. showed that endothelial cells did not arise from tumor-initiating cells in human hepatocellular carcinoma [32]. But Yao et al. pointed out that the conclusion made by Ghanekar et al. was not convincing due to too little data and the uncommon method they used to identify CSCs [33]. These mean whether liver CSCs have the ability to differentiate into endothelial cells remains unclear. In the present study, we examined the ability of liver CSCs to transdifferentiate into endothelial cells and explored the role of ROS in regulating this process, together with the molecular mechanism involved therein. Our results suggested that cancer stem-like sphere cells enriched from Hep G2 are able to differentiate into endothelial cells.

It is well documented that the differentiation process of CSCs involves multiple signal pathways [34]. Different type

(a)

(b)

(c)
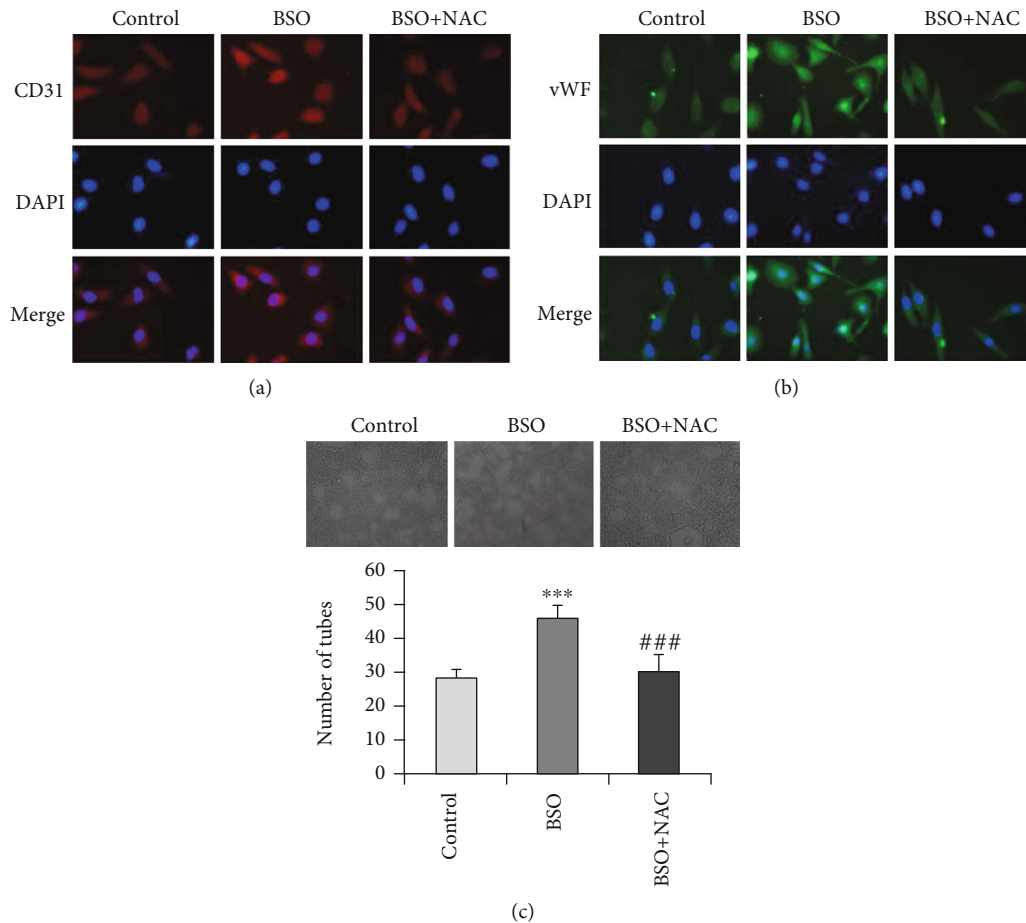
Figure 6: BSO promotes the endothelial differentiation of sphere cells. Endothelial differentiated sphere cells cultured in the presence or absence of 1 mM NAC for 30 min were further treated with 1 mM BSO for 24 h. The cells were then subjected to immunofluorescence analysis for the expression of CD31 (a) and vWF (b) (200×). (c) Endothelial differentiated sphere cells were seeded on Matrigel in the presence or absence of 1 mM NAC for 30 min and then further treated with or without 1 mM BSO for 24 h. The photographs of tube formation were taken (100×). The number of formed tubes was calculated. The data were presented as mean ± SD ($n = 3$). ***$P < 0.001$ vs. control group and ###$P < 0.001$ vs. BSO treatment alone.

of CSCs may activate distinct signal pathways during the endothelial differentiation process. For example, stem-like ovarian cancer cells differentiate into endothelial cells in a VEGF-independent but IKKβ-dependent manner [12]. Similarly, endothelial differentiation of other CSCs such as glioblastoma [9], breast cancer [11], and renal carcinoma [35] is all VEGF-independent. Moreover, endothelial differentiation of glioblastoma CSCs is also dependent on NOTCH signaling pathway [8]. In our study, neither Bevacizumab nor DAPT inhibits the tube formation of endothelial differentiated sphere cells, indicating that endothelial differentiation of cancer stem-like sphere cells from Hep G2 was independent of VEGF and NOTCH signaling.

Akt is a serine/threonine protein kinase. Once activated, Akt regulates many physiological cell processes including proliferation, differentiation, apoptosis, and motility through phosphorylating a series of protein substrates [36]. Recent studies have revealed that PI3K/AKT signaling pathway is upregulated in CSCs, which is related to the maintenance of CSCs phenotype in several tumors [37]. NF-κB is a transcription factor. In resting cells, NF-κB is sequestered in cytosol

via interaction with IκB. Once activated by upstream signaling molecules, IκB kinase (IKK) phosphorylates IκB, tagging it for degradation. As a result, NF-κB is liberated from IκB, translocates to the nucleus, and proceeds to activate the expression of a cohort of target genes which are related to a series of cellular processes, including the inflammatory response, cellular adhesion, differentiation, proliferation, autophagy, senescence, and apoptosis [38]. Elevated or constitutive NF-κB activity, which has been found in CSCs from many types of cancers, participates in the self-renewal, proliferation, survival, and differentiation of CSCs [39]. In liver CSCs, NF-κB signaling is frequently activated [40]. Based on the fact, we then explored the effect of Akt1/2 kinase inhibitor and BAY 11-7082, an IKK-β inhibitor, on the tube formation of endothelial differentiated sphere cells. We found that either Akt1/2 kinase inhibitor or BAY 11-7082 dose-dependently blocked tube formation of endothelial differentiated sphere cells, suggesting that sphere cells from Hep G2 differentiate into endothelial cells in an Akt- and IKK-dependent manner. Collectively, our findings provided an important evidence that liver CSCs have the ability to

(a)

(b)

(c)

(d)
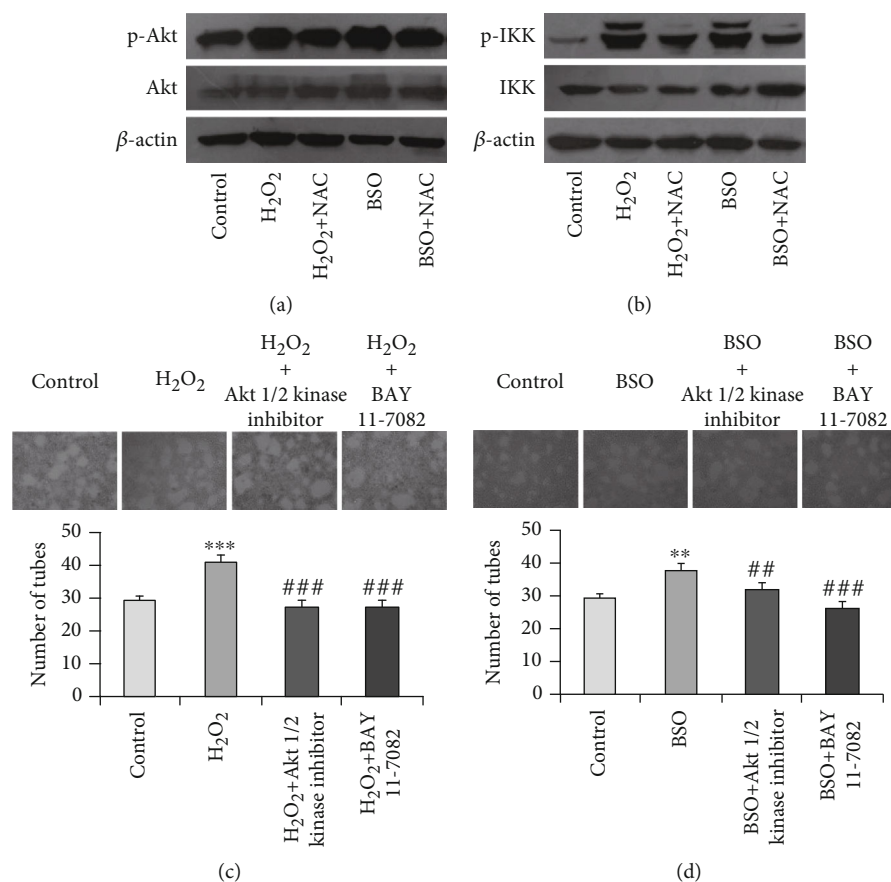
Figure 7: ROS induced endothelial differentiation of sphere cells through the activation of Akt/IKK signaling pathway. Endothelial differentiated sphere cells cultured in the presence or absence 1 mM NAC for 30 min were further treated with $100\,\mu M\ H_2O_2$ or 1 mM BSO for 24 h. The cells were then subjected to western blotting for detecting the indicated proteins (a, b). Endothelial differentiated sphere cells were seeded on Matrigel in the presence or absence of Akt1/2 kinase inhibitor ($10\,\mu M$) or BAY 11-7082 ($2.5\,\mu M$) for 30 min and then further treated with or without $100\,\mu M\ H_2O_2$ (c) or 1 mM BSO (d) for 24 h. The photographs of tube formation were taken (100×). The number of formed tubes was calculated. The data were presented as mean ± SD ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group and $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. H_2O_2 or BSO treatment alone.

differentiate into endothelial cells, as well as the underlying mechanisms of this process.

Studies have shown that CSCs contain lower levels ROS, which contributes to their stemness and resistance to chemotherapy and radiation therapy [41]. In our experiments, cancer stem-like sphere cells derived from Hep G2 also have lower levels of ROS than nonsphere cells, which are consist with the previous reports from Haraguchi et al. [25].

Accumulative evidences have demonstrated that ROS and ROS-dependent signaling pathways play an important role in self-renewal and differentiation of CSC. Sato et al. reported that ROS promote the differentiation of glioma-initiating cells through the activation of p38 MAPK signaling pathway [42]. However, it remains poorly explored whether ROS are involved the transdifferentiation process of cancer stem cells into endothelial cells. In the present study, we found that both exposure to exogenous ROS and elevating endogenous ROS by inhibiting the cellular antioxidant mechanism promote endothelial differentiation of cancer stem-like sphere cells. These indicate that ROS play an essential role in regulating the differentiation of liver cancer stem cells into endothelial cells.

Emerging evidences indicate that ROS play an essential role in the process of self-renewal and differentiation of CSCs through the activation of multiple ROS-dependent signaling pathways, such as PI3K/AKT, ATM, and Notch pathway [43]. Our results indicate that Akt/IKK signaling pathway is critical for the differentiation cancer stem-like sphere cells into endothelial cells. So we then examined the effects of ROS on the activation Akt/IKK signaling pathway. We found that treatment with either hydrogen peroxide or BSO induced the phosphorylation of both Akt and IKK. This increased phosphorylation could be blocked by NAC. Accordingly, both Akt1/2 kinase inhibitor and BAY 11-7082 blocked the tube formation induced by hydrogen peroxide or BSO. These data suggest that ROS promote the differentiation of cancer stem-like sphere cells into endothelial cells through the activation of Akt/IKK signaling pathway.

However, it is important to note that although many studies have confirmed the CSC properties of sphere-formed cells from liver cancer [23, 26, 30], it would be more convincing to examine some of CSC markers such as CD133 and CD44 variant expressions and tumorigenicity of sphere-formed cells to validate the CSC phenotype. In addition,

sphere cells in the present study were isolated only in one HCC cell line Hep G2, which is thought to be derived from hepatoblastoma. Other HCC-derived cell lines will be needed in the future to further confirm the findings.

5. Conclusions

Collectively, the results of the present study demonstrate that cancer stem-like sphere cells from Hep G2 are able to differentiate into endothelial cells both morphologically and functionally, and this process is independent of VEGF and NOTCH signaling but dependent on the activation of Akt and IKK. ROS promote endothelial differentiation of cancer stem-like sphere cells through activation of Akt/IKK signaling pathway. Therefore, our study reveals a new mechanism of angiogenesis in liver cancer that might be related to the resistance to conventional antiangiogenic treatments. Moreover, our data also indicate that targeting ROS-dependent Akt/IKK signaling pathway may provide a novel therapeutic strategy for liver cancer treatment.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Zhengbin Zhao and Jing Gao contributed equally to this work.

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Review Article

Mechanisms of the Regulation and Dysregulation of Glucagon Secretion

Arnold N. Onyango [iD]

*School of Food and Nutrition Sciences, Jomo Kenyatta University of Agriculture and Technology, P. O. Box 62000,
00200 Nairobi, Kenya*

Correspondence should be addressed to Arnold N. Onyango; arnold.onyango@jkuat.ac.ke

Glucagon, a hormone secreted by pancreatic alpha cells, contributes to the maintenance of normal blood glucose concentration by
inducing hepatic glucose production in response to declining blood glucose. However, glucagon hypersecretion contributes to the
pathogenesis of type 2 diabetes. Moreover, diabetes is associated with relative glucagon undersecretion at low blood glucose and
oversecretion at normal and high blood glucose. The mechanisms of such alpha cell dysfunctions are not well understood. This
article reviews the genesis of alpha cell dysfunctions during the pathogenesis of type 2 diabetes and after the onset of type 1 and
type 2 diabetes. It unravels a signaling pathway that contributes to glucose- or hydrogen peroxide-induced glucagon secretion,
whose overstimulation contributes to glucagon dysregulation, partly through oxidative stress and reduced ATP synthesis. The
signaling pathway involves phosphatidylinositol-3-kinase, protein kinase B, protein kinase C delta, non-receptor tyrosine kinase
Src, and phospholipase C gamma-1. This knowledge will be useful in the design of new antidiabetic agents or regimens.

1. Introduction

The hormone glucagon, produced by pancreatic alpha cells,
contributes to the regulation of blood glucose by promoting
hepatic glucose production in response to declining blood
glucose. However, its excessive secretion contributes to the
development of type 2 diabetes [1, 2]. Moreover, in both type
1 and type 2 diabetes, its secretion is dysregulated; with
hypersecretion at moderate and high glucose, aggravating
hyperglycemia; and failure of secretion at low glucose, leading
to life-threatening hypoglycemia [2, 3]. The mechanisms of
such alpha cell dysregulations are not well understood. This
article discusses the related literature to present an up-to-
date understanding of these processes, beginning with an out-
line of the pathways of glucagon secretion. The mechanism of
induction of hyperglucagonemia in otherwise healthy individ-
uals and how this contributes to type 2 diabetes is discussed. A
synthesis of the literature unveils a signaling pathway that
contributes to glucose- and/or hydrogen peroxide-induced
glucagon secretion. Excessive activation of this pathway in

diabetes dysregulates glucagon secretion through alpha cell
oxidative stress and reduced ATP synthesis. The relevance of
such a pathway to the antihyperglycemic and antihypoglyce-
mic effects of some antidiabetic agents is discussed.

2. Glucagon Secretion Pathways

Glucagon synthesis involves transcription of the preproglu-
cagon (*Gcg*) gene to produce proglucagon mRNA, which is
translated to proglucagon, whose cleavage by prohormone
convertase 2 produces glucagon [4]. The synthesized gluca-
gon molecules are packaged into secretory vesicles (SVs),
which need to be translocated to the plasma membrane
(PM) where they get docked through protein-protein inter-
actions [5, 6]. Before secretion, the vesicles get primed for
exocytosis through protein interactions that promote their
rapid calcium-dependent fusion with the PM [5].

   As illustrated in Figure 1, one of the hypothesized path-
ways leading to primed glucagon granule exocytosis begins
with potassium efflux through ATP-dependent K^+ channels
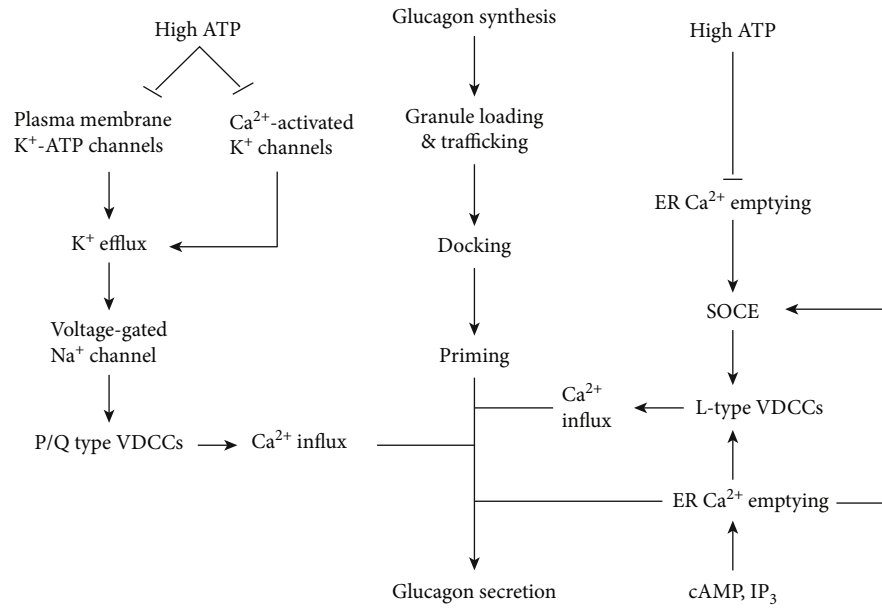
FIGURE 1: Pathways of glucagon secretion. VDCC: voltage-dependent calcium channel; ER: endoplasmic reticulum; SOCE: store-operated calcium entry.

(K-ATP channels) [7, 8]. According to this hypothesis, glucagon secretion requires the closure of most of these channels, allowing limited K^+ efflux to alter the membrane potential to a range that permits the opening of voltage-dependent Na^+ channels; the resulting Na^+ influx causes subsequent opening of P/Q type voltage-gated calcium channels (VDCCs); calcium influx through these VDCCs is coupled to fusion of glucagon vesicles with the plasma membrane, resulting in glucagon secretion; and too low or too high ATP levels induce excessive opening or closure of the K-ATP channels, respectively, leading to the inhibition of this pathway [7–9]. K^+ channels activated by intracellular calcium (calcium activated K^+ channels) were recently found to contribute to glucagon secretion and were suggested to be useful in limiting voltage-dependent inhibition of P/Q type VDCCs during prolonged periods of low glucose [10]. Nevertheless, this K-ATP channel hypothesis is not fully accepted [7, 11]. For example, at low glucose, reduction in alpha cell ATP by inhibition of fatty acid oxidation was found not to affect K-ATP channel conductance, and no membrane hyperpolarization due to K^+ efflux was observed; instead, there was membrane depolarization due to Na^+/K^+ ATPase inhibition [11]. Likewise, as reviewed by Gylfe [7], it has been reported in some studies that glucose, which increases ATP, promotes membrane hyperpolarization rather than the expected depolarization due to K-ATP channel closure.

Other glucagon secretion pathways depend on the reduction of ER calcium content (Figure 1). For example, at low glucose, the ER calcium pump SERCA is relatively inhibited, and the low ER calcium levels cause the ER transmembrane protein, stromal-interacting molecule 1 (STIM 1), to oligomerize and move to interact with the plasma membrane calcium channel orai1, thus activating this 'store-operated channel' and inducing store operated calcium entry (SOCE) [7, 12]. SOCE depolarizes the membrane, thus inducing calcium entry

through L-type VDCCs which promote exocytosis [7, 12]. High glucose inhibits SOCE by reverse translocation of STIM 1 to the ER, and this effect is maximal by 3 mM glucose [7, 12].

ER calcium release and glucagon secretion can be induced even at high glucose subsequent to the intracellular increase in cyclic adenosine monophosphate (cAMP) or inositol triphosphate (IP_3) [6, 13, 14]. For example, fatty acids promote glucagon secretion at both low and high glucose by binding to the FFAR1 receptor, which is coupled to phospholipase C activation and generation of IP_3, which induces the release of calcium from the ER, thus raising the cytosolic calcium, which is amplified by SOCE and L-type VDCCs to promote glucagon exocytosis [11, 14–16]. Besides, fatty acids are also metabolized to produce ATP, which is required for a variety of processes, including glucagon synthesis, glucagon vesicle trafficking, docking and priming, maintaining Na^+/K-ATPase activity for membrane repolarization, and preventing excessive opening of K-ATP channels [8, 11, 14, 17].

3. Isolated Alpha Cells Exhibit a V-Shaped Glucagon Secretion Curve in Response to Increasing Glucose: The Influence of ATP and a Signaling Pathway Leading to Phospholipase C Gamma-1 Activation and ER Calcium Release

Isolated alpha cells have a V-shaped glucagon secretion curve in response to increasing glucose concentrations from 0 mM, with maximal suppression at moderate glucose concentrations of 5-7 mM [6, 18]. At low glucose concentrations, ATP plays a signaling role in glucagon secretion through cAMP elevation, which is important for accelerating the mobilization of glucagon granules to the readily releasable

pool [13, 19]. Suppression of glucagon secretion in response to glucose has been attributed to increasing ATP concentrations and resultant closure of K-ATP channels, membrane hyperpolarization through increased Na^+/K^+ ATPase activity, inhibition of ER calcium release, and reduction of cAMP concentrations [6–8, 13]. cAMP level reduction may be explained by ATP-induced ER filling and reverse translocation of STIM to the ER, since, at the plasma membrane, STIM 1 activates adenylyl cyclase [12, 13]. The increasing ATP also coincides with decreasing activation of adenosine monophosphate kinase (AMPK), a promoter of glucagon secretion by an unknown mechanism [20, 21].

The reason for increasing glucagon secretion above the 5-7 mM glucose range is less well understood. However, as suggested hereafter, a signaling pathway beginning with sodium-glucose cotransporter 1 (SGLT-1) and involving the generation of reactive oxygen species (ROS) can explain this phenomenon (Figure 2). This is partly because ROS released from beta cells at 16.7 mM glucose were found to increase alpha cell glucagon content and secretion and alpha cell proliferation [22]. Similarly, hyperglycemia induces alpha cell hydrogen peroxide production, PI3K-Akt signaling, cell proliferation, and glucagon secretion [23]. This is in contrast to the hyperglycemia- and hydrogen peroxide-induced inhibition of PI3K-Akt in beta cells [23]. In the Goto-Kakizaki diabetes-prone rat model, the elevation in pancreatic islet PI3K-Akt is associated with increased activation of the nonreceptor tyrosine kinase Src and related ROS production, which can be inhibited by Src inhibitors [24, 25]. Src activation has similarly been found in pancreatic islets of db/db mice [26]. Inhibitors of the epidermal growth factor receptor (EGFR) were found to reduce ROS in islets from Goto-Kakizaki rats, and it was postulated that Src may transactivate this receptor [25]. In addition, an increase in glucagon secretion in hyperglycemia is associated with an increased activity of protein kinase C delta (PKC-δ) [27]. Thus, PI3K-Akt, PKC-δ, Src, and EGFR should be important components of the suggested signaling pathway (Figure 2).

According to Figure 2, transport of glucose and sodium (Na^+) through SGLT-1 is responsible for initiating signaling, through PI3K activation that leads, via PKC-δ and Src, to NADPH oxidase (Nox) and the production of hydrogen peroxide (H_2O_2). This is based on the analogy that in cardiomyocytes exposed to high glucose, glucose transport through SGLT-1 induces Nox2 activation in a process dependent on sodium and glucose transport but not metabolism, and which is associated with PKC activation [28]. SGLT1 was reported to contribute to glucagon secretion when islets were incubated for 2 hrs with both 5 mM and 20 mM glucose, by a mechanism dependent on transport rather than glucose metabolism [29]. Membrane depolarization, as can be induced by Na^+ entry through SGLT-1, can trigger activation of PI3K and Akt, upstream of Nox2 [30]. Akt promotes alpha cell proliferation via mammalian target of rapamycin (MTOR) [31] and also activates CREB [32], which promotes glucagon synthesis [4].

Human alpha cells express the melatonin 1 receptor (MT1) [33]. Melatonin signaling through this receptor induces PI3K-Akt signaling [34] and promotes glucagon secretion via PI3K and PLC-γ1, even at high glucose such as 16.7 mM [35].

PI3K activates PLC-γ1 through production of phosphatidylinositol 3-phosphate, but Akt can also activate this phospholipase, especially when EGFR is also activated [36]. PLC-γ1 generates IP3, which causes ER calcium release and glucagon secretion as already described in Glucagon Secretion Pathways. PKC-δ promotes trafficking of glucagon secretory granules to sites close to L-type VDCCs that participate in ER-dependent glucagon secretion [37]. Thus, by activating PI3K-Akt, glucose can induce glucagon secretion similarly to melatonin. At increasing glucose above the 7 mM glucose, the ATP level in alpha cells remains constant and maximal [20, 38]. Therefore, the increase in glucagon secretion with increasing glucose may be due to increasing activation of the signaling pathway in Figure 2 rather than changes in ATP. Nox activity may increase with increasing glucose because of higher NADPH availability from the pentose phosphate pathway, since this pathway was found to be required for hyperglycemia-induced Nox activity elevation in cardiomyocytes [28]. Moreover, with increasing glucose, there is increased nonenzymatic protein glycation, which further promotes the activation of Nox and Src [28, 39, 40].

PI3K activates PKC-δ [41], which activates Akt, Nox, and Src [41–43]. Src activates Nox, PLC-γ1, and EGFR [25, 44, 45]. EGFR activates both PLC-γ and PI3-K [36]. Nox produces superoxide anions that are converted by superoxide dismutase to hydrogen peroxide (H_2O_2). Hydrogen peroxide, via Src, activates PI3K [46], thus establishing a positive feedback loop for sustained P13-Akt activation and hydrogen peroxide generation. This also explains the fact that hydrogen peroxide can promote glucagon secretion, increase glucagon content, and cell proliferation [22].

Hydrogen peroxide-mediated Src activation depends on sulfenylation of two cysteine residues [47]. ROS-mediated carbonylation of specific proline and threonine residues of Na^+/K^+ ATPase additionally promotes Src signaling by freeing the latter from an inhibitory interaction with the former, and this has been reported to be involved in the pathogenesis of obesity and cardiovascular dysfunctions [48, 49]. Although it has been suggested that such carbonylation involves hydroxyl radicals generated by the Fenton reaction between hydrogen peroxide and ferrous ions [48], this is unlikely due to the very high reactivity of hydroxyl radicals, which makes them react unselectively [50]. Singlet oxygen ($^1O_2$) is a more selective ROS, which can be formed by the reaction of hydrogen peroxide with glucose [51], oxidizes amines [52], and such oxidation was recently suggested as being involved in the formation of biologically relevant amide-type adducts such as Nε-(hexanoyl) lysine [53]. Thus, it is proposed that the carbonylation of Na^+/K^+ ATPase may be mediated by singlet oxygen according to Figure 3.

4. Elevated Plasma Nonesterified Fatty Acids (NEFA) Induce Alpha Cell Insulin Resistance and Associated Dysfunctions That Promote the Pathogenesis of Type 2 Diabetes

Dysregulation of glucagon secretion starts before the development of type 2 diabetes [54, 55]. The path towards
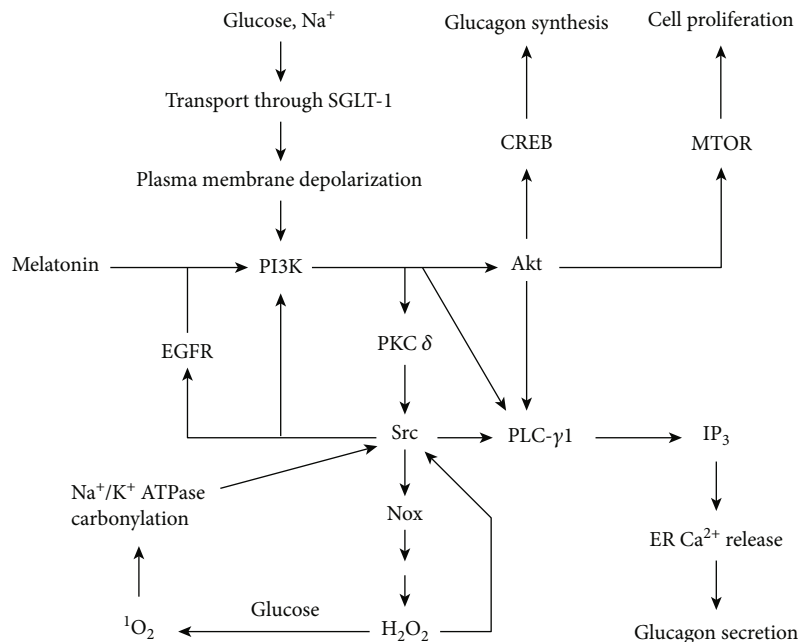
FIGURE 2: Suggested signaling pathway for glucose-induced increase in glucagon synthesis, glucagon secretion, and alpha cell proliferation. Melatonin and hydrogen peroxide (H_2O_2) also initiate the pathway.
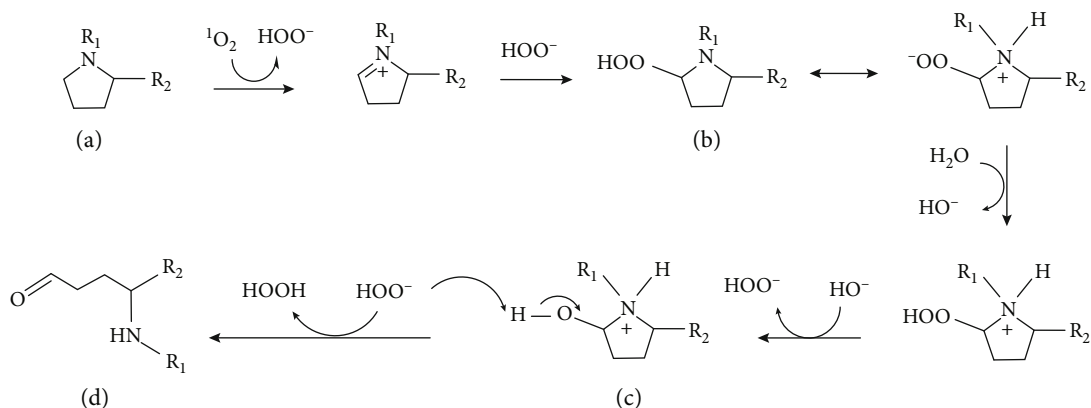
FIGURE 3: Suggested reaction of proline residue (a) with singlet oxygen ($^1O_2$) to form hydroperoxide (b), which reacts with H_2O to form hydroxy-derivative (c) and hydroperoxide anion (HOO –), followed by reaction of the latter two to form glutamate 5-semialdehyde residue (d).

type 2 diabetes involves two major types of prediabetic states, namely, impaired fasting glucose (IFG, defined by fasting glucose of 5.6-6.9 mM) and impaired glucose tolerance (IGT, defined by 2-hour glucose of 7.8-11 mM after oral consumption of 75 g equivalent of glucose) [56]. Elevated hepatic glucose production is the key characteristic of IFG and decreased suppression of postprandial hepatic glucose production contributes to IGT [57]. Fatty acids induce a dose-dependent elevation of glucagon secretion at both low- and moderate-glucose concentrations [14, 16, 58]. Thus, conditions such as obesity that elevate plasma NEFA expose alpha cells to the latter's glucagon-elevating effects [59]. However, both glucagon and fatty acids induce insulin secretion [16, 60], which inhibits glucagon secretion. Hence, elevated fatty acids may initially promote fasting hyperinsulinemia but not hyperglucagonemia (Figure 4). Sustained NEFA eleva-

tion and resultant hyperinsulinemia can induce insulin resistance in alpha cells, hepatocytes, and other cell types [61–63]. Palmitate induces both insulin resistance and ER stress in alpha cells [61, 64]. ER stress upregulates glycogen synthase kinase 3 (GSK3) [65], which causes insulin resistance by phosphorylating insulin receptor substrate 1 (IRS1), which subsequently undergoes ubiquitination and proteosomal degradation [66].

Insulin inadequately inhibits glucagon secretion in insulin-resistant alpha cells [61], resulting in fasting hyperglucagonemia, which promotes fasting hepatic glucose production and IFG, especially in the setting of hepatic insulin resistance (Figure 4). Fasting hyperglucagonemia may promote muscle wasting and is associated with IGT due to decreased postprandial uptake of glucose by muscles [1, 67]. Likewise, alpha cell insulin resistance reduces postprandial glucagon suppression, and thus sustains hepatic glucose
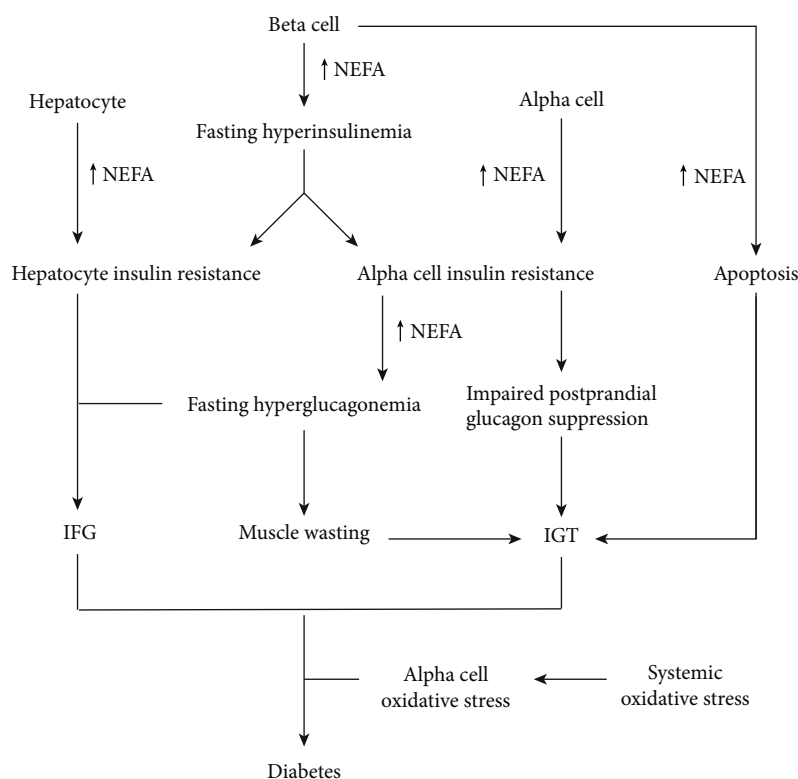
FIGURE 4: Fatty acid- and oxidative stress-induced alpha cell dysfunction upstream of type 2 diabetes.

production in the postprandial state, which contributes to IGT [68]. Chronic exposure of beta cells to palmitate induces ER stress and apoptosis [64], which reduces insulin production, thus contributing to IGT. Although alpha cells are subject to ER stress, they are resistant to apoptosis [69]. Thus, the alpha cell to beta cell ratio with associated glucagon to insulin ratio may increase with time, further elevating blood glucose [31].

Prediabetic individuals either revert to normoglycemia or progress to diabetes. Systemic oxidative stress is an important factor associated with progression to diabetes [70–73]. Hypertension is also strongly related to the progression to diabetes [74], and this can be linked to oxidative stress [75]. During systemic oxidative stress, alpha cells may be chronically exposed to hydrogen peroxide from beta cells and endothelial cells, and this may lead to chronic activation of PI3K-Akt signaling in alpha cells according to Figure 2. Although Akt ordinarily phosphorylates and inhibits GSK3, chronic Akt activation desensitizes GSK3 from this inhibition [76]. GSK3 activation promotes mitochondrial damage, including inhibition of complex 1, mitochondrial fission, dissolved cristae, and overall change in morphology [77–80]. Alpha cells with such mitochondrial damage may be under elevated superoxide anion production even at basal glucose [81]. As described in the next section, oxidative stress and mitochondrial alterations increase glucagon secretion at normal and high glucose and are therefore likely to accelerate the occurrence of frank hyperglycemia characteristic of diabetes. Hence, chronic infratherapeutic treatment of Goto-Kakizaki young rats with the GSK3 inhibitor, lithium, prevented islet inflammation and diabetes [82].

5. Alpha Cell Insulin Resistance, Mitochondrial Abnormalities, and Chronic Oxidative Stress Dysregulate Glucagon Secretion in Diabetes

In normal pancreatic islets, unlike isolated alpha cells, the rise in glucagon secretion at glucose concentrations above 7 mM is suppressed by paracrine action of somatostatin and insulin produced by beta cells and delta cells, respectively, and by gap junction coupling between these cells [11, 83]; but, this paracrine suppression is lost in diabetes because of alpha cell insulin and somatostatin resistance [18, 55]. Chronic exposure of alpha cells to high glucose upregulates the expression of SGLT-1 [9, 29] and overactivates the signaling pathway in Figure 2, as evidenced by increased activation of PI3K-Akt, PKC-δ, Src, and ROS generation in diabetic islets [24–27, 81]. Oxidative stress and mitochondrial abnormalities cause reduced ATP production in alpha cells [9, 84, 85]. Decreased ATP increases glucagon secretion at both moderate and high glucose [9, 85]. On the other hand, at low glucose, ATP can drop below the level required for glucagon secretion, thus leading to failure of glucagon counterregulation and hypoglycemia [9, 84, 85]. Accordingly, the antioxidant epigallocatechin-3-gallate prevented oxidative stress and restored glucagon secretion in a TC1-6 pancreatic alpha cell line, leading to the suggestion that combining conventional antihyperglycemia therapy with antioxidant therapy may avert hypoglycemia in clinical treatment of diabetes [86]. Oral administration of glucose restores glucagon secretion [87]. Apart from its role as a substrate for ATP synthesis, glucose

is metabolized through the pentose phosphate pathway to generate NADPH for reduction of oxidized glutathione, and hence alleviation of oxidative stress [88]. Notably, in response to hypoglycemia, activation of the hypothalamic-pituitary-adrenal axis occurs, resulting in release of catecholamines that greatly increase plasma free fatty acids, yet this does not necessarily resolve the hypoglycemia [89]. Although fatty acids may supply energy to prevent hypoglycemia [11], it is likely that they do not efficiently promote hypoglycemia recovery because they cannot resolve the oxidative stress and might even aggravate it.

6. Antioxidant Antidiabetic Agents Improve Glucagon Hypersecretion and Hyposecretion

Glucagon-like peptide 1 (GLP-1) and GLP-1 receptor agonists such as exendin reduce hyperglycemia and are associated with lower risk of hypoglycemia [25, 90, 91]. They promote cAMP formation, which, through Epac2, inhibits Src signaling and induces Nrf2 antioxidant response [25, 92]. Thus, by reducing ROS formation, GLP-1 and its receptor agonists increase ATP production [25, 93], to improve glucagon hyposecretion and hypersecretion. G protein-coupled receptor 119 (GPR119) agonists induce glucagon secretion during hypoglycemia but not hyperglycemia in diabetic mice [94]. Like GLP-1R signaling, GPR119 signaling involves cAMP production; and this receptor has the advantage of being robustly expressed in alpha cells, unlike the GLP-1 receptor [94].

Exogenous insulin administration promotes somatostatin secretion, which aggravates hypoglycemia by reducing cAMP formation; while somatostatin receptor antagonists improve hypoglycemia by increased cAMP [3], and associated reduction in oxidative stress. It is likely that, at high glucose, somatostatin only lowers glucagon when in collaboration with insulin, which activates Akt, thus inhibiting GSK3 and promoting Nrf2-associated expression of antioxidant enzymes. Otherwise, somatostatin alone, by reducing cAMP, might induce oxidative stress. Accordingly, diabetes patients, with low insulin secretion, experience hyperglucagonemia although their somatostatin secretion is even upregulated [95].

Although according to Figure 2, SGLT-1 inhibitors should inhibit PI3K-Src-ROS signaling and thus prevent hypoglycemia, this is not the case in [29]. This can be explained by their reduction of glucose entry for ATP synthesis and glutathione reduction. A related result has been reported that in cardiomyocytes, SGLT-1 induces ROS generation at high glucose but promotes survival at low glucose by replenishing ATP stores through enhanced glucose availability [96].

There has been increasing interest in the antidiabetic effects of the flavonoid quercetin, but this has mainly been limited to animal studies [97]. Quercetin has been demonstrated to inhibit glucagon secretion through PKC-δ inhibition [27], and it is also known to be an antioxidant and inhibitor of Src [98]. Further studies of this flavonoid and related phytochemicals in the prevention or management of diabetes are warranted. The same applies to the peptide pNaKtide which inhibits Na$^+$/K$^+$ ATPase-dependent Src activation and has been found to be beneficial against various other metabolic disorders [49].

7. Conclusion

In healthy individuals, glucose increases ATP to promote glucagon secretion in hypoglycemia and to suppress glucagon secretion at higher glucose levels to prevent hyperglycemia. At high glucose, such as in the postprandial state, glucagon secretion is suppressed by paracrine action of somatostatin and insulin produced by beta cells and delta cells, respectively. Alpha cell dysfunctions such as insulin resistance, mitochondrial alterations, and oxidative stress contribute to the pathogenesis of type 2 diabetes and glucagon dysregulation in diabetes. A signaling pathway that can be initiated by glucose and sodium transport through SGLT-1 or by hydrogen peroxide promotes glucagon secretion and, if overactivated, may induce oxidative stress and ATP reduction as key contributors to glucagon dysregulation in diabetes. This pathway can be targeted in the search for new antidiabetic agents.

Conflicts of Interest

The author declares that there is no conflict of interests regarding the publication of this paper.

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Review Article

Evolution of the Knowledge of Free Radicals and Other Oxidants

Sergio Di Meo [ID] and Paola Venditti [ID]

Università degli Studi di Napoli Federico II Dipartimento di Biologia, Complesso, Universitario Monte Sant'Angelo, Via Cinthia, I-80126 Napoli, Italy

Correspondence should be addressed to Paola Venditti; venditti@unina.it

Free radicals are chemical species (atoms, molecules, or ions) containing one or more unpaired electrons in their external orbitals and generally display a remarkable reactivity. The evidence of their existence was obtained only at the beginning of the 20th century. Chemists gradually ascertained the involvement of free radicals in organic reactions and, in the middle of the 20th century, their production in biological systems. For several decades, free radicals were thought to cause exclusively damaging effects . This idea was mainly supported by the finding that oxygen free radicals readily react with all biological macromolecules inducing their oxidative modification and loss of function. Moreover, evidence was obtained that when, in the living organism, free radicals are not neutralized by systems of biochemical defences, many pathological conditions develop. However, after some time, it became clear that the living systems not only had adapted to the coexistence with free radicals but also developed methods to turn these toxic substances to their advantage by using them in critical physiological processes. Therefore, free radicals play a dual role in living systems: they are toxic by-products of aerobic metabolism, causing oxidative damage and tissue dysfunction, and serve as molecular signals activating beneficial stress responses. This discovery also changed the way we consider antioxidants. Their use is usually regarded as helpful to counteract the damaging effects of free radicals but sometimes is harmful as it can block adaptive responses induced by low levels of radicals.

1. Introduction

The term "radical" was first introduced by Guyton de Morveau in 1786 and later used by Gay-Lussac, Liebig, and Berzelius to indicate groups of atoms which were found unchanged in many substances (see Solov'ev [1]). The introduction of the term is generally attributed to Liebig and Wöhler, who in 1832 published a paper reporting that, in the various transformations of the essence of bitter almonds (the benzoic aldehyde) and of its derivatives containing chlorine and bromine, the radical, to which the formula $C_{14}H_{10}O_2$ was attributed, remained unchanged [2]. The work of Liebig and Wöhler exercised a considerable influence on the development of organic chemistry. After the classification of organic compounds in homologous series, the radicals were considered as groups of atoms, belonging to molecules of organic compounds, which are kept unaltered in reactions involving the functional group of the compounds and which

can be replaced by other radicals without modifying these reactions substantially.

Despite the interest in the radicals and their chemistry, their isolation was considered impossible, and, until the end of the nineteenth century, no direct evidence of their independent existence was found. In 1840, Berzelius believed that the inability to isolate the radicals did not depend on the fact that they did not exist but on the fact that they were combined too quickly and that the methods available in that time were insufficient for their isolation (see Solov'ev [1]).

Presumably, the first reaction involving free radicals was that of Fenton in 1894 [3]. He noted that when to a small amount of a tartaric acid solution a drop of diluted solution of ferrous sulphate was added, followed by a drop of hydrogen peroxide and finally an excess of caustic alkali, a light violet colour was obtained. The observed change was proposed as a distinguishing test for tartaric acid. Free radicals were not known at that time, and only three decades later, the

Table 1: Steps in evolving knowledge of free radicals.

Toxic effects of oxygen on central nervous system (Paul Bert effect)	Bert [61]
Pulmonary toxicity of oxygen (Lorrain Smith effect)	Smith [62]
Preparation of the triphenylmethyl radical, $(C_6H_5)_3C^.$	Gomberg [5]
Properties of xanthine oxidoreductase (XOR)	Schardinger [100]
Oxygen effect on radiosensitivity	Schwartz [44]
Isolation of the atomic hydrogen	Wood [11]
Preparation of the free radical methyl ($^.CH_3$)	Paneth and Hofeditz [13]
"Activated solvent" hypothesis for indirect action of ionizing radiation	Risse [40]
Discovery of free radicals as biochemical intermediates in biological systems	Michaelis [29]
First utilization of X-rays for cancer treatment	Grubbe [218]
Discovery of the "peroxide effect"	Kharasch and Mayo [14]
Generation of the hydroxyl radical	Haber and Weiss [27]
Suggestion of a link between retinopathy and excess of oxygen	Campbell [64]
Involvement of free radical in oxygen toxicity	Gerschman et al. [68]
Observation of free radicals in biological systems by ESR	Commoner et al. [69]
Detection by ESR of a semiquinone during the riboflavin oxide-reduction	Beinert [31]
Implication of free radicals in biological aging	Harman [70]
Formation of H_2O_2 by microsomal NADPH oxidase	Gillette et al. [86]
Spin restriction in oxygen reactivity	Taube [67]
Discovery of the superoxide dismutase (SOD)	McCord and Fridovich [71]
"Superoxide theory" of oxygen toxicity	McCord et al. [74]
Generation of H_2O_2 by pigeon heart mitochondria	Loschen et al. [82]
Mitochondrial formation of H_2O_2 under hyperbaric conditions	Boveris and Chance [85]
Superoxide as initial product of respiratory burst	Babior et al. [95]
H_2O_2 mimics the signaling activity of insulin	Czech et al. [238]
Stimulation of NADPH oxidase by insulin	Mukherjee and Lynn [240]
Activation by $^.OH$ radical of guanylate cyclase	Mittal and Murad [237]
Oxygen radical involvement in reperfusion injury	Granger et al. [102]
Observation by ESR of ROS production during exercise	Davies et al. [199]
Formation of peroxynitrite from nitric oxide and superoxide	Blough and Zafiriou [129]
Definition of "oxidative stress"	Sies [157]
Increase in lipid peroxidation in hyperthyroid rat liver	Fernández et al. [181]
Identification of bacterial oxyR gene	Christman et al. [260]
Identification of endothelial-derived relaxing factor (EDRF) in $NO^.$	Ignarro et al. [121]; Khan and Furchgott [122]; Palmer et al. [123]
Purification of nitric oxide synthase (NOS)	Bredt and Snyder [125]
Relationship between free radicals and muscle fatigue	Reid et al. [249]
Training slows down peroxidative processes during acute exercise	Venditti and Di Meo [331]
Discovery of Nrf2	Itoh et al. [265]
Training decreased free radical activity	Venditti et al. [337]
Mechanisms by which ROS initiate cellular signaling	Thannickal and Fanburg [295]
Antioxidant supplementation prevents training-induced useful adaptations for muscular cells	Gomez-Cabrera et al. [339]
ROS generation promotes healthy aging	Ristow and Schmeisser [254]

involvement of the hydroxyl radical ($^.OH$) was proposed by Haber and Wilstätter [4]. Currently, oxidation processes that use H_2O_2 activation by iron salts, classically referred to as Fenton's reagent, are known to be very effective in destroying many dangerous organic pollutants in water. Furthermore, the Fenton reaction plays a very important role in free radical biology and medicine.

However, the path taken to recognize the possibility of the independent existence of free radicals and their fundamental importance for living systems has been long and not without obstacles. This review is aimed at retracing this path by highlighting the steps that have made a fundamental contribution to understanding the role played by free radicals in biological systems (Table 1).

2. Free Radical Isolation

Only at the beginning of the last century, evidence was found that isolation of organic free radicals with a measurable lifetime was possible following the preparation of the triphenylmethyl radical, $(C_6H_5)_3C^·$, carried out by Gomberg [5].

In this compound, obtained during the attempt to synthesize the hydrocarbon hexaphenylethane, $(C_6H_5)_3$-C-C-$(C_6H_5)_3$, the central carbon is trivalent since it is combined with three substituents instead of four and presents an unshared electron. Free radicals of the triphenylmethyl type are stable only in certain organic solvents; they are rapidly destroyed by irreversible reactions in the presence of air, water, or strong acids.

A whole range of other aryl-substituted analogous compounds was prepared soon afterwards, and Gomberg's inference that the hexaphenylethane dissociated into two free radicals was apparently substantiated by carrying out molecular weight determinations [6, 7].

It is interesting that this discovery involved stable free radicals. Generally, most free radicals are extremely reactive and, consequently, short-lived species. The great chemical reactivity of the free radicals is to be associated with the available combining energy of the odd electron and their reactions, whenever possible, resulting in the completion of electron pairs. The reason for the relative stability of triphenylmethyl and its analogues, which favoured their isolation, was not an easy problem to solve for theoretical chemists. However, the application of wave mechanics to organic chemistry led to an extended conception of *resonance* within complicated molecules, and it was realized that the domain of the odd electron of triphenylmethyl, like that of the aromatic sextet of benzene, may extend over a large region of intramolecular space. In consequence, much less intrinsic energy is associated with the free valence electron in the complex molecule of the triphenylmethyl than in more simple compounds. However, a relatively stable species, such as triphenylmethyl, is not commonly found, and the technology in that year could not handle transient entities with a very short life so that the research on free radicals had to await further developments.

In many quarters, Moses Gomberg's announcement of the triaryl methyl type free radicals was greeted, even as late as 1930, with disbelief or, at least, disinterest. Still, in 1915, von Richter wrote: "The assumption of the existence of free radicals, capable of existing alone and playing a special role in chemical reactions, has long been abandoned" [8]. A decade later Porter, at Berkeley, said: "Negative results gradually established the doctrine that a free carbon radical was incapable of independent existence" [9], even though the evidence of the existence of free radicals continued to increase.

Although measurements of the vapour densities of gases at high temperatures had indicated that diatomic molecules could dissociate into free atoms, the possibility of the independent existence, at normal temperatures, of free atoms such as hydrogen, oxygen, or chlorine received almost no consideration until 1913, when Bohr showed that the spectrum emitted from a hydrogen discharge tube could be inter-

preted as an emission spectrum of an atomic form and not of a molecular form of hydrogen [10].

In 1922, Wood [11] first isolated and described the properties of atomic hydrogen, which he produced in an electrical discharge tube. The chemical properties of atomic hydrogen were investigated more fully by Bonhoeffer in 1924 [12].

Subsequently, Paneth and Hofeditz [13] prepared the free radical methyl ($^·CH_3$), by pyrolysis of tetramethyl lead using an adaptation of the system used by Bonhoeffer to study atomic hydrogen.

In the subsequent years, the extensive and careful work of Gomberg and other authors who carried on in his field, together with the work on gas-phase free radicals initiated by Paneth, led to a body of experimental evidence for stable as well as short-lived free radicals, so that free radicals gained respectable status in chemical circles.

3. Free Radical in Chemical Reactions

Considering the significant progress made in the field of free radical isolation, it is not surprising that chemists would start to introduce free radical hypotheses into their reaction mechanisms. In 1933, Kharasch and Mayo [14] invoked a free radical mechanism for the addition of hydrogen bromide to olefins which was subsequently applied to other chemical systems.

Before 1933, the orientation of the addition of hydrogen bromide to alkenes was the subject of much confusion. Sometimes, the addition took place according to Markovnikow's rule [15], which established that the acidic proton adds to the less substituted carbon of the double bond. This is because the carbocation obtained from this addition is stabilized by the presence of electron repellent alkyl group(s):

$$CH_3 - CH = CH_2 + H^+ \longrightarrow CH_3 - \overset{+}{C} - CH_3 \xrightarrow{Br^-} CH_3 \\ - CHBr - CH_3 \tag{1}$$
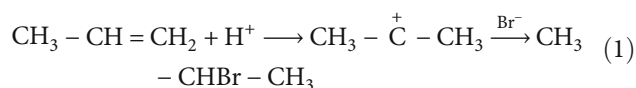
However, at other times, addition took place in the opposite way as the hydrogen added to the most substituted carbon. The mystery was solved in 1933 by the research of Kharasch and Mayo who explained how an anti-Markovnikow orientation could be achieved via free radical addition [14]. The factors able to explain this process turned out to be organic peroxides present in the alkenes-peroxides that were formed by the action of atmospheric oxygen on the alkenes. Indeed, Kharasch and Mayo found that when alkenes that contained peroxides or hydrogen peroxide reacted with hydrogen bromide, anti-Markownikoff addition of hydrogen bromide occurred. Thus, they proposed that the anti-Markownikoff addition of HBr was due to the presence of peroxides and termed this the "peroxide effect," which was thought to proceed through a free radical chain addition reaction in which the intermediate carbon-centred radical was stabilized by the adjacent alkyl group(s):

TABLE 2: Researchers in the field of free radicals and antioxidants awarded with the Nobel Prize.

Fritz Haber	1918 Chemistry	For the ammonia synthesis process
Albert Szent-Györgyi	1937 Medicine	For his discoveries on biological combustion processes, with particular reference to vitamin C and fumaric acid catalysis
Linus C. Pauling	1954 Chemistry	For his researches in the field of the molecular attraction and its applications for the explanation of the structure of complex substances
Nikolaj N. Semënov	1956 Chemistry	For his researches on mechanisms of chemical reactions
Cyril N Hinshelwood	1956 Chemistry	For his researches on mechanisms of chemical reactions
Gerhard Herzberg	1971 Chemistry	For his contributions to the knowledge of electronic structure and the geometry of molecules, in particular free radicals
Louis J Ignarro	1998 Medicine	For the discovery of NO· as signal molecule
Ferid Murad	1998 Medicine	For the discovery of NO· as signal molecule
Robert F. Furchgott	1998 Medicine	For the discovery of NO· as signal molecule

$$R - O - O - R \longrightarrow 2R - O^{\cdot} \xrightarrow{2HBr} 2R - OH + 2Br^{\cdot}$$

$$CH_3 - CH = CH_2 + Br^{\cdot} \longrightarrow CH_3 - \overset{\bullet}{C}H - CH_2 \qquad (2)$$

$$- Br \xrightarrow{HBr} CH_3 - CH_2 - CH_2\text{-}Br + Br^{\cdot}$$
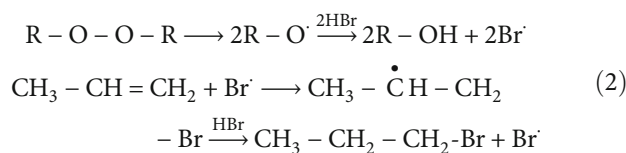
Kharasch also suggested that radical intermediates and chain reactions can play an important role in many organic reactions, and over the following years, he succeeded in developing synthesis reactions, including sulfonation [16], chlorination [17], and carboxylation [18] of hydrocarbons and paraffin.

His work paved the way to the development of synthetic materials ranging from rubber to plastics. Conventional polymerization for condensation continued to be used to produce nylon and other products. But free radical polymerization had advantages such as high tolerance of chemical impurities and extreme temperatures and the ability to be used with a wide range of monomers (organic molecules). Today, free radicals are used to produce nearly half the polymers we use—materials used in everything from food wrapping to paint, adhesives, film, carpeting, piping, and more.

In this context, the work of Semenov, whose researches fall into the field of chemical kinetics and the processes of combustion, is significant [19]. The outcome of these researches is the certainty that many chemical reactions are aided by free radicals which are produced during the process. Semenov's work also opened a new path for understanding the connection between the reactivity and the structure of particles entering a chemical reaction and created the possibility of rationally regulating the rate and direction of chemical changes. This, in turn, had profound consequences for the improvement of consolidated industrial processes and for the development of new processes, for example, in the fields of polymerization and direct oxidation as well as hydrocarbon cracking. Interestingly, some concepts of Semenov concerning chain reactions were also formulated by the renowned English kinetic chemist C. N. Hinshelwood [20], who was awarded the Nobel Prize in Chemistry jointly with Semenov in 1956 (Table 2).

It is worth noting that antioxidants were used for the first time in the nineteenth century in the rubber industry when it

was observed that some molecules, identified empirically, could slow the degradation and allow optimization of the process of vulcanization. Moureau and Dufraisse [21] were the first to propose an inclusive theory which should account for the effectiveness of almost infinitesimally small amounts of material in retarding or accelerating the processes of oxidation.

It has also been suggested [22] that Cummings and Mattill [23], who in 1931 realized the idea of the function of vitamin E as an *in vivo* lipid antioxidant, were perhaps inspired by the work on autoxidation by Moureau and Dufraisse [21].

In terms of antioxidants, a milestone in the evolution of knowledge was the work of Albert Szent-Györgyi, who, in the thirties, isolated a strongly reducing substance that he called "hexuronic acid" [24], and that was subsequently identified as vitamin C [25].

The same researcher and his collaborators studied the antioxidant effect of polyphenols found in plants governing vascular permeability and proposed the name vitamin P [26] but could not withstand the criticism of the absence of deficiency syndrome, as fundamental to the definition of a function vitamin.

A further significant contribution to the free radical chemistry was the concept found in Haber's final paper [27] that the highly reactive hydroxyl radical could be generated from an interaction between superoxide ($O_2^{\cdot}$) and hydrogen peroxide (H_2O_2):

$$H_2O_2 + O_2^{\cdot -} \longrightarrow O_2 + {}^{\cdot}OH + OH^- \qquad (3)$$

The authors recognized that the reaction is thermodynamically unfavourable in biologic systems, having a second-order rate constant of zero in aqueous solution, and would require some sort of catalyst to proceed. Thus, they discussed the need for a metal ion catalyst and illustrated that the net reaction creating the hydroxyl radical can be broken down into two chemical reactions (Equations (4) and (5)).

The former predicts that hydrogen peroxide is reduced at the iron centre with the generation of the hydroxyl free radical. This reaction is commonly referred to as Fenton reaction

although Fenton never wrote it:

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + {}^{\cdot}OH + OH^- \qquad (4)$$
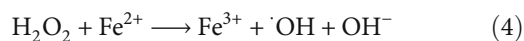
The second indicates that superoxide recycles oxidized iron to the ferrous form:

$$O_2{}^{\cdot -} + Fe^{3+} \longrightarrow Fe^{2+} + O_2 \qquad (5)$$
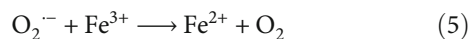
It is interesting that, although a lot of research was done to determine the nature of the species involved in Fenton reaction, the nature of these species is still under discussion. In fact, the popular theory, due to Haber-Weiss, involving the formation of ${}^{\cdot}OH$ radicals, has been questioned by studies suggesting an alternative interpretation of the Fenton reaction including the formation of the ferryl ion as active oxidizing species [28].

Chemical studies involving free radicals were ongoing for many years before it was recognized that such reactive species are produced in biological systems. At that point, it was quickly realized that the Haber-Weiss reaction might represent the major mechanism by which the highly reactive hydroxyl radical is generated in biological systems.

4. Free Radicals in Biological Systems

It is widely believed that the prolific work of Leonor Michaelis was responsible for the interest in free radicals as naturally occurring biochemical intermediates in biological systems. In investigations started in the 30s, Michaelis observed that the curves of oxidation-reduction potentials, obtained by adding increasing amounts of an oxidant to hydroquinone, initially showed the loss of an electron and then, in a subsequent stage, the loss of another electron [29]. He believed that the loss of the first electron corresponded to the formation of the free semiquinone radical. The second electron was given by the semiquinone with the formation of the completely oxidized form, the quinone.

Since the intermediate radicals are very unstable and quickly lose the second electron, their concentration in the reaction medium is very low, so it is very difficult, or even impossible, to identify them. Therefore, it is not always possible to differentiate oxidation reactions, which proceed in one step and involve radicals, from those that proceed by transferring two electrons at a time, relying exclusively on the products that are formed. However, Michaelis enunciated the theory that all the oxidations of organic molecules, which involve the exchange of two electrons, proceed through two successive stages, the intermediate stage being constituted by a free radical [30].

We know today that this theory is inaccurate in that there are biochemical reactions in oxidative reactions with loss of two electrons, which occur in a single step and which do not involve free radicals. However, Michaelis's ideas gave rise to research that indicated that some intermediate reagent can sometimes be identified in enzymatic oxidation-reduction reactions of biological molecules, even though there was no evidence that the intermediate was a free radical.

However, starting in 1954, using sensitive methods for detecting free radicals, such as electronic spin resonance (ESR), which uses the paramagnetism of free radicals, some researchers showed that in some enzyme-substrate systems, it was possible to identify a paramagnetic intermediate compound. One of the areas in which the ESR was validly used was the study of enzymatic oxidation. In such reactions, an electron is first removed by an enzyme from the substrate and then transferred to a coenzyme. An important example is the formation of a semiquinone during the riboflavin oxide-reduction (vitamin B2), which constitutes the flavin coenzymes, flavin mononucleotide (FMN), and the flavin adenine dinucleotide, involved in many oxidation-reduction reactions [31].

Later, it was shown that free radicals were much more widespread in biological systems than previously assumed. Free radicals were found not only in the case of oxidation-reduction processes but also in many reactions of biological interest such as photochemical reactions, photosynthesis, and bioluminescence.

The path, which has led to the onset and acceptance by the scientific community of the idea that free radicals are continuously formed in the cell as collateral products of normal metabolic reactions, can be initiated by the discovery of the mechanisms underlying the effects of oxygen toxicity and ionizing radiation.

5. Effects of Radiation

Evidence is available that physical or chemical environmental perturbations, of which radiation emission is perhaps the main contributor, can induce pathological effects through free radical mechanisms.

In 1895, the German physicist Röentgen discovered "a new kind of ray" which could blacken photographic films enclosed in a light-tight box [32]. He named these rays as X-rays, which meant unknown rays. In 1896, radioactivity was discovered by Antoine Henri Becquerel [33]. In the same year, Pierre and Marie Curie discovered and isolated radium successfully [34].

The potential hazards of radiations or radioactive materials were not adequately acknowledged at the beginning of their discoveries, although there was evidence that radiation produces biological damage. In fact, the first cases of human injury were reported in the literature just a few months following Röentgen's original paper announcing the discovery of X-rays (see Sansare et al. [35] for a review). Furthermore, as early as 1904, the first case of death occurred due to metastatic carcinoma induced by X-ray exposure.

Early human evidence of harmful effects because of exposure to radiation in large amounts existed in the 1920s and 30s, based upon the experience of early radiologists, miners exposed to airborne radioactivity underground, persons working in the radium industry, and other special occupational groups. However, the long-term biological significance of smaller, repeated doses of radiation, however, was not widely appreciated until relatively recently, and most of our knowledge of the biological effects of radiation has been accumulated since World War II. In fact, much of what we know today about the relationship between ionizing and

cancer stems from studies conducted on people who survived the atomic bombs of Hiroshima and Nagasaki. Since these were very particular exposure conditions, it was difficult for a long time to understand whether those conclusions could be applied even at the most common levels of exposure. In recent years, several studies have confirmed that even low levels of exposure can give rise to the transformation of cells that leads to the development of cancer. The quantification of this risk, however, is very complex: it depends on several factors, including the dose to which one is exposed and the duration of exposure, the type of radiation, the areas of the body irradiated, and the age at which one has entered in contact with radiation.

In the first part of the last century, much progress was also made in understanding the effects induced by ionizing radiation on individual components of biological systems and the basic mechanism underlying such effects.

Early studies concerning the action of radiation on enzymes had led to the conclusion that X- and γ-rays only influence enzymes when the dose is enormous [36]. However, in 1940, Dale found that the reason for these failures was the use of large amounts of enzyme and of impure preparations [37]. Indeed, by decreasing the concentration of the enzyme, the carboxypeptidase, he obtained inhibition with relatively low doses of X-rays. Subsequent studies showed that when dilute solutions of enzymes were irradiated with X-rays, the enzymes requiring -SH groups for enzymatic activity were more susceptible to inhibition than enzymes that did not require –SH groups for activity [38]. Furthermore, the enzymes can be reactivated by the addition of glutathione. Thus, it was suggested that inhibition of sulfhydryl enzymes was due to oxidation of sulfhydryl groups, whereas inhibition of nonsulfhydryl enzymes, which required larger amounts of X-rays, was attributed to protein denaturation. The mechanism of –SH groups was explained by following Weiss's suggestion [39], of the liberation of free radicals resulting from the interaction of X-rays with the water molecules. In fact, ionizing radiation can directly damage different biologically essential macromolecules, such as DNA, membrane lipids, and proteins, through ionizations that create sites of electron loss (radical cations), electron gain (radical anions), and excitations. However, the action of ionizing radiation can also be indirect involving the primary formation of free radicals, such as H$\cdot$ and $\cdot$OH, with subsequent formation of H_2O_2, atomic oxygen, and $HO_2\cdot$, which react with the macromolecules.

This indirect action already suggested by Risse [40] in his "activated solvent" hypothesis, and later developed by Fricke [41], had been resumed by Dale, who had postulated that carboxypeptidase enzyme molecules were not directly affected by the ionizing radiation, but indirectly through collision with a labile product resulting from water ionization [31].

The significant consequences of various types of radiation-induced DNA damages show that DNA is the principal target for the biological effects of radiation.

In the early days, radiation-induced DNA damages were studied under two different conditions, irradiating the DNA molecule either directly or in a dilute aqueous solution. These studies allowed to establish that, as for proteins, ionizing radiation damages DNA either directly by deposition of energy in the DNA resulting in the ejection of electrons leading to the formation of a free radical (R) or indirectly by the ionization leading to the formation of hydroxyl radicals and indirectly by reactions with radicals produced by ionizing H_2O molecules.

It was also hypothesized that the free radical-mediated covalent modifications leading to oxidative damage to critical biomolecules during and immediately following irradiation result in most, if not all, of the biological effects of ionizing radiation. The most compelling evidence in favour of this hypothesis came from observations that manipulations of antioxidants (i.e., thiols, hydroxyl radical scavengers, and hydroperoxide metabolizing enzyme systems) at the time of irradiation appeared to alter the reactions of free radicals (and reactive oxygen species) leading to alterations in oxidative damage as well as alterations in the biological effects of IR [38, 42, 43].

It is worth noting that the radiosensitivity of the tissues is strongly dependent on oxygen concentration, a phenomenon named oxygen effect. This effect was first demonstrated by Schwartz in 1909 [44]. Using X- and γ-rays, he showed that firm pressure applied to the skin during irradiation greatly reduced the subsequent reaction as compared to irradiation under normal conditions. Later, Jolly found reduced damage in the guinea pig and rat thymus irradiated when the blood supply was occluded by a ligature [45], and Mottram observed similar protection from radiation damage by occluding the blood supply of the rat's tail by ligature of the vascular connections [46]. These findings were later confirmed by other researchers who used different animal species and tissues [47].

For a long time, the oxygen fixation hypothesis, developed in the late 1950s, has been widely regarded as the most satisfactory explanation of the oxygen effect [48]. Central to this hypothesis was the belief that most DNA damage produced by X-rays can be repaired, but that repair is more difficult or impossible when caused by the product of a radical and an oxygen molecule, so that the amount of stable DNA damage and the extent of lethality from a given dose increase [48]. In fact, the actual role of oxygen in the inactivation mechanism represents still an open problem; in particular, it has been shown that oxygen fixation hypothesis cannot be regarded as maintainable more and, on the other hand, has argued that the oxygen effect can be hardly a simple consequence of greater reactivity of oxygen radicals [49]. Such an explanation of the oxygen effect is based on the possibility that the primary radicals, H$\cdot$ and e^-_{aq} react with O_2, generating $HO_2\cdot$ and $O_2^{\cdot-}$, respectively:

$$H\cdot + O_2 \longrightarrow HO_2\cdot$$
$$e^-_{aq} + O_2 \longrightarrow O_2^{\cdot-} \tag{6}$$
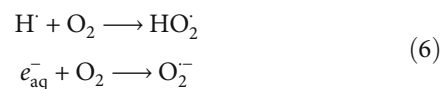
This should lead to the generation of new free radicals which amplify the radiation effect. On the other hand, it is not possible to exclude that a free radical generated from a biological molecule, by direct effect or by extraction of one hydrogen by $\cdot$OH, can bind to O_2 forming a peroxyl radical, which can fix the damage in a relatively unrepairable

condition, and extract one hydrogen from another molecule causing a chain reaction.

It is also worth noting that, while classical radiation toxicity models identify DNA damage as the universal critical lesion in cells [50], studies now support that the survival of many organisms is governed by the level of oxidative protein damage caused during irradiation [51, 52], which limits the functionality and efficiency of enzymes, including those needed to repair and replicate DNA.

The presence of oxygen also increases the damage to lipids. Indeed, unsaturated lipids are liable to undergo a process known as lipid peroxidation, a chain reaction, initiated by reactive free radicals such as ˙OH, which, in the presence of oxygen, leads to the formation of lipid peroxides and other derivatives [53]. Peroxidation of membrane lipids alters their structure and interferes with membrane function. Studies beginning in the 1960s showed that several halogenated hydrocarbons exert their toxic effects by stimulating lipid peroxidation and gave early emphasis on the biological role of free radical reactions [54–56]. However, although detection of end products of lipid peroxidation is the evidence more frequently quoted for a role of free radicals in human disease or tissue injury by toxins, lipid peroxidation has not been well exploited to understand the biological effects of radiation. Probably, this occurred because increases in protein and DNA damage are often more important events in causing cell injury than peroxidation of membrane lipids, which is often a late event accompanying rather than causing final cell death.

6. Oxygen Toxicity

For a large part of living beings, oxygen is an essential molecule for survival, being the basis of biological oxidations, which meet most of the energy needs of aerobic organisms. Although oxygen is essential for these organisms, it can also act as a toxic agent and pose a threat to their existence. This contradictory aspect of aerobic life was defined by Davies as the "paradox of aerobic life" [57]. All organisms can survive in the presence of oxygen because, in adapting to the oxidizing atmosphere of the earth, they have developed an elaborate antioxidant defence system. However, this system is only suitable for oxygen pressure in the atmosphere (about 156 mmHg), and it is widely demonstrated that exposure to oxygen pressures greater than the atmospheric one causes severe damage.

Historically, reports of harmful effects of oxygen followed soon after the discovery and purification of the gas in the late 18th century, which permitted scientists to expose animals to oxygen-enriched atmospheres.

Usually, priority in the discovery of oxygen is given to Priestley who called it "dephlogisticated air" [58], even though the oxygen had been discovered in 1772 by Carl W. Scheele, a Swedish apothecary, who, however, did not publish his findings until 1777 [59].

Anyway, Priestley was one of the first to suggest that there may be adverse effects of this "pure air" when he observed a candle burn out faster in oxygen than in air and wondered if "the animal powers are too soon exhausted in this pure kind of air" [58].

On the other hand, the toxicity of atmospheric oxygen had already been exploited by our ancestors for therapeutic purposes, such as the treatment, by exposure to air, of sites infected with anaerobic bacteria.

Immediately after the promulgation of the combustion theory of respiration, the pertinent literature recorded a considerable number of investigations in connection with the question of oxygen toxicity. According to various authors, definite effects were obtained by breathing pure oxygen. The respiratory exchange was increased, the circulation quickened, and congestion of the lungs, or even inflammation and death, occurred. The theory was that the addition of oxygen increased the pulmonary combustion and thereby produced these pathological changes. This result was controverted by Regnault and Reiset in their classical investigation [60]. They showed that no increase in oxidation occurred and no pathological changes ensued on the exposure of animals to atmospheres rich in oxygen.

The first important contribution in the field of oxygen toxicity was by Paul Bert who, in 1878, first demonstrated convulsions in larks exposed to 15-20 ATA (atmosphere absolute) air [61], and the toxic effects of oxygen on the central nervous system are hence called "Paul Bert effect." Furthermore, in a large series of experiments, he showed that the effects on all living organisms arising from variations in barometric pressure are entirely the result of the tensions at which the oxygen is maintained in the various atmospheres. By exposing an animal to four atmospheres of oxygen, the same effect is brought about as that caused by increasing the barometric pressure of the air 20 times [61].

In 1899, J Lorain Smith, while trying to reproduce "Bert effect," noticed fatal pneumonia in rats after 4 days of exposure to 73% oxygen at 1 ATA [62]. This marked the discovery of pulmonary toxicity of oxygen, called the "Lorrain Smith effect."

The signs of oxygen toxicity are detectable in various tissues, even if the most worrying ones are those of the central nervous system and of the lung, which can be considered real target organs.

However, when after 1940 the spread of a syndrome currently called "retinopathy of prematurity" was found in preterm infants, this syndrome was not related to oxygen toxicity. Retinopathy was characterized by the formation of new abnormal blood vessels in the retinal periphery and could result in real blindness due to retinal detachment. Furthermore, the lower the gestation time and the weight of the newborn at birth, the greater the risk. Retinopathy initially described as retrolental fibroplasia by Terry in 1942 [63] was the leading cause of blindness in children in the United States. Only in 1951, it was suggested that the retinopathy was due to an excess of oxygen in the incubators where premature infants were placed to promote their development [64].

6.1. Mechanisms of Oxygen Toxicity. The first hypothesis made to explain the toxic effects of oxygen was that it inhibits cellular enzymes. There were several examples of enzymatic inactivation linked to oxygen in anaerobic organisms [65]. However, there was a reluctance to conclude that the inhibitory effects of oxygen on metabolism were the direct cause of the symptoms of the oxygen toxicity on the intact animal.

Haugaard, who reviewed the pertinent literature in 1968 [66], gave two reasons why the hypothesis did not gain wider acceptance. First, the rates at which enzymes were inactivated in vitro were too slow to account for the rapidity of toxic effects in intact animals. Furthermore, in aerobic cells, most of the enzymes are completely insensitive to oxygen, and those inactivated are quite low.

Despite being a free radical, O_2 does not present a high reactivity. The reactions in which it is involved do not normally occur at ordinary temperatures or in the absence of catalysts, although its high oxidizing power makes most of the substances of biological interest thermodynamically unstable in its presence. This apparent contradiction is explained by the electronic configuration of the O_2 in the ground state, which has two electrons with parallel spins in the two outermost orbitals. For this reason, in the oxidation processes, it would be necessary to make available, by the molecules to be oxidized, two electrons with spin parallel to each other and opposite those of the unpaired electrons of oxygen. Since the molecules of stable organic compounds contain valence electrons with opposite spin, the need to operate spin inversion before electrons are accepted in the oxygen orbitals slows or precludes the reaction with such molecules, a phenomenon called spin restriction [67].

Since the energy necessary for vital processes in aerobic organisms derives from oxidation reactions in which oxygen is consumed, it is evident that in such organisms, processes are working through which the spin restriction is, in some way, eliminated with consequent increase of the reactivity of oxygen.

It is possible to put enough energy into oxygen to elevate one of its parallel spinning electrons to a higher orbital and in the process to invert its spin. Such an excited state of oxygen is referred to as singlet oxygen, and for singlet oxygen, the spin restriction has been eliminated and it is much more reactive than is ground state oxygen [67].

The energy inherent in visible light is enough to convert ground state oxygen into singlet oxygen, but oxygen does not absorb visible light. Dyes such as methylene blue or rose Bengal do absorb visible light and then, upon collision with oxygen, can transfer the energy from that light to oxygen. That is one basis of photosensitized oxidations. There is a way that the spin restriction can be circumvented, and that is by adding the electrons to oxygen one at a time at a rate that allows electronic spin inversions to occur between collisional events. The univalent pathway of oxygen reduction requires that intermediates of oxygen reduction be generated, which are reactive and can damage biological molecules.

On this basis, an important turning point in understanding the mechanism of toxicity occurred when in 1954 Gerschman and associates published an article hypothesizing that oxygen poisoning and radiation injury have at least one common basis of action, possibly through the formation of oxidizing free radicals [68]. The hypothesis was based on similarities of the effects of irradiation and exposure to hyperbaric oxygen, on the synergism between radiation and hyperbaric O_2 in decreasing the survival of exposed mice, and on observations that substances of varied chemical nature known to increase resistance to irradiation exhibited protective action against oxygen poisoning.

In the same year, Commoner et al. made the first observation of free radicals in biological systems using electron paramagnetic resonance methods [69].

Two years later, Harman hypothesized that oxygen radicals may be formed as by-products of enzymatic reactions in vivo. He proposed that traces of iron would catalyse oxidative reactions in vivo and that peroxidative chain reactions were possible by analogy to the principle of in vitro polymer chemistry. Harman also implicated that free radicals are produced during aerobic respiration in cellular damage, mutagenesis, cancer, and degenerative process of biological aging [70].

The theory then led to a significant corollary on the role of antioxidants: if the oxidative reactions are a chain, one molecule that intercepts the radical initiator or propagator prevents the entire chain. It follows that only the reactions of radicals that trigger chains may be the subject of a functionally valid inhibition by antioxidants: a concept that, although simple, is today very often forgotten.

7. The Superoxide Dismutase Discovery

Harman's idea did not capture the imagination of most life scientists, until the discovery of the superoxide dismutase (SOD) enzyme by McCord and Fridovich [71]. Toward the end of the 60s, these authors investigated the oxygen-dependent reduction of cytochrome c by xanthine oxidase [72]. Oxygen would be expected to oxidize cytochrome c rather than to facilitate reduction, so that McCord and Fridovich suggested that xanthine generated an unstable reduced form of oxygen, presumably the superoxide anion, and that this radical was the agent which directly reduced cytochrome c. Because some proteins inhibited the reduction of cytochrome c by xanthine oxidase, they realized that inhibitory effects were due to novel protein contaminating the other proteins tested, which catalysed the superoxide dismutation. They purified from bovine erythrocytes a protein that inhibited the reduction of cytochrome c. This protein had been previously purified as a copper-binding protein [73], but McCord and Fridovich showed that this protein was an efficient scavenger of superoxide, thus identifying the first enzyme known to act on a free radical substrate [71].

Similar enzymes were soon recovered from a wide range of air-tolerant bacteria but, significantly, were scarce in anaerobes [74].

A protein antioxidant catalyst which had as its substrate a small inorganic free radical was something novel to biochemistry at that time, but, paradoxically, the observation of the widespread distribution of SOD family of enzymes in cells was the major hint of the wide occurrence of free radical activity in the body.

Despite this, initially, the role of SOD was greeted with scepticism by some. Not all were prepared to accept that SOD was exclusively a biological enzyme and preferred to view it more as a copper transport protein.

In these conflicting opinions, an important role played the idea that the radicals were too reactive and uncontrollable to participate in any reaction involving an enzyme.

However, the discovery of the superoxide dismutase made real the existence of radicals in living systems and led to the "superoxide theory," which foresaw that the O_2 toxicity was due to the generation of anion radical superoxide [74]. Interestingly, subsequent observations performed modifying SOD activity showed that superoxide was at least partially responsible for the mechanisms leading to the well-known effects of oxygen on radiosensitization [75].

Subsequent studies showed that the superoxide is a moderately reactive radical, which under physiological conditions generally behaves as a mild reductant rather than an oxidizing agent. This is because superoxide exists naturally as a small anion, which is more prone to give its electron than to accept a second electron from another biological molecule. The limited chemical reactivity of superoxide created considerable controversy about the role of superoxide in cellular toxicity [76].

However, superoxide can be considered the "primary" oxidant species, whose generation can lead to the formation of more reactive "secondary oxidant species" [77].

To explain how a more potent oxidant might be generated by superoxide, the iron-catalysed formation of hydroxyl radical from hydrogen peroxide was proposed. Indeed, superoxide can undergo SOD-catalysed dismutation, producing hydrogen peroxide:

$$2O_2^{\cdot -} \xrightarrow{\text{SOD}} O_2 + H_2O_2 \qquad (7)$$
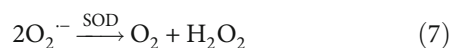
Hydrogen peroxide reactivity is reduced by the stability of its oxygen-oxygen bond. However, in the presence of reduced transition metals (e.g., Fe^{2+} or Cu^+), it can be converted into the hydroxyl radical (Fenton reaction) (Equation (4)). Conversely, the hydroxyl radical has high reactivity, which makes it a very dangerous radical with a very short in vivo half-life of approx. 10^{-9} s [78].

The hydroxyl radical oxidizes most organic molecules at diffusion-limited rates, but its most significant impact is likely to be upon DNA since even a single DNA lesion is potentially mutagenic or lethal.

Because of its strong oxidant capacity and importance in radiation-induced damage to biological molecules, hydroxyl radical became widely accepted as the major toxin produced in vivo.

In the last quarter of the 20th century, the scientific community began to accept the fundamental principle that many of the same free radicals that were formed as a result of ionizing radiation interacting with biological material were also formed as by-products of oxidative metabolism.

8. Reactive Oxygen Species

It is worth noting that until the mid-1970s, the literature almost exclusively refers to free radicals. Later, it became evident that not only free radicals but also nonradical products, such as H_2O_2 or hypochlorous acid (HOCl), which are also powerful oxidizing agents, participate in free radical reactions. To consider both the radical and the nonradical species, the more general term "reactive oxygen species" (ROS) was introduced [79]. Later, species containing nitrogen, such as nitric oxide (NO$^{\cdot}$) and peroxynitrite (ONOO$^-$), were shown to be biological molecules and were termed reactive nitrogen species (RNS) [80].

This view that ROS were generated in biological systems was also supported by the finding of the cellular sources of such species. A mitochondrial H_2O_2 production had been first observed in 1966 by Jensen, who found that antimycin-insensitive oxidation of nicotinamide adenine dinucleotide (NADH) and succinate by bovine heart submitochondrial particle was coupled with H_2O_2 production [81]. Soon after, it was demonstrated, for the first time, that under aerobic condition, H_2O_2 was generated by pigeon heart mitochondria in the presence of succinate [82]. The discovery that mitochondria contain their own SOD, MnSOD [83], and the subsequent detection of the mitochondrial generation of superoxide radical anion [84] confirmed that H_2O_2 generated within mitochondria arose from the dismutation of superoxide ($O_2^{\cdot -}$) and the biological significance of mitochondrial $O_2^{\cdot -}$ production. Since then, a huge literature has developed on the sources and consequences of mitochondrial production of reactive oxygen species. In particular, the discovery that electron transfer along the inner mitochondrial membrane carriers is associated with the formation of reactive oxygen species suggested the mitochondrial involvement in degenerative processes linked to several diseases and aging.

In this regard, it is worth noting that a detailed study evaluating the variations of formation of H_2O_2 under different metabolic conditions showed a marked increase in such a formation and its immediate onset under hyperbaric conditions [85], thus providing an explanation at the molecular level for "O_2 poisoning."

Although mitochondria are responsible for the continuous production of reactive oxygen species, they are not the only source at either the organism or the cellular level. In fact, in the 70s, evidence was already available that generation of active oxygen species can also occur as a by-product of other biological reactions in other cellular organelles.

For example, it was long known that liver microsomes contained an enzyme system, NADPH (TPNH) oxidase, which catalyses the oxidation of NADPH by oxygen to yield NAD$^+$ and hydrogen peroxide [86]. In later years, this result was confirmed repeatedly, and the H_2O_2 formation was believed to be linked to the monooxygenation system for xenobiotics since pretreatment of the animals with phenobarbital increased the rate of H_2O_2 formation [87] and inhibitors of cytochrome P450 affected the production of H_2O_2 [88].

It was also recognized that NADPH-dependent redox chain of microsomes generated hydrogen peroxide and superoxide radicals [89], the latter being possibly precursors of the former. Moreover, superoxide formation involved autooxidation of NADP-specific flavoprotein dehydrogenase (NADPH-cytochrome c reductase) [88] and dissociation of oxycomplex of cytochrome P450 [90].

Other examples of cellular organelles which were found to have high oxidative activity include peroxisomes, which

contain oxidases, which reduce oxygen to hydrogen peroxide at the expense of the oxidation of a substrate RH_2, and large amounts of catalase (CAT), an enzyme able to reduce hydrogen peroxide to water [91]. The idea that the peroxisomes generate ROS as an integral feature of their normal metabolism was further exemplified by the fact that peroxisomes in the rat liver may be responsible for as much as 20% of the oxygen consumption and 35% of the H_2O_2 production [92].

In 1961, Iyer et al. [93] had shown that the phagocyte respiratory burst results in the generation of hydrogen peroxide; in 1964, Rossi and Zatti [94] had correctly proposed that an NADPH oxidase was responsible for the respiratory burst; and in 1973, Babior et al. [95] reported that the initial product of the respiratory burst oxidase was superoxide and not hydrogen peroxide. The phagocyte NADPH oxidase was the first identified example of a system that generates ROS not as a by-product but rather as the primary function of the enzyme system.

The next steps were the identification of proteins responsible for ROS production in phagocytes [96] and the cloning of the gene coding for the catalytic subunit of the phagocyte NADPH oxidase, commonly referred to as gp91^{phox} [97], which in the novel NOX terminology was called NOX2. In parallel with the progress toward understanding the phagocyte NADPH oxidase, a series of observations suggested that enzyme systems like the phagocyte NADPH oxidase exist in many other cell types (see Bedard and Krause [98] for a review).

Several soluble cell components, including thiols, hydroquinones, catecholamines, and flavins, were found to be able to undergo redox reactions and contribute to intracellular ROS production [99]. To these, it occurs to add several cytosolic enzymes that produce ROS during their catalytic activity. Among the enzymes producing ROS, the researchers paid close attention to xanthine oxidase (XOR).

XOR is a flavoenzyme, which was extensively studied for its biochemical and structural properties ever since 1902 [100]. The enzyme catalyses the oxidation of hypoxanthine to xanthine and xanthine to uric acid using $NADP^+$ or O_2 as an electron acceptor. The mammalian enzymes exist in the NAD^+-dependent form (xanthine dehydrogenase (XDH)) in freshly prepared samples from organs under normal conditions, i.e., they exhibit high xanthine/NAD^+ reductase activity, even in the presence of O_2 [101]. In oxidatively damaged tissues, the XDH is converted, due to proteolysis or oxidation of the thiol groups of cysteine, into xanthine oxidase (XO) [101], which, when acting on its substrates, is able to transfer electrons to molecular oxygen generating the superoxide [72].

Some years after, it was proposed that xanthine oxidase-derived oxidants mediate the microvascular injury associated with reperfusion of the ischemic intestine [102], and subsequently, this idea was extended to several organs and systems [103].

9. Reactive Nitrogen Species

The discussions above have focused on oxygen and oxygen-derived species. However, there are other relevant radicals, nitric oxide, NO$^{.}$, and its derivatives.

Nitric oxide is the first gaseous species unequivocally identified as an endogenously generated cell signalling/effector agent. Furthermore, superoxide is considered the primary ROS from which more strongly oxidizing species originate, so nitric oxide is considered the primary RNS.

Nitric oxide, first identified as a gas by Joseph Priestley, is a simple molecule consisting of just one atom of oxygen and one atom of nitrogen [104]. For a long time after this discovery, NO$^{.}$ was thought to be simply an atmospheric pollutant. It is produced above all during high-temperature combustion processes, like those that happen in the engines of cars, together with nitrogen dioxide ($NO_2^{.}$), another free radical. It is then oxidized in the atmosphere by oxygen and more rapidly by ozone-producing nitrogen dioxide. The toxicity of nitrogen dioxide is significant, contrary to that of nitric oxide, which is instead limited. Indeed, nitrogen dioxide plays a fundamental role in the formation of photochemical smog as it is the basic intermediate to produce a whole series of very dangerous secondary pollutants such as ozone, nitric acid, nitrous acid, alkyl nitrate, and peroxyacetyl nitrates.

The proposal that NO$^{.}$ was a biological molecule was rather controversial because early indications of its presence in biological systems were ignored for many decades and even because nitrovasodilators had been used clinically for a century without understanding their mechanism of action. In fact, at that time, drugs such as nitroglycerine were given to patients for heart conditions like angina to promote vasodilation and reduce blood pressure, but no one knew how these drugs worked.

The discovery of NO$^{.}$ as a biological molecule involved in vasodilation is linked to the studies dealing with cyclic guanosine monophosphate (cGMP) and endothelial-derived relaxing factor (EDRF).

Cyclic GMP began to emerge as a second messenger during the late 1960s and early 1970s. The existence of endogenously produced cGMP was demonstrated by its isolation and identification from rabbit urine [105]. Later, this was confirmed by another study from the same laboratory [106], which suggested that cGMP is synthesized in a reaction catalysed by a cyclase. In 1966, a phosphodiesterase specific for cGMP was isolated and partially purified from a dog heart [107], and subsequently, guanylyl cyclase activity was described [108]. Thus, it became apparent that steady-state levels of cGMP in cells and tissues were determined by the balance of cGMP synthesis by guanylyl cyclase and cGMP degradation by phosphodiesterases. Increased levels of cGMP produced by guanylate cyclase within vascular smooth muscle allowed blood vessels to relax and thus increase blood flow.

In 1977, two groups demonstrated independently that organic nitrates induced a dose-dependent increase in the levels of cGMP in smooth muscle [109, 110]. Subsequently, biochemical experiments showed that all the nitrovasodilators and nitric oxide (NO) activate the soluble guanylate cyclase [111, 112]. However, the possibility that NO might be synthesized in mammals and was able to function as an endogenous regulator was considered to be too far-fetched for another decade.

Progress was made when Furchgott and Zawadzki [113] found that without the endothelial cells the smooth

muscle cells were not able to cause vasodilation. This suggested that a factor produced by the endothelial cells was required for relaxation of the blood vessels acting on an unidentified target in skeletal muscle. The diffusible factor later referred to as the endothelium-derived relaxing factor (EDRF) [114] was quickly inactivated by oxyhaemoglobin and was inherently unstable in the perfusion cascades used to study the vasorelaxation. It was soon shown that this mysterious factor, termed endothelial-derived relaxing factor (EDRF), increased cGMP synthesis in isolated blood vessels and increased protein phosphorylation in smooth muscle [115, 116].

Widespread speculation about the chemical nature of EDRF developed soon after its discovery [117, 118], but only in 1988, Furchgott [119] and Ignarro et al. [120] independently in papers presented at a symposium in Rochester, suggesting that EDRF produced by endothelium may be $NO^{\cdot}$.

Soon after the 1986 symposium, three laboratories compared the biological and chemical characteristics of EDRF and NO, and all found EDRF released upstream and NO infused upstream to have similar rates of decay, similar susceptibility to inhibitors like Hb and superoxide generators, and similar stabilization by SOD [121–123]. A year later, Palmer and coworkers [124] made the major finding that the source of endothelial NO was guanidinium hydrogen of L-arginine.

In 1990, Bredt and Snyder purified oxide nitric synthase (NOS), the enzyme responsible for NO synthesis from a rat cerebellum (NOS) [125]. Because this NOS was from the rat cerebellum, it was named the neuronal NOS isoform (nNOS or NOS-1). The discovery of constitutively expressed neuronal NOS was quickly followed by the identification of endothelium-derived NOS (eNOS or NOS-3), constitutively expressed [126], and inducible NOS (iNOS or NOS-2) not constitutively expressed [127].

The discovery of $NO^{\cdot}$ as an enzymatically generated free radical was paralleled by the recognition that it could readily react with $O_2^{\cdot-}$ [128]. It was known that the combination reaction leads to proximities [129], a peroxy acid originally studied in the chemical literature as a strong oxidizing and nitrating compound [130]. A paper on the unusual properties of a mixture of hydrogen peroxide and nitrous acid by Baeyer and Villiger from 1901 [131] can be regarded as the first report on peroxynitrite. However, as an oxidant, peroxynitrite attracted little attention because it produced a bewildering array of products even with simple starting substrates such as phenol. In 1970, several investigators more thoroughly characterized the chemistry of peroxynitrite showing that it decomposed to form hydroxyl radical and nitrogen dioxide [132].

At the end of the 80s, when $NO^{\cdot}$ was discovered, the controversy about the reactivity of superoxide and its biological effects was still open. Therefore, it is not surprising that $NO^{\cdot}$ was viewed by some researchers as a protective factor because of its capacity to scavenge radical superoxide thus acting as an antioxidant [133, 134]. However, in 1990, Beckman et al. suggested that the reaction product of superoxide and nitric oxide was peroxynitrite, which decomposes when protonated to form the potent oxidants, hydroxyl radical

and nitrogen dioxide [135]:

$$O_2^{\cdot-} + NO^{\cdot} \longrightarrow ONOO^-$$
$$ONOO^- + H^+ \longrightarrow ONOOH \longrightarrow NO_2^{\cdot} + {}^{\cdot}OH \tag{8}$$
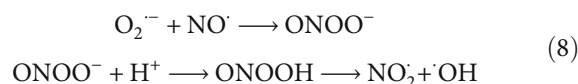
These results were confirmed by Darley-Usmar and collaborators [136] using systems to cogenerate superoxide and $NO^{\cdot}$.

The reaction of $NO^{\cdot}$ with $O_2^{\cdot-}$ occurs biologically even in the presence of superoxide dismutase (SOD), indicating that it is extremely fast to outcompete the enzyme-catalysed dismutation. Thus, nitric oxide may substantially increase the toxicity of superoxide converting a relatively mild reductant into at least two potent oxidants. The recognition that the homolysis of ONOOH could yield $^{\cdot}OH$ led to the postulation of a new biologically relevant mechanism of oxygen radical-mediated molecular damage, which is more effective than the widely accepted reaction of reduced iron with hydrogen peroxide (known as the Fenton reaction or the iron-catalysed Haber-Weiss reaction).

On the other hand, although Fenton chemistry was known to occur *in vitro*, its significance under physiological conditions was and remains a matter of debate today, noting particularly the negligible availability of "free catalytic iron" due to its effective sequestration by the various metal-binding proteins. However, organisms overloaded with iron (as in the conditions of haemochromatosis, β-thalassemia, and haemodialysis) contain higher amounts of "free available iron", and this can have deleterious effects.

10. Oxidative and Nitrosative Damage

In the last quarter of the 20th century, following the discovery of the superoxide dismutase enzymes by McCord and Fridovich [71], the scientific community began to accept the fundamental principle that free radicals were formed as byproducts of oxidative metabolism When ROS were initially integrated into biomedical concepts, it was thought that they caused exclusively toxic effects and were associated with pathologies. In fact, ROS avidly interact with many molecules including other small inorganic molecules as well as proteins, lipids, carbohydrates, and nucleic acids. Through such interactions, ROS may irreversibly destroy or alter the function of the target molecule.

Moreover, after its early description as an endothelial-derived relaxing factor, $NO^{\cdot}$ emerged not only as a fundamental signalling device but also as a potent mediator of cellular damage in a wide range of conditions, and nitrosative stress has been implicated in the pathogenesis of a large variety of disorders.

Although $NO^{\cdot}$ has often been described as highly toxic and reactive, it is not, and most of the cytotoxicity attributed to $NO^{\cdot}$ is rather due to peroxynitrite produced from the diffusion-controlled reaction between $NO^{\cdot}$ and superoxide anion. Indeed, although not a free radical in nature, peroxynitrite is much more reactive than its parent molecules $NO^{\cdot}$ and $O_2^{\cdot-}$.

Kinetic studies indicated that peroxynitrite oxidized target molecules through two distinct mechanisms. First, peroxynitrite and its protonated form peroxynitrous acid (ONOOH) exerted direct oxidative modifications through one- or two-electron oxidation processes. Only a few chemical groups directly reacted with peroxynitrite, which favoured selective reactions with key moieties in proteins, such as thiols [137] and iron/sulphur centres [138]. The second mechanism involved peroxynitrite indirectly mediating oxidation by decomposing into highly reactive radicals. Indeed, peroxynitrite decomposition produces the highly reactive $^{.}OH$ radicals, as well as nitrogen dioxide, which is a strong oxidant with significant cytotoxic potential [139, 140]. Nitrogen dioxide is also formed when peroxynitrite reacts with carbon dioxide. Such a reaction also leads to the formation of the radical anion carbonate ($CO_3^{.-}$):

$$ONOO^- + CO_2 \longrightarrow CO_3^{.-} + NO_2^. \tag{9}$$

Carbonate radical is more selective than hydroxyl radical but can initiate many of the damaging reactions commonly attributed to hydroxyl radical in the biological literature and is perhaps equally significant as a biological oxidant [141]. An important aspect of peroxynitrite-mediated toxicity is its capability of promoting protein tyrosine nitration through nitrogen dioxide and anion carbonate considered as a central aspect of peroxynitrite-mediated cytotoxicity [142].

10.1. Antioxidant System. Interestingly, the observations regarding free radicals were accompanied by the discovery that biological systems were equipped with an integrated antioxidant defence system capable of counteracting the damaging effects of ROS and RNS.

Catalase had been first noticed in 1818 by Louis Jacques Thénard, who discovered H_2O_2 and suggested that its breakdown was caused by an unknown substance [143], to which, in 1900, Oscar Loew gave the name catalase and found it in many plants and animals [144].

In 1957, Mills discovered an enzyme (GPX) using the reducing properties of glutathione for the protection of human erythrocytes against degradation of haemoglobin by hydrogen peroxide [145].

Previously, a heat-labile system capable of reducing GSSG was discovered in the liver by Hopkins and Elliott [146], and the next year, Mann [147] found that the hepatic GSSG reduction was linked to glucose oxidation by what was later identified as NADPH production in the pentose phosphate pathway. The enzyme directly involved in the reduction of GSSG, glutathione reductase (GR), was subsequently demonstrated in the rat liver by Rall and Lehninger [148]. Thus, it was realized that GPX and GR are the most important enzymes in maintaining cell redox homeostasis, since their combined action is the major determinant of reduced glutathione (GSH) content of tissues because GR serves to regenerate the active glutathione from its oxidized form produced in the GPX reaction.

Thioredoxin (Trx) was discovered in 1964 and characterized as a hydrogen donor for the enzymatic reduction of ribo-nucleotides in *Escherichia coli* [149]. The reduction reaction of thioredoxin required NADPH and was catalysed by a specific enzyme, which, in an accompanying work, was called thioredoxin reductase (TrxR) [150].

Subsequent investigation showed that the Trx/TrxR system is the major ubiquitous disulphide reductase responsible for maintaining proteins in their reduced state [151].

Until quite recently, CAT and GPX were thought to be the major peroxide-reducing enzymes protecting cells. The shift began in 1994 when protein sequence comparisons led to the recognition of a third abundant and widespread group of peroxidases [152]. The name peroxidoxins initially proposed for this group was quickly morphed to become the currently used peroxiredoxins (Prxs) [153]. These enzymes were quite distinct from catalases and GPXs especially in that they had no special cofactor but simply used cysteine residues for catalysis. Moreover, while for many Prxs the physiological reductant appears to be thioredoxin [152, 153], for some there exists a specific peroxiredoxin reductase (PrxR) that contains a thioredoxin-like domain [154]. As Prxs can also show high catalytic reactivity with peroxynitrite, they may also be important for defence against reactive nitrogen species [155].

11. Oxidative Stress

It is worth noting that in healthy organisms, the production of free radicals is low, and the antioxidant defence systems quickly remove ROS and the RNS before they cause structural and functional damage to the cell. The balance is not perfect so that some ROS and RNS-mediated damage occurs continuously, and damaged molecules must be repaired or replaced. This is demonstrated by the finding of ongoing oxidative damage in vivo in animals, including humans. For example, low levels of oxidative base damage products are present in DNA isolated from all aerobic cells. Low levels of carbonyls and certain products resulting from the attack of ROS upon proteins are detected in healthy animal tissues and body fluids. Age pigments accumulate in tissues, and specific end products of lipid peroxidation are present in body fluids [156]. These observations indicated that ROS-removing systems keep cellular levels of ROS in tissues rather than removing them completely. Moreover, it was understood that, in addition to exposure to high oxygen pressure and ionizing radiation, there were several different pathophysiological conditions in which the imbalance between the speed of production of free radicals and the capacity of the cellular defence systems becomes wider.

In 1985, Sies introduced the term "oxidative stress" to indicate this imbalance between prooxidants and antioxidants in favour of the former [157]. Severe oxidative stress produces DNA damage, rises in intracellular free Ca^{2+} and iron, damage to proteins (including membrane ion transporters), and lipid peroxidation.

Among the conditions that lead to the establishment of oxidative stress, there are, to name just a few, carbon tetrachloride toxicity; ischemia-reperfusion injury; hypermetabolic states, such as hyperthyroidism; and physical activity.

11.1. Carbon Tetrachloride Toxicity. It was long known that inhalation of vapours of carbon tetrachloride (CCl_4), a chlorinated hydrocarbon used as a solvent for oils and fats, as a refrigerant and as a dry-cleaning agent could depress central nervous system activity and cause degeneration of the liver and kidneys [158]. However, there was a paucity of information concerning mechanisms of its action. Only in 1961, it was proposed that the hepatotoxic effects of CCl_4 were due to the free radicals formed by homolytic scission of the carbon halogen bond [159]. In 1975, the existence of free radicals during CCl_4 metabolism was proven by an electron spin resonance study [160]. Subsequent investigation showed that reductive dehalogenation of CCl_4 was catalysed by cytochrome P450, the terminal oxidase of the hepatic mixed-function oxidase system [161]. Moreover, it was found that low partial pressure of oxygen in tissue resulted in the predominant formation of $CCl_3^{\cdot}$ and $CHCl_2^{\cdot}$ radicals, whereas high partial pressure of oxygen shifts CCl_4 metabolism toward the formation of the $CCl_3OO^{\cdot}$ radical with consequent lipid peroxidation [162].

11.2. Ischemia-Reperfusion Injury. It is long known that, although restoration of blood flow is the sole method for salvaging ischemic tissues, the extent of injury often increases when the blood supply is restored. One example of reperfusion injury is the phenomenon of stunning that was described by Heyndrickx and colleagues in the dog heart in 1975 [163]. They showed that a myocardium reversibly injured by ischemia does not contract as efficiently as the control myocardium after reperfusion. The finding of the paradoxical enhancement of the injury response following reperfusion (or reoxygenation) of ischemic (or hypoxic) tissue led to the proposal that the sudden reintroduction of molecular oxygen to energy (and oxygen)-starved tissue results in a unique type of injury response that is not manifested during the period of hypoxic stress [164, 165]. Since its inception, the concept of reperfusion injury steadily gained attention likely for the implication of this mechanism of tissue injury in a growing list of organs. In the early 1980s, ROS were proposed as potential mediators of reperfusion injury because of the detection of chemical products generated by the reaction of ROS [166]. The idea that ROS could account for reperfusion injury was quickly embraced, mainly because it was consistent with the observation that interventions that enhanced ROS scavenging and/or detoxification protected against reperfusion injury following intestinal [102], myocardial [167, 168], and skeletal muscle [169] ischemia.

The oxidative stress elicited in tissues/cells following ischemia-reperfusion was linked to a variety of different sources of ROS, including xanthine oxidase, NADPH oxidase, and mitochondria. The hypothesis that xanthine oxidase is a major source of ROS was initially proposed by Shlafer et al. [167], which predicted that the accumulated hypoxanthine, arising from ATP metabolism, would react with the readmitted oxygen to produce a burst of superoxide, because of the conversion of the xanthine dehydrogenase to xanthine oxidase [170].

NADPH oxidase is another source that accounts for an important part of the ROS formed during ischemia-reperfusion.

Studies implicating neutrophils in reperfusion injury provided some of the earliest evidence suggesting the involvement of NOX as a source of ROS in postischemic tissue [171, 172]. However, there are several lines of evidence that support a role for nonphagocytic NOX as a source of ROS following I/R [173].

Mitochondria have been implicated as a major source of I/R-induced ROS production in a variety of organs. The proposal that the respiratory chain is a major source of ROS during reperfusion of the ischemic myocardium [174] was supported by the observation that a generation of oxygen radicals was induced *in vitro* upon reoxygenation of mitochondria isolated from hearts that had been subjected to ischemia [175]. Further support was obtained demonstrating by electron paramagnetic resonance that resumption of mitochondrial oxidative phosphorylation upon postischemic reflow can be a source of oxygen radicals in intact rabbit hearts [176]. It was proposed that, upon the respiration resumption, ROS generation is promoted by O_2 interaction with ubisemiquinone, which accumulates in mitochondria during ischemia because of respiratory chain inhibition [177]. The observation that the increase in ischemia duration was directly related to a gradual increase in lipid peroxidation and decline in mitochondrial respiration and inversely related to functional recovery of the tissue [178] supported the idea that heart performance is strongly conditioned by mitochondrial functionality. Further support was provided by the observation that the antioxidant protection of mitochondrial function was associated with decreased impairment of cardiac function following ischemia-reperfusion [179].

11.3. Hyperthyroidism. It is long known that thyroid hormones are key regulators of growth, development, and metabolism. It is also well known that elevated circulating levels of thyroid hormones are associated with modifications in the whole organism and several body districts. The most studied modification found in hyperthyroid animals is the increase in their basal metabolic rate (BMR) due to an increase in the rate of O_2 consumption in target tissues. The idea is well established that, like other long-term effects, thyroid calorigenesis is achieved by thyroid hormone influence on transcription of T_3-responsive genes, which are mediated through nuclear thyroid hormone receptors (TRs) [180].

In the 80s, it was found that the hypermetabolic state in hyperthyroidism is associated with increases in lipid peroxidation in the rat liver [181], heart, and skeletal muscle [182]. Subsequent work demonstrated that products of lipid, protein, and DNA oxidation were found in several target tissues (see Di Meo and Venditti [183] for a review). More recent works showed stimulation of mitochondrial H_2O_2 generation following T_3 treatment in the rat liver, heart, and skeletal muscle [184–186].

It is worth noting that the long-term effects of thyroid hormone on mitochondrial respiration are obtained by

increasing the content of electron carriers, such as cytochromes [187, 188] and ubiquinone [189, 190], and their percent reduction [188, 191]. There is also indirect evidence that even mitochondrial concentration of oxidizable electron carriers increases in hyperthyroid tissues [184, 185]. Thus, it is conceivable that the increase in mitochondrial ROS generation, underlying cellular oxidative damage, is a side effect of the thyroid hormone-induced biochemical changes by which animal tissues increase their metabolic capacity.

Moreover, significant evidence that tissue oxidative stress underlies some dysfunctions produced by hyperthyroidism has been obtained exploring the application of antioxidants, in particular, vitamin E, as therapeutic agents in thyroid-related disorders (see Venditti et al. [192] for a review).

11.4. Physical Activity. As regards the physical activity, we all had the experience that any form of exercise, if carried out vigorously enough, can become painful. But only one form of exercise, eccentric exercise, if unaccustomed to it, leaves us stiff and sore the next day. This is because acute exercise could produce significant damage, including structural and functional alterations in skeletal muscle as well as in other tissues. According to some reports, mitochondrial swelling is provoked in rat gastrocnemius muscle by running [193], and extensive mitochondrial swelling is provoked in the myocardium of both running and swimming rats [194]. Thus, an increase in the total area occupied by mitochondria and sarcoplasmic reticulum is found in middle gluteal muscle from treadmill-exercised horses [195].

Moreover, the extent of tissue damage mainly depends on the intensity and duration of exercise [184]. Serum elevation of creatine kinase and lactate dehydrogenase, universally accepted as a marker of tissue damage, is found in runners following an 80 km race [196].

Skeletal muscle injury also depends on the way how the muscle is used, because the contraction-induced injury is much more severe following eccentric rather than concentric or isometric contraction [197].

Although several metabolic factors have been proposed as mechanisms of primary damage during acute exercise, in the late 1970s, it was proposed that ROS can be implicated in the damage that develops in the tissues owing to long-lasting aerobic exercise [198], even though, until now, direct evidence of ROS production during physical activity increase is lacking. The first demonstration that an increase of free radicals verified in both muscle and liver of rat subjected to an exhaustive running was obtained by Davies et al. [199] using electron spin resonance. After which, with the same method, it was reported that exhaustive endurance exercise also increases the signal related to free radical generation in the rat heart [200] and in human serum [201]. Final confirmation that free radical production is enhanced by high muscular activity was obtained in experiments performed stimulating electrically skeletal muscles [202].

Muscle contraction also affects NO production as first demonstrated by the increase in NO release from muscles exposed to prior electrical stimulation [203] and NO· concentrations in expired air during physical activity [204].

The changes in the content of the markers of lipid, protein, and DNA and in the redox state after acute exercise supply indirect information on the enhanced ROS production during acute exercise.

Ever since Dillard and his colleagues showed that in a subject exercised on a bicycle ergometer there is an increase in the exhaled air content of pentane, an index of lipid peroxidation [198], the information on the formation of oxidative damage markers after exercise in the tissues of various animals, among which humans, has increased [205, 206].

An important practical consequence of the demonstration that free radicals are involved in tissue damage caused by exhaustive exercise is that it is possible to minimize the effect of such radicals by the administration of antioxidants such as beta carotenes, vitamin C, vitamin E, glutathione, or N-acetylcysteine.

Since Brady et al. reported in 1979 that a 4-week vitamin E-reinforced diet (50 IU/kg diet) inhibited increases in rat liver TBARS levels immediately after exhaustive swimming exercise [207], the inhibitory effect of antioxidant intake on exercise-induced oxidative damage has been studied extensively. Most research has demonstrated that antioxidant administration has beneficial effects against the damaging effects of intense physical exercise (see Kawamura and Muraoka [208] for a review).

Apart from the protective role against damage caused by free radicals during exhaustive exercise, antioxidants might have a positive effect on performance and the prevention of fatigue. In 1994, Reid et al. used NAC, a precursor of GSH, to investigate the effects of free radicals on muscle fatigue and found that intravenous infusion of NAC inhibited tibialis anterior muscle fatigue induced by low-frequency electrical stimulation [209]. This study was the first human study to prove that free radicals can induce muscle fatigue and that supplementary intake of antioxidants can reverse it.

Based on these studies, nutritional interventions and antioxidant integration began to be used frequently in athletic populations to reduce oxidative damage, ameliorate the performance, and accelerate the recovery of muscle function.

12. Free Radicals and Diseases

Owing to increased understanding of the damaging effects of ROS, the concept of ROS as agents of cellular damage in biological organisms became widely accepted in theories of aging. Moreover, a growing number of diseases/disorders were gradually linked either directly or indirectly with ROS. That is, while some of these disorders are primarily due to free radicals, others may be only secondarily involved. In this latter group, tissue injured by various processes, such as trauma, toxic substances, and infectious processes, may undergo free radical damage more rapidly than healthy tissues. Tissue destruction and degeneration can result in increased oxidant damage, by such processes as metal ion release, phagocyte activation, and disruption of mitochondrial electron transport chains (so that more electrons "escape" to oxygen to form $O_2^{\cdot-}$). It follows that almost any

disease is likely to be accompanied by increased formation of reactive oxygen species.

The protective effects of iron chelation in several disorders [210], superoxide dismutase and catalase in ischemia-reperfusion [170], and the protection by vitamins, such as vitamin C [211, 212], and antioxidants, such as probucol [213], under a variety of experimental conditions clearly indicated a key role for free radicals in many disorders. The list of diseases in which the ROS formation was implicated was already long in 1987 (it contained over 50 diseases) [214], and it was destined to grow in the following years.

In Table 3, several human diseases/disorders are reported for which a role for oxidative stress has been suggested. Among these disorders, the aging process has been included. More than 300 theories have been proposed to explain the aging process [215], but none has yet been generally accepted by gerontologists. However, the initial proposal by Harman that free radicals are causally related to the basic aging process [70] has received increasing acceptance as a possible explanation of the chemical reactions at the basis of aging [216].

The identification of free radical reactions as promoters of the aging process implies that interventions aimed at limiting or inhibiting them should be able to reduce the rate of formation of aging changes with a consequent reduction of the aging rate and disease pathogenesis. However, even though dietary antioxidants appear to be important in delaying/preventing certain human diseases, especially cardiovascular disease and some types of cancer [217], available evidence does not allow to recommend antioxidant supplementation as a useful means to prevent age-related pathophysiological modifications and clinical conditions. Furthermore, several concerns are present not only about their efficacy but also on their safety.

TABLE 3: Selected diseases/disorders for which a role of free radicals has been suggested.

Adult respiratory distress syndrome	Gout
Allergic encephalomyelitis	Haemachromatosis
Alzheimer's disease	Hearing loss
Amyotrophic lateral sclerosis	Hypertension
Asbestosis	Insulin resistance
Autism	Keshan disease
Autoimmune vasculitis	Lypofuscinosis
Bloom syndrome	Malaria
Bronchopulmonary dysplasia	Multiple sclerosis
Burns	Muscular dystrophy
Cancer	Myasthenia gravis
Cataract	Pancreatitis
Chronic autoimmune gastritis	Parkinson disease
Chronic granulomatous disease	Psoriasis
Cirrhosis	Psychosis
Contact dermatitis	Retinal degeneration
Depression	Retrolental fibroplasias
Dermamyositis	Rheumatoid arthritis
Dermatomyositis	Schizophrenia
Diabetes mellitus	Sickle cell anemia
Emphysema	Stroke
Emphysema	Systemic lupus erythematosus
Favism	Thalassemia
Glomerulonephritis	Ulcerative colitis

13. Useful Effects of ROS

Although free radicals were considered harmful to the living organism for their damaging effects, it was long known that these effects could be exploited for the benefit of the organism.

14. Radiation Therapy

As previously mentioned, during early practical work and scientific investigation, experimenters noticed that prolonged exposure to X-rays created inflammation and, more rarely, tissue damage on the skin. The biological effect attracted the interest of several physicians, among which Emil Grubbe, who, only a month or two after Röentgen's announcement also before understanding the physical properties and biological effects of X-rays, was the first physician known to use them for cancer treatment [218]. Interestingly, at that time, Grubbe was a student at the Hahnemann Medical College of Chicago. Because radiation was determined to be the cause of tumours, one of the professors at the homeopathic college suggested its therapeutic use in cancers. Thus, this incident has been considered by the proponents of homeopathic medicine as a further example from history in which an insight from a homeopathic perspective has provided an important breakthrough in medical treatment [219].

Radiation therapy uses particles or waves moving at a high frequency to target the DNA of cancer cells in the body and change the way they can replicate. Malignant tumours are characterized by unlimited cellular proliferation intimately connected with nucleic acid synthesis. If the DNA required for mitosis and replication is damaged, the cells are unable to replicate as usual and the growth of a cancerous tumour is inhibited. Of course, the mechanisms of the harmful effects of therapeutic exposure to ionizing radiation on tumour cells are the same as the effects of nontherapeutic exposure to radiation on normal cells. Therefore, radiation-induced ionizations can act directly on the cellular molecules and cause damage and can act indirectly producing free radicals which are derived from the ionization or excitation of the water component. Thus, hydroxyl radical generation is, paradoxically, the major mechanism by which malignant cells are killed during radiotherapy.

15. Phagocytosis

Another demonstration that free radicals could perform useful functions derived from the discovery that phagocytes

(neutrophils, macrophages, and monocytes) release free radicals to destroy invading pathogenic microbes as part of the body's defence mechanism against disease.

In normal phagocytosis, polymorphonuclear leukocytes and macrophages consume large quantities of oxygen when engulfing their prey. This "extra respiration of phagocytosis" had already been described by the first half of the 20th century [220]. However, the unusual nature of the process was only revealed in 1959 when it was discovered that it was not inhibited by classical mitochondrial poisons such as cyanide and azide [221] indicating that it was not simply a reflection of the enhanced energy requirements of phagocytosis. In 1964, Rossi and Zatti [94] correctly proposed that an NADPH oxidase was responsible for the respiratory burst, which was soon discovered to be a requirement for the efficient killing of bacteria by neutrophils [222]. This observation was rapidly reinforced by the recognition that a syndrome identified by Berendes et al. in 1957 [223] "fatal granulomatosis of childhood" (now referred to as chronic granulomatous disease) (CGD), which was characterized by a severe predisposition to pyogenic infection, was associated with the complete absence of this oxidase activity from the patient phagocytes [224].

In 1973, soon after its discovery, superoxide dismutase was used to show that the product generated by activated neutrophils was superoxide [95]:

$$NADPH + O_2 \xrightarrow{NOX2} NADP + O_2^- + H^+ \qquad (10)$$

Moreover, the next year, it was demonstrated that this process was lacking in CGD [225].

Thus, the importance of ROS production by the immune system was clearly exemplified by multiple and persistent infections arising in patients with CGD in which the defective membrane-bound NADPH oxidase system made them unable to produce the superoxide anion radical (O_2^{-}) and therefore to kill bacteria.

This important development provided a direct link between free radical chemistry and biology. At the time, most free radical chemistry was conducted by radiation biologists in test tubes, and its application to biology was purely theoretical. This new discovery was thought to prove that the production of free radical reactions in a biological process was toxic enough to kill organic structures as tough as bacterial and fungal spores. Soon, these observations were extrapolated to implicate free radical reactions in a host of pathological processes involving neutrophil infiltration and tissue damage

Subsequently, several additional highly reactive oxygen-derived metabolites, including hydroxyl radical, singlet oxygen ($^1O_2$), and hypochlorous acid (HClO), were identified or predicted to exist as a result of activation of phagocytic cells [226].

The observation that SOD, CAT, and $\cdot$OH scavengers, such as benzoate and mannitol, inhibited the phagocytic killing suggested that both O_2^{-} and H_2O_2 were required for the phagocytic bactericidal event and that $\cdot$OH generated by the interaction of O_2^{-} and H_2O_2 in the Haber-Weiss reaction was the toxic agent for microorganisms [227].

On the other hand, another possible explanation for the requirement for both O_2^{-} and H_2O_2 in the phagocytic activity might involve $^1O_2$, which could be formed by a reaction between O_2^{-} and $\cdot$OH:

$$O_2^- + \cdot OH \longrightarrow OH^- + {}^1O_2 \qquad (11)$$
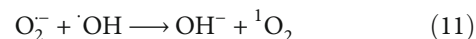
The role of $^1O_2$ in the killing of phagocytized bacteria had been suggested by the finding that *Sarcina lutea* containing carotenoid, a scavenger of $^1O_2$, were protected against phagocytic bactericidal activity compared to a colourless mutant [228].

However, other reactive metabolites can be formed as a result of the metabolism of H_2O_2 by cellular enzymatic systems. The cytotoxicity of hydrogen peroxide itself is considerably enhanced in the presence of myeloperoxidase (MPO), which is simultaneously released from the azurophil granules into the phagocytic vacuoles. The enzyme-hydrogen peroxide complex that is formed can oxidize various halides to produce, for example, HClO, which has a potent bactericidal action [229]:

$$H_2O_2 + Cl^- + H^+ \xrightarrow{MPO} H_2O + HClO \qquad (12)$$
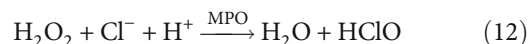
Singlet oxygen can, in turn, be formed by the interaction of H_2O_2 with hypochlorite [230]:

$$HOCl + H_2O_2 \longrightarrow H_2O + HCl + {}^1O_2 \qquad (13)$$
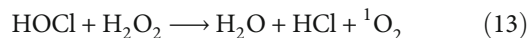
About a decade later, it was discovered that, in addition to NOX2-dependent production of oxygen radicals, murine macrophages have the capacity to produce nitrogen-based radicals that can contribute to microbial killing through an L-arginine-dependent biochemical pathway [231]. Such a study is proved to be more difficult to extrapolate to humans. Indeed, some studies showed low but detectable NO$\cdot$ output [232, 233] that can be further increased by activating agents such as the chemotactic peptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine [232]. Conversely, other studies failed to show NOS activity or the production of the NO$\cdot$ metabolite nitrite and nitrate by human neutrophils, even after activation with proinflammatory cytokines [234, 235]. However, it is now indisputable that human macrophages produce NO$\cdot$ and that NOS2, which is not expressed in macrophages at "rest," is significantly enhanced in response to appropriate inflammatory stimuli [236]. The activity of this enzyme is required for the formation of nitrotyrosine around phagocytosed bacteria, most likely through the intermediate production of peroxynitrite [236].

16. Free Radicals as Signalling Molecules

According to several researchers, a new era of free radicals in biological systems began in the 1970s when Mittal and Murad provided evidence that hydroxyl radical induced the activation of guanylate cyclase and the genesis of the

"second messenger" cyclic guanosine monophosphate (cGMP) [237]. However, as early as 1974, it had been reported that exogenously added hydrogen peroxide could mimic the signalling activity of insulin [238] and oxidation of key fat cell sulfhydryl's in response to insulin receptor interaction playing a role in mediating the glucose transport activation [239].

This revelation remained within small free radical research circles until, at the end of the 70s, Mukherjee and Lynn [240] described a NADPH oxidase in membranes isolated from fat cells, the activity of which was stimulated by preincubation of cells with insulin. This provided the basis for an attractive hypothesis for the mechanism of action of insulin, according to which the hormone, through the stimulated H_2O_2 production, would mediate membrane sulfhydryl oxidation and thus promote glucose transport. In fact, a few years later, insulin was shown to activate a plasma membrane enzyme system with the properties of a NADPH oxidase resulting in the downstream production of H_2O_2 [241], which plays a role in facilitating normal signal transduction by insulin.

Moreover, in the 1980s, publications reported that low concentrations of superoxide and hydrogen peroxide H_2O_2 could also stimulate cell proliferation in hamsters [242] and human [243] fibroblasts in culture. Since then, a large body of evidence has been accumulated that, at the cellular level, ROS, besides acting as a "second messenger" of the insulin and stimulating growth responses in a variety of mammalian cell types [244], also regulate a wide variety of physiological functions.

Indeed, ROS were found to play crucial roles in the activation of genes under the control of the transcription factor NF-κB [245], be mediators in the biosynthesis of prostaglandins [246], function in embryonic development [247], and act as signalling molecules within the individual cell and among cells during their lifespan [248].

Evidence has been also obtained that ROS are involved in normal muscle contraction. This idea dates back to the 90s when Reid and collaborators [249] reported that low levels of ROS that are present in skeletal muscle under basal conditions are a requirement for normal movement and that antioxidant-mediated depletion of ROS from unfatigued skeletal muscle results in the inhibition of their contraction [250].

Furthermore, ROS contribute to complex functions, including blood pressure regulation [251] and cognitive function [252]. It has been also shown that, although an increased ROS formation rate has been postulated to be the major determinant of lifespan [253], several longevity-promoting interventions increase the generation of ROS that activate cellular stress pathways to dampen tissue degeneration to promote healthy aging [254]. This has suggested that living systems have not only adapted to a coexistence with free radicals but also developed various mechanisms for the advantageous use of free radicals in various physiological functions.

An important development in the field of the ROS beneficial effects was the discovery that, in organisms from simple bacteria to complex mammals, ROS can induce redox-sensitive signal cascades leading to increased expression of antioxidant enzymes.

For example, it was found that, following mouse [255] and rat [256] irradiation, cells and tissues appeared to respond by increasing the expression of cellular antioxidant defences, and this increased antioxidant capacity has been hypothesized to be at least partially responsible for radiation-induced adaptive responses. The increase in the effectiveness of the antioxidant defence system provided by this genetic response enables cells to survive an oxidant exposure that would normally be lethal.

Several studies showed that bacteria have evolved sophisticated molecular mechanisms to monitor oxidant levels and to activate antioxidant defence genes. In particular, the enteric bacterium *Escherichia coli* provided an excellent model to study gene regulation in response to oxidative stress. In fact, the basic principles of the oxidative stress response are universally conserved from bacteria to eukaryotes, although the exact mechanisms by which they sense ROS vary depending on the type and severity of stress condition and organism.

Thus, *E. coli* can stimulate the production of enzymes that scavenge the superoxide radical or various peroxides, of DNA repair enzymes, and of other proteins that mitigate the toxic effects of oxidative mutagens [257–259]. These protective inductions represent adaptive responses to oxidative stress because they are triggered by nontoxic levels of oxidizing agents or ionizing radiation and protect cells against a subsequent challenge with otherwise lethal levels of those oxidants.

The bacterial oxyR gene was identified in *Salmonella typhimurium* as a regulator of acquired resistance to oxidative stress, and it was found to be required for the expression of 9 of the many stress proteins, including catalase, alkyl hydroperoxide reductase, glutathione reductase, and MnSOD, synthesized in response to sublethal levels of hydrogen peroxide [260].

Subsequent researches defined a genetic locus, the soxR regulon [261, 262], which is part of the multigene response to superoxide generators and positively regulates 9 of the ~40 polypeptides distinct from those induced by H_2O_2 [263].

It was discovered that in mammal genes, transcription determining cell survival can be activated by ROS in two ways: either via transcription factors, which can interact directly with specific DNA motifs on promoters of target genes, or via activation of mitogen-activated protein kinase cascades, which in turn activate transcription factors that trigger target gene transcription [264].

Indeed, clear evidence was obtained that ROS cellular levels are strongly linked to the regulation of cellular antioxidant levels. One of the main examples of this effect is the control of the expression of several genes that codify for antioxidant and detoxifying enzymes by the nuclear factor erythroid 2-related factor 2 (Nrf2). Its action is mediated through the bond to the antioxidant response element (ARE) together with the small musculoaponeurotic fibrosarcoma (Maf) proteins, of the promoter region of the target genes [265]. The activity of Nrf2 depends on its subcellular localization that is, in turn, dependent, at least in part, by

its bond with specific residues of cysteine of the protein Kelch ECH-associating protein 1 (Keap1) [266]. Furthermore, ROS can interact and activate several cellular processes among which the proliferation and survival through the interaction with signalling molecules such as mitogen-activated protein kinases (MAPKs), phosphoinositide 3-kinases (PI3K), phosphatase and tensing homolog (PTEN), and protein tyrosine phosphatases [267].

Similarly, the RNS also exert a dual role being harmful or beneficial to the living systems. Nitric oxide, which was initially discovered as a signalling molecule able to modulate in blood vessel diameter [121], was subsequently identified as a molecule able to induce cellular toxicity and damage metabolic enzymes and to generate, reacting with superoxide, the peroxynitrite [268], as well as a regulator of important physiological processes [269].

Nitric oxide is probably the smallest and most versatile bioactive molecule identified. Early studies concerning the role of NO$^\cdot$ in mammalian organisms showed that, besides inducing vasorelaxation, NO$^\cdot$ inhibited platelet aggregation [270]. However, it was soon evident that the effects and function of NO$^\cdot$ went beyond its ability to regulate vascular tone and cell adhesion.

For example, it was found that nitric oxide influences considerably the central and peripheral nervous systems. In fact, stimulation of cerebellar slices with excitatory amino acids led to the release of a labile mediator with pharmacologic properties like those of NO$^\cdot$, including the ability to raise cGMP in cerebellar cells that did not themselves respond to excitatory amino acids and the ability to relax vascular smooth muscle [271]. Subsequent studies on the peripheral nervous system provided the best evidence for a transmitter role of NO$^\cdot$. NOS inhibitors selectively blocked nonadrenergic noncholinergic- (NANC-) mediated relaxation of the gastrointestinal tract [272]. These data coupled with the selective localization of nNOS to the myenteric plexus indicated that NO$^\cdot$ functions as the NANC neurotransmitter [273].

Interestingly, the observation that lipid peroxidation can be inhibited by excess NO$^\cdot$ and yield a variety of nitroso- and nitro-fatty acid-derived products [274] also demonstrated that NO$^\cdot$ can also function as an antioxidant since it terminates free radical processes and stops radical chain propagation reactions.

17. Mechanisms of Redox Signalling

Even though the ROS and RNS were involved in both cellular damage and signal transduction was well established, several controversial questions remained open.

Differences in the concentrations of the ROS and RNS could, at least in part, be responsible for the dual role of such species as essential molecules for the regulation of cellular functions and as harmful by-products of metabolism. Indeed, at low concentrations, ROS played an important role as regulatory mediators in signalling processes, whereas, at moderate or high concentrations, they were harmful to cell organisms inactivating important cellular molecules. This was analogous to the effects of nitric oxide, which had both

regulatory functions and cytotoxic effects depending on the enzymatic source and relative amount of NO$^\cdot$ generated [275]. Thus, NO$^\cdot$ worked as a signalling molecule mediating vasodilation when produced in low concentrations by the constitutive isoform of nitric oxide synthase (eNOS) in vascular endothelial cells [276]. Conversely, it worked as a source of highly toxic oxidants, such as peroxynitrite and nitrogen dioxide (NO$_2$), utilized for microbicidal killing when produced in high concentrations by iNOS in macrophages [277].

Although these results suggested that the cellular levels of reactive species determine the shift from their beneficial to harmful effects, the concentrations to which this shift happens were and still remain generally unknown today.

Moreover, there was an apparent contrast between the specificity that is required for signalling and the general reactivity of cellular ROS that renders them indiscriminate and potentially lethal oxidants, so that it was unclear how any specificity in their opposite actions could be achieved. Specificity in signalling is achieved through the noncovalent binding of a ligand to its cognate receptor due to the complementarity of macromolecular shapes. By contrast, ROS operate in signalling through chemical reactions with specific atoms of target proteins that lead to covalent protein modifications. Therefore, ROS molecular recognition occurs at the atomic level and not at the macromolecular level, which necessarily expands the potential number of ROS-specific receptors. Thus, it was unclear how the specificity was achieved in ROS signalling.

The cell type, duration of oxidant production, reactive species produced, and localization of their source and their targets were suggested as contributing factors [216], but the information on this topic was still scarce and discordant.

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17.1. ROS Source. Regarding the ROS source, mitochondria are considered the main source of ROS in the cell in both physiological and pathological conditions [278]. This is why mitochondria are considered to play the main role in many diseases and in the aging process [279]. In fact, the available data show that the evidence of mitochondria as the main source of ROS is lacking [280]. Conversely, they show that there is a strong interaction among the different sources of cellular ROS [281, 282], and this renders it more difficult to define the main source of reactive species in different physiological and pathological conditions.

17.2. Colocalization of Sources and Targets of ROS. Another primary layer of control for ROS signalling is the colocalization of sources and targets of ROS by the generation of the small-molecule oxidant in proximity to a given substrate. This form of regulation can directly influence the kinetics of a putative chemical signalling reaction by controlling the local concentrations of the participating molecular reactants. For example, NOX proteins that influence receptor tyrosine kinase signalling are often colocalized with their putative physiological targets, such as phosphatases and kinases, at the plasma membrane. This also prevents oxidation of pathological targets such as nucleotides that are confined to other

parts of the cell [283, 284]. Indeed, recent data show that ROS generation is localized for signalling in various cell types [285]. Other examples of colocalization of ROS signalling sources and targets are ER localization of NOX4 and its target, the protein tyrosine phosphatase 1B (PTP1B) [286] and the localized generation of HOCl by myeloperoxidase in phagosomes for pathogen defence [287].

17.3. Species Involved in Signalling. Most of the ROS cannot diffuse far from the site of their production due to their high instability and reactivity and to the antioxidant cellular capacity. This is the case of the radical $^{.}OH$ which possesses a half-life of about 10^{-9} s for its high reactivity, differently from H_2O_2 which has a half-life of about 1 ms [288]. This explains why H_2O_2 can diffuse far from its source, differently from $^{.}OH$ which has a mean effective radius of action of about 30 A and reacts with or very near to the biomolecule that produced it. Thus, a hydroxyl radical formed in the mitochondria will be unlikely to have a direct effect on other parts of the cell, for example, DNA in the nucleus.

Moreover, $^{.}OH$ has indiscriminate reactivity toward biological molecules, whereas other ROS, such as $O_2^{.-}$ and H_2O_2, each has preferred biological targets. Despite this, hydroxyl radical has also been thought to be involved in signalling by Mittal and Murad [237], who suggested that stimulation of guanylate cyclase by SOD was due to the formation of H_2O_2 which, reacting with superoxide, produced $^{.}OH$ radicals that activated guanylate cyclase. This idea was refuted by Friebe et al. [289] who found that stimulation of guanylate cyclase by SOD was not influenced by $^{.}OH$ and proposed that it was due to elimination of superoxide, thereby preventing its reaction with $NO^{.}$. Thus, the idea is widely shared that $^{.}OH$ radical cannot play any specific role in signalling.

This does not seem to apply also for the derivatives of the nitric oxide. It had been assumed that peroxynitrite has an impact on pathways, which, under physiological conditions, are regulated by tyrosine phosphorylation and dephosphorylation. Although peroxynitrite does not react directly with tyrosine, all secondary radicals arising from peroxynitrite promote protein tyrosine oxidation and nitration [290]. The tyrosine nitration blocks the respective signalling cascades so that the fact that NO_2 irreversibly binds to proteins seemed to have a pathological impact on cellular function, rather than contributing to physiological intracellular signalling. This agreed with the idea that the biological impact of primary and secondary reactive species is different. Primary ROS and RNS are regulated by SOD, CAT, GPX, and NOS, respectively, and are predominantly associated with signalling having a specific physiological function for the regulation of intracellular signalling. Conversely, the secondary species are associated with oxidative stress. They should be evolutionarily developed for extracellular actions, predominantly as part of the innate immune system for killing bacteria. The intracellular release of such secondary species leads to deleterious consequences, as these are highly active, without a reliable control system for intracellular levels. It was, therefore, generally assumed that, in evolution, the primary species were developed for intracellular physiological signalling whereas the secondary species, which can cause damage

to the cell, were developed for extracellular actions, such as the killing of bacteria. Nevertheless, publications are dealing with the signalling role of peroxynitrite [291].

17.4. H_2O_2 Signalling. Most of the papers on ROS-mediated intracellular signalling suggested either $O_2^{.-}$ or H_2O_2 as the major signalling molecules.

It was known that superoxide can oxidize thiols to thiyl radicals, which can initiate a chain reaction, but the rate constants for this reaction are rather low, about $10^3 M^{-1} s^{-1}$ at pH 7.4 [292], which are insignificant in comparison to the rate constants (about $10^9 M^{-1} s^{-1}$) for the cytosolic and mitochondrial superoxide dismutases [293]. When $O_2^{.-}$ is generated by the NADPH oxidase isoform, NOX2, on the outside of cells, such as endothelial ones, it can enter cells, resulting in signalling [294]. However, inside the cell, it is rapidly dismuted to H_2O_2 and O_2 and targets that react with which have not been demonstrated to do so in vivo so that it is likely that superoxide acts as a precursor of H_2O_2.

H_2O_2 enzymatic production and degradation, which provide specificity for time and place, and its chemistry, which provides specificity for the oxidation of thiols, qualify the peroxide a suitable second messenger.

It has been proposed that H_2O_2 initiates cellular signalling through modifications of target protein molecules or changes of the intracellular redox state [295], even though the distinction between these mechanisms is not easy.

The main targets of H_2O_2 are thiol groups of cysteine residues, and the best-described modification of the protein molecules involves the oxidation of such residues. Oxidation of –SH groups results in the formation of several different products, including sulfenic acid derivatives (-SOH) [296], which can subsequently undergo further oxidation to sulfinic (-SO$_2$H) and eventually sulfonic (-SO$_3$H) acid. Furthermore, they can form disulphide bonds with nearby cysteines (-S-S-) [297] and be transformed into several adducts by reaction with $NO^{.}$ (S-nitrosylation) or GSH (S-glutathiolation) [298]. Except for sulfonic acid and to a lesser degree sulfinic acid, the modifications are reversible by reducing systems such as thioredoxin and peroxiredoxins [299].

The sulfinic acid formation requires a second reaction with H_2O_2 which seems more likely to occur under severe oxidative stress than during physiological signalling. The same is true for the sulfonic that practically does not exist in biological conditions. It follows that the oxidation states of a cysteine residue relevant for signalling are likely only the sulfenic acid derivative and the disulphide.

The oxidation-linked modifications of thiol groups result in changes in the structure and function of the protein, which may modify the activity of an enzyme if the critical cysteine is located within its catalytic domain [300] or the ability of a transcription factor to bind DNA if it is located within its DNA-binding motif [301]. Several proteins, including transcription factors, molecular chaperones, and protein tyrosine phosphatases, are regulated via redox processes. The redox state of protein thiols represents a "molecular switch" able to reversibly activate/deactivate protein function. This process resembles the phosphorylative regulation in which the addition of a phosphate group by a protein kinase and its

removal by a protein phosphatase reversibly change protein activity [302].

On the other hand, crosstalk and/or sequential partnership between the two signalling mechanisms occur in some cases of cell regulation. Evidence linking the cellular redox state to a phosphorylative process came from the study of the Trx/ASK1 system [303]. Apoptosis signal-regulating kinase-1 (ASK1) is a member of the family of mitogen-activated protein kinase kinase kinases (MAPKKK) that is involved in the activation of stress-activated protein kinases, such as p38 and JNK [304]. Thioredoxin, an antioxidant protein, was found to form a complex with ASK1. When thioredoxin was complexed to ASK1, the activity of ASK1 was inhibited [303]. The rise in ROS levels that occurred following tumour necrosis factor stimulation resulted in the dissociation of ASK1 from thioredoxin and the subsequent activation of ASK1 activity [303].

As for how the changes in the redox cellular state initiate cellular signalling should be noted that, in comparison with the extracellular environment, the cytosol is normally maintained under strong "reducing" conditions. In normal conditions, the maintenance of the cytosol redox state is due to the "redox-buffering" capacity of intracellular thiols, such as GSH and Trx, which oppose cellular oxidative stress by reducing H_2O_2. The activity of GSH reductase and Trx reductase maintains the high ratios of reduction to oxidized forms of GSH and Trx, respectively. Such substances can participate in cell signalling processes: GSH can regulate redox signalling by alterations both in the level of total GSH and in the ratio between its reduced and oxidized forms, while Trx can regulate the activity of some proteins by directly binding to them [295].

17.5. NO Signalling. The well-known effect of NO˙ on smooth muscle cells is due to the capacity of nitric oxide to bind to the heme moiety of guanylyl cyclase to induce a conformational change which activates the enzymes and results in the formation of the second messenger cyclic guanosine monophosphate (cGMP) from guanosine $5'$-triphosphate (GTP). cGMP in turn, regulates protein kinases (cGK), phosphodiesterases (PDE), and ion-gated channels. Since the initial elucidation of the effects of the ˙NO/cGMP system on blood pressure regulation, platelet aggregation, and neurotransmission [305], other examples of the alternative target for NO˙ came to light. Some examples are the activation of another heme-containing enzyme, cyclooxygenase, which leads to an increase in prostaglandin formation [306], and the inhibition of cytochrome oxidase, which regulates mitochondrial respiration [307].

Many biological effects of NO˙ are mediated through heme-independent pathways by which S-nitrosylation of protein-associated targets of NO includes cysteine and tyrosine residues, and it is apparent that S-nitrosylation of target proteins is another potentially important regulatory system accounting for some of NO˙ physiologic actions.

It was found that eNOS and iNOS are susceptible to NO-induced thiol modifications [308]. S-nitrosylation of NOS enzymes regulates the NO˙ production suggesting a feedback mechanism to control its activity [308]. NO˙ also targets the mitochondrial proteins complex I and complex IV involved in respiration. Although the inhibition of complex IV remains reversible throughout the time of exposure, the inhibition of complex I, which seems to be due to S-nitrosylation of this enzyme, becomes progressively persistent as the concentration of reduced glutathione (GSH) in the cell decreases [309].

Like NO˙, peroxynitrite easily crosses biological membranes, and despite a relatively short half-life (~10 ms), it can interact with target molecules in adjacent cells within one or two cell diameters [269]. Peroxynitrite can indirectly trigger the nitration of tyrosine residues in proteins, forming 3-nitrotyrosine, via the generation of highly reactive radicals formed by its reaction with carbon dioxide [310].

Protein tyrosine nitration is a covalent modification resulting from the addition of a nitro ($-NO_2$) group onto one of the two equivalent ortho carbons of the aromatic ring of tyrosine [310]. The nitration may produce three distinct effects on the affected proteins: loss of function, the gain of function, or no effect [311]. Widespread tyrosine nitration occurred in cells during *in vitro* exposure to peroxynitrite, affecting structural proteins, ion channels, metabolic enzymes, and proteins involved in apoptosis, to name only a few [311–313]. The relevance of such observations *in vivo* remained, however, to be established, given that the yield of nitrotyrosine formation under conditions of elevated peroxynitrite generation *in vivo* (nitrooxidative stress) remained largely smaller than what can be achieved *in vitro* with direct peroxynitrite exposure.

However, in most reported studies, nitration of tyrosine has been associated with a significant loss of function of the nitrated protein. An important example of the loss of enzyme activity is that of mitochondrial MnSOD, the first protein to be found nitrated in vivo [314]. Nitration of a single tyrosine residue (Tyr-34) leads to complete enzyme inactivation, with the possible consequence of favouring peroxynitrite generation in this organelle, due to the impaired dismutation of $O_2^{˙-}$ [315].

A major aspect of tyrosine nitration by peroxynitrite is the possibility of impairment of cellular processes depending on the generation of phosphotyrosine [269]. It is worth noting that the hypothesis that tyrosine nitration would essentially inhibit phosphotyrosine-dependent cell signalling has been largely reviewed following a series of investigations concluding that peroxynitrite rather promoted the process of tyrosine phosphorylation in a variety of cell types [291]. Proposed mechanisms involve an imbalance between tyrosine phosphorylation and dephosphorylation, related to the activation of tyrosine kinases or the inhibition of tyrosine phosphatases, respectively, by peroxynitrite. However, the scarcity of *in vivo* results that confirm *in vitro* data indicates that the relevance of peroxynitrite-dependent signalling in pathological states remains to be specified.

18. Stress Antioxidative

The aforementioned results indicate that metabolic pathways of aerobic organisms lead to the obligate production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are not necessarily bad. Reactive species are

actually needed as signal transduction elements in processes that are essential for life.

Just as reactive species are not necessarily bad, antioxidants are not necessarily good. It is well accepted that oxidative stress is produced when the equilibrium between reactive species and antioxidants is tilted in favour of the former. However, it is now well established that the equilibrium can be broken also if antioxidant levels exceed those of the reactive species, and Dündar and Aslan [316] proposed the term "antioxidative stress" for such disequilibrium.

First, antioxidants play the role of blocking free radical production and oxidative stress, but they are not able to distinguish among radicals which produce damage and those that play a physiological role. Moreover, these compounds not only function as antioxidants but also have prooxidant action. For example, vitamin C is a well-known antioxidant, and Linus Pauling reported a pronounced effect of vitamin C in decreasing the incidence and delaying the onset of malignant lesions in rats [317] and mice [318] exposed to ultraviolet light. However, by now, a vast number of nutritional intervention trials using vitamin C, as well as other antioxidants, have shown no efficacy in preventing gastrointestinal cancer and delaying death [319]. Rather, they seem, to slightly shorten the lives of those who take them.

On the other hand, there is evidence that ascorbic acid can also act as a prooxidant. Vitamin C does not affect itself, but the combination with iron causes intense oxidation of polyunsaturated fats.

The superoxide ion is not a particularly reactive radical, and its toxicity is linked to the fact that it can reduce the Fe^{3+} ion to Fe^{2+} ion restoring a species, H_2O_2, which can produce the radical ˙OH. Ascorbic acid also converts Fe^{3+} to Fe^{2+} thus allowing another cycle of ˙OH generation from the renewed ferrous ion [320]. Integration with ascorbic acid leads to a constant supply of reduced ascorbic acid, thus leading to a repetitive generation of ˙OH radicals by iron [320, 321]. Haematologists who studied iron overload due to transfusion therapy of patients with thalassemia or sickle cell disease reported that vitamin C can mobilize iron from their body iron stores to overwhelm the iron-binding capacity of iron-binding proteins and the resultant free iron-producing death within minutes to hours from iron-induced cardiac failure [322, 323]. Thus, vitamin C supplements can cause rapid progression to death of the otherwise more slowly progressive congestive cardiomyopathy of haemochromatosis [324]. Three young athletes so died, and no one thought of assessing their iron status before letting them take vitamin C supplements [325].

Moreover, because at low doses ROS possess a crucial role in many physiological functions, the balance between oxidant production and antioxidant protection is believed to be critical in maintaining healthy biological systems. Therefore, antioxidants at high doses besides acting as prooxidants, could also disrupt the redox balance following their potential to interact with ROS present at physiological concentrations required for optimal cellular functioning, leading to cellular dysfunction [326].

Furthermore, antioxidant supplementation can inhibit the adaptive response to ROS. A paradigmatic example is provided just by exercise. Indeed, a single session of strenuous or prolonged exercise leads to the production of a high number of radicals and other reactive oxygen species (ROS), which cause tissue damage and dysfunction. Conversely, the single sessions of a training program produce low amounts of ROS which can induce adaptive responses beneficial for the organism [327].

Interestingly, regular exercise appears to decrease the incidence of a wide range of ROS-associated diseases, including heart disease, T2DM, rheumatic arthritis, Alzheimer's and Parkinson's diseases, and certain cancers [328, 329].

The main adaptations occur in the trained skeletal muscle which can differ with the type of exercise but seem to be nevertheless dependent on ROS production. Thus, aerobic physical activity induces skeletal muscle adaptive responses [328], which increase resistance to conditions leading to increased ROS production including prolonged or strenuous exercise [330–332]. Conversely, heavy resistance exercise results in hypertrophy of the muscle cells with an increase in strength, without major changes in biochemical makeup.

An important concept developed over the past decade is that the responses to training are likely the result of the acute but cumulative effects of the responses to single exercise bouts [333]. Thus, each bout of exercise initiates acute and transient changes in gene transcription which are reinforced by repeated exercise stimuli, leading to altered, chronic expression of a variety of nuclear and mitochondrial DNA (mtDNA) gene products, which ultimately form the basis of skeletal muscle training adaptation and improvements in exercise capacity [334]. Training also slows down peroxidative processes [331, 335] and the appearance of other signs of free radical generation [331] induced by acute exercise in rat skeletal muscle. This effect is associated with increased antioxidant defences [331, 336] and decreased free radical activity [337, 338].

It was suggested that the ROS generated during the single exercise sessions act as signals regulating molecular events crucial for the adaptive responses to training. It was also proposed that antioxidant supplementation, decreasing ROS formation, prevents useful adaptations for muscular cells [339]. Such an idea was tested determining the effects of the antioxidant supplementation on exercise-induced adaptive responses. Investigations concerning the effects of higher intakes of vitamin C and/or E on exercise performance and redox homeostasis have supplied contrasting results [340]. However, antioxidant supplementation has been more consistently reported to prevent health-promoting effects of physical exercise in humans [341, 342].

19. Conclusions

Our excursion into the field of free radicals has led us to point out that the scientific community has had to revise its ideas around these substances in quite a short time. Initially seen as substances that cannot be isolated due to the limitations of the available methods, they were then considered as short-lived substances, but important for their involvement in the combustion and organic synthesis processes. Subsequently, they were considered biochemical intermediates

naturally occurring in biological systems, but impossible to identify. Thanks to the technique of electron spin resonance it was shown that free radicals were much more widespread in biological systems than previously assumed. The next step was the recognition that these species were responsible for the toxicity of oxygen and the deleterious effect of ionizing radiation.

Then came the evidence that free radicals were by-products of normal cellular metabolism and that their harmful effects were counteracted by an antioxidant defence system.

When the balance between free radical production and the capacity of antioxidant systems to neutralize them was disturbed in favour of the former, oxidative stress arose. This led to oxidative modification of biological macromolecules with consequent cell structural and functional dysfunction leading to diseases and aging.

The most recent stage has been the recognition that radicals at low concentrations can perform important functions in biological systems and that antioxidants can prevent such actions.

If subsequent researches will confirm such results, they will lead to a definitive rethinking of the idea that there has been for a long time about radicals and antioxidants. Above all, the still widespread habit of considering such substances as good and bad, respectively, would fall. Conversely, the idea would be affirmed that the goodness and badness of these substances are relative and are dependent on a series of factors, among which their concentration is very important.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

The figure is a graphical abstract. *(Supplementary Materials)*

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