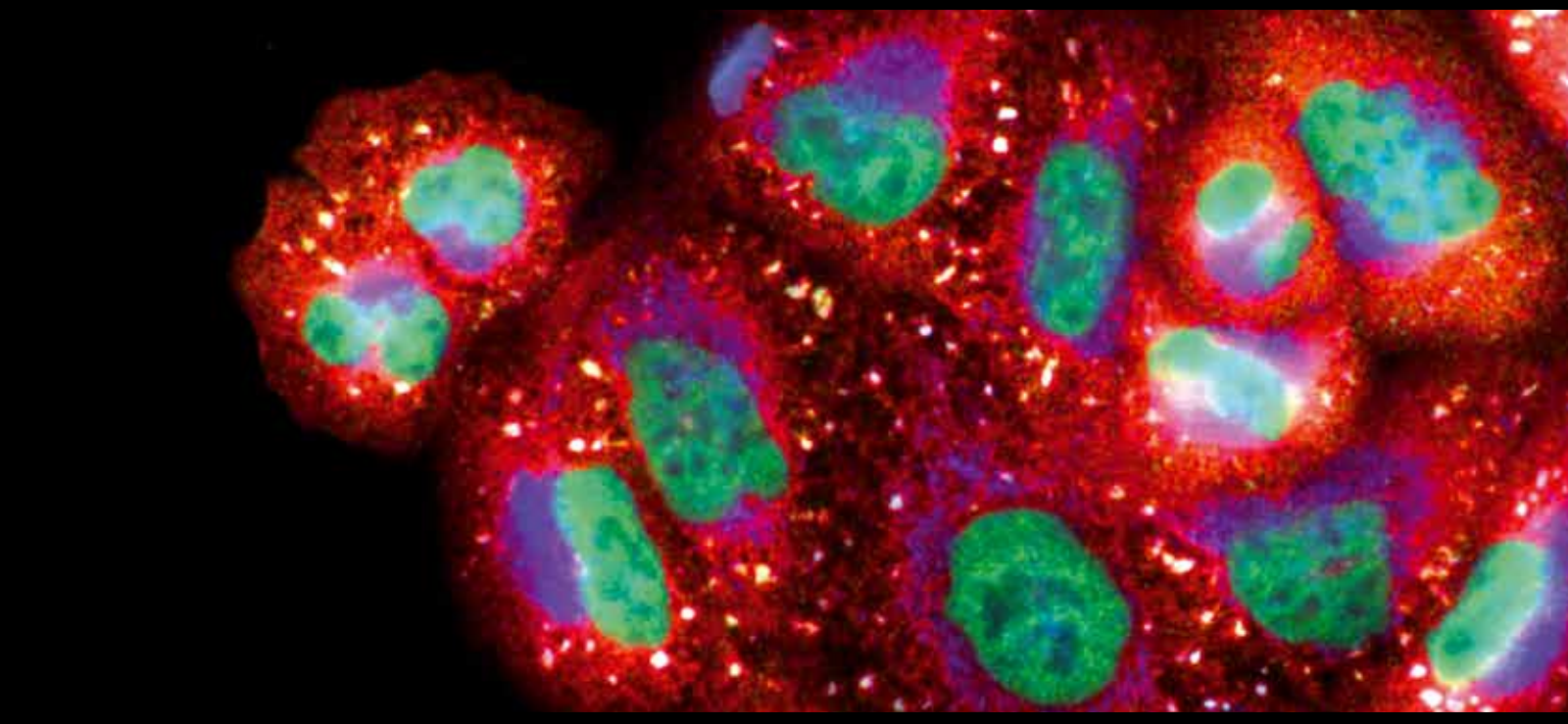
Oxidative Medicine and Cellular Longevity

Hydrogen Peroxide in Adaptation

Guest Editors: Ivan Spasojević, David R. Jones, and Michael E. Andrades



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Editorial

Hydrogen Peroxide in Adaptation

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The perception of the roles of H_2O_2 in living systems has come a long way, transcending from H_2O_2 being considered as (i) exclusively damaging; (ii) a necessary evil "unwanted but an inevitable product of aerobic metabolism"; (iii) important for specific biological processes that involve ROS aggressiveness, such as the battle of the innate immune system with pathogens; and (iv) signalling species [1–6]. The list does not end here. A number of studies have illustrated that at concentrations in the high physiological range, H_2O_2 induces more permanent, modifying changes, adaptations, increasing the resistance of biological systems to the same stimulus (hormesis) or other stressors (cross-adaptation), or enabling the adaptation to altered ecology. The capability of H_2O_2 to induce the synthesis of a large number of proteins and to provide cross-resistance implies that living systems may intentionally produce H_2O_2 as a component of adaptation in response to different fluctuations and perturbations shifting the system away from homeostasis [7–9]. Some data even implicate an important role of H_2O_2 in interspecies communication and in the development of multicellularity [10].

Examples of signalling roles of H_2O_2 emerge at progressing pace, although the field of redox research is yet to take a fully organised and systematised profile. To date it has been shown that H_2O_2 participates in signalling pathways responsible for the regulation of cell differentiation, proliferation, migration, survival and apoptosis, and mitochondrial relocalisation and of various biological processes, such as vascularisation, angiogenesis, vascular tone control, maintenance of glucose level, oxygen tension regulation, calcium metabolism regulation, immune system control, and wound healing [3–6]. Redox signalling was first described in *prokaryotes*. An excellent example of an H_2O_2 sensor is the transcription factor OxyR which is activated by H_2O_2 oxidation of one specific cysteine (thiol) residue. The thiol residue is modified to –SOH, –S–SG, or –S–S– (with another cysteine that emerges onto the protein surface due to conformation changes). Activated OxyR targets multiple genes involved in peroxide removal and the regulation of iron metabolism (the latter is aimed at preventing the Fenton reaction). An example of H_2O_2 signalling in bacteria which is involved in the regulation of processes other than redox control is the *Staphylococcus aureus* virulence factor MgrA, the oxidation of which results in increased resistance to antibiotics. A system composed of the enzyme Orp1 and the transcription factor Yap1 is yeast's analog of OxyR. A specific –SH residue in Orp1 is oxidised by H_2O_2 to –SOH, further reacting with a cysteine on Yap1 to form a disulfide bridge. The consequent change in conformation leads to nuclear accumulation of Yap1 which promotes the expression of a number of redox enzymes. A similar system is found in another fungus, *Schizosaccharomyces pombe*, but with an additional feature that Pap1 (the Yap1 homologue) is not activated if H_2O_2 is present at concentrations exceeding 1 mM. In this case, the critical cysteine residue in the activator Tpx1 (the Orp1 analogue) is oxidised to $–SO_2H$ resulting in Tpx1's inability to oxidise Pap1 and to provoke the formation of a specific intramolecular disulphide bond in Pap1 [1–3]. Cells and tissues in mammals and other complex organisms are much more relaxed with respect to an external prooxidative environment as they have specialised organs to deal with the hazards. As a consequence, they generally do not need rapid redox responses and are in position to develop redox signalling pathways that are involved in the regulation

of processes other than feedback activation of antioxidative defence.

The basic principles of H_2O_2 signalling in mammals have been described [3–6]. They are as follows. (i) The inactivation of phosphatases. The oxidation of a specific thiol residue to –SOH prevents it from accepting phosphate. In some cases a disulphide bond is formed thus preventing further oxidation to $–SO_2H$ or $–SO_3H$ and irreversible inactivation. (ii) The activation or inhibition of kinases. ASK1 is activated by H_2O_2-mediated oxidation of two cysteine residues to –SOH which leads to intermolecular disulphide bond formation and multimerisation. In vascular smooth muscle cells, Src kinase is activated by H_2O_2, which results in cofilin and myosin light-chain kinase inactivation and consequent F-actin stabilisation and promotion of actin-myosin interactions, thus enabling migration. (iii) The activation/derepression of transcription factors. NF-κB, p53, HIF-1α, and AP-1 have redox sensitive thiol groups in DNA-binding domains. In addition, it has been proposed that H_2O_2-provoked oxidation of specific thiol residues in DJ-1 and the Nrf2-inhibitor KEAP1 results in derepression of Nrf2-regulated transcription of a set of enzymes involved in antixenobiotic and cytoprotective response. NF-κB is activated by thiol oxidation, while IκB kinase can be inhibited by H_2O_2, which together makes a biphasic redox-sensing mechanism. (iv) The activation of ion channels such as ATP-sensitive K^+ channels or TRPA1. (v) The modification of activity of a number of other proteins. Very recent findings show that H_2O_2 is essential for keeping the immune system under control. The oxidation of T-cell surface thiol switches by H_2O_2 results in suppressed activity and proliferation of T cells. In order to activate T cells, dendritic cells release glutathione which is then cleaved to cysteine to reduce –SOH groups back to –SH, thus turning the redox switch-on [11]. It is important to note that H_2O_2 signalling pathways are intertwined with the effects of other signalling species, such as NO and CO.

On the other hand, excessive production of H_2O_2 or some other ROS, and a consequential supraphysiological level of oxidation (i.e., oxidative stress), has been related almost to all human diseases that one may think of [12]. Pertinent to this, the isolation of "natural" or the creation of synthetic antioxidants has become a very lucrative activity, which may explain the "explosion" of antioxidative research in the past four decades. However, promising *in vitro* data have not been translated into success in human clinical trials. If we put aside the fact that many researchers in the field neglect important issues such as (i) which ROS is/are overproduced in the particular condition they are trying to treat? (ii) At which site(s) are ROS produced? (iii) Are ROS important for pathophysiology, or do they merely represent byproducts (in other words, a cause or consequence)? (iv) What are the metabolic properties and targets of the proposed antioxidants (e.g., ascorbate seems to exert some beneficial effects in cancer and sepsis treatment via its prooxidative interplay with iron, but not due to its antioxidative effects)?; the key problem is that the importance of redox signalling has not been appreciated [13]. Such careless, almost concept-free approach is giving antioxidants a bad name and erases the belief of the importance of redox processes in (patho)physiology in the general (scientific) population, thus compromising the entire redox field. This frustrates the experts and represents the subject of concern for authorities. Barry Halliwell and coworkers have made some extreme examples to point out where this carelessness may end up. They have shown that human urine and feces possess reasonable antioxidative capacities [14]. Although those are "natural products," there seems to be no interest on the market yet. On the other hand, the European Food Safety Authority recently presented negative scientific opinions on a staggering number of health claims that various compounds and products, some of which are widely accepted "antioxidants," exert antioxidative/beneficial effects in humans [13]. The explanation for *in vivo* ineffectiveness of many *in vitro* antioxidants hides in the fact that redox signalling is too precious for the cells and tissues to allow exogenous meddling. In addition, the intrinsic enzymatic antioxidative system is more efficient than any supplementary antioxidants (of course, if there is no vitamin deficiency), so cells see the later more as a threat to normal redox signalling and less as a help [13, 14]. In order to maintain a flexible redox poise living systems have developed refractory mechanisms [15]. A good illustration of the activity of refractory mechanisms represents the fact that one may consume huge amounts of ascorbate or vitamin E, but the level of these in the blood will not rise above a specific level [15]. When exposed to an excess of exogenous antioxidants, the cells are even prepared to suppress the intrinsic enzymatic antioxidative defence in order to preserve redox homeostasis [16]. From the evolutionary point of view, the refractory system had to be developed or otherwise signalling pathways involved in the regulation of crucial biological processes would have been diet sensitive (e.g., excessive consumption of fruits and vegetables would result in proreductive conditions and the obstruction of redox signalling pathways). Do these facts altogether imply that there is no future for antioxidative therapy? Of course not! We still can adjust the redox milieu with more sophisticated approaches such as the modulation of enzyme and transcription factor activities, or we may offer compounds that have antioxidative effects but are not recognised by cells as antioxidants *per se* (examples being pyruvate, fructose, fructose 1,6-(bis)phosphate, oxaloacetate, fumarate, and metal chelators), to help the organism to fight intracellular oxidative stress against its own "will."

H_2O_2 is important for the development and differentiation of multicellular communities of unicellular yeast and bacteria and for their adaptation to the ever-changing environment [17]. In yeast, H_2O_2 at moderate concentrations increases longevity. More importantly, H_2O_2 has been proposed to regulate the initial steps of ammonia production which synchronises the development of colonies. Finally, H_2O_2 is involved in the programmed cell death that develops in the centre of a colony. The dead cells in the colony centre are likely to release nutrients that are then used by the younger prosperous cells at the edge of the colony to survive and colonise other localities [17]. It has been shown that many bacterial species express H_2O_2-producing enzymes L-amino acid oxidase, lysine oxidase, and pyruvate

oxidase [18]. In developing colonies, bacteria seem to be programmed to produce H_2O_2 which cleaves DNA resulting in genetic rearrangements via double-strand DNA break repair and the release of extracellular DNA fragments that are incorporated into the chromosome by competent cells. In this way, bacteria acquire genetic flexibility and advantageous gene variations which enable them to adapt to altered environments during initial phases of the development of biofilms [19]. In multispecies oral biofilms, streptococci use H_2O_2 (at concentrations above 1 mM) during active growth, as a mean of biochemical warfare against competitors, particularly in times of low carbohydrate (food) availability. However, some species recognise streptococcal-produced H_2O_2 as a signal to upregulate the resistance to host innate immune system responses, which also uses H_2O_2 [20]. It is noteworthy that the two host-derived peroxidases in the human oral cavity, salivary peroxidase and myeloperoxidase, use streptococcal-generated H_2O_2 to produce an antimicrobial substance, hypothiocyanite [21]. So in summary what we have here looks like a love/hate redox triangle. It has been documented in complex organisms that exposure to mild oxidative stress initiated by ROS or low level radiation increases resistance to later challenges with higher concentrations or doses of the initial stressor [22]. For example, nematodes exposed to low level oxidative stress cross-adapt, developing increased resistance to other stressors, such as heavy metals [23]. The key players in the adaptive response of nematodes seem to be antioxidative defence, heat-shock proteins and metal binding proteins, which are regulated by a branched gene pattern. It has been reported that nematodes activate intrinsic ROS production when exposed to environmental stress, such glucose deficiency [24]. This implies that oxidative stress/other stressor cross-adaptation may be based upon the role of H_2O_2 signalling in adaptive processes in general. Some insects seem to use H_2O_2 in a rather creative manner in order to survive subzero temperatures. It has been shown recently that the Arctic springtail (*Megaphorura arctica*) suppresses catalase activity and increases H_2O_2 production when exposed to freezing temperatures [25]. Combined with cryoprotective dehydration, this results in the accumulation of H_2O_2 in body fluids to surprisingly high level (15 to 20%, v/v). If we take into account that the mixture of H_2O and H_2O_2 represents a eutectic system, where the freezing point of 20% (v/v) H_2O_2 solution is at -15°C [26], it is clear how arctic insects may use H_2O_2 to prevent deadly freezing in a harsh environment. These findings could also represent the basis for new concepts in cryopreservation research. In mammals, the increased production of H_2O_2 in cardiomyocytes is actively involved in hypoxia-induced preconditioning to ischemia [27]. The preconditioning protects ischemic cardiomyocytes through upregulation of antioxidative defence and by H_2O_2-provoked opening of mitochondrial K^+-ATP channels. The importance of H_2O_2 in the development of increased resistance to ischemia by intermittent hypoxia preconditioning is implicated by the fact that treatment with the antioxidant N-acetylcysteine in the preconditioning phase may completely prevent the development of cardioprotection [27]. It has been suggested that H_2O_2 plays a key role in the endothelial adaptation to exercise by stimulating an upregulation of endothelial NO synthase [28]. Finally, H_2O_2 has been reported to provoke hormesis in mitochondria of pancreatic β cells [29]. An important role in this process seems to be played by the increased expression of mitochondrial uncoupling protein UCP2. Even more, H_2O_2 promotes the secretion of insulin from pancreatic β cells, which generally exhibit low levels of antioxidative defence and are therefore highly redox-sensitive [29]. Complimentary to the cellular level where mitochondria play a dual role (H_2O_2-producing/energy-converting organelles), there may be a link between the redox and energy status of the living system as a whole.

In this special issue we present you seven (excellent, in our honest opinion) papers providing various examples of adaptive and signalling roles of H_2O_2. We would like to invite you to find others in the literature, and there will be, for sure, more to come.

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David R. Jones
Michael E. Andrades

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Research Article

Reversible Oxidation of Myometrial Voltage-Gated Potassium Channels with Hydrogen Peroxide

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The uteri, spontaneously active or Ca^{2+} (6 mM) induced, were allowed to equilibrate, and to inhibit voltage-gated potassium (K_V) channels 1 mM 4-amino pyridine (4-AP) was applied for 15 min before adding H_2O_2 . H_2O_2 was added cumulatively: 2 μM, 20 μM, 200 μM, 400 μM, and 3 mM. Average time for H_2O_2 concentrations (2, 20, 200, and 400) μM to reach its full effect was 15 min. H_2O_2 3 mM had a prolonged effect and therefore was left to act for 30 min. Two-way ANOVA showed significant differences in time dependency between spontaneous and Ca^{2+}-induced rat uteri after applying 3 mM H_2O_2 (type of contraction, $P = 0.0280$), but not 400 μM H_2O_2 ($P = 0.9271$). Our results indicate that H_2O_2 oxidises channel intracellular thiol groups and activates the channel, inducing relaxation. Cell antioxidative defence system quickly activates glutathione peroxidase (GSHPx) defence mechanism but not catalase (CAT) defence mechanism. Intracellular redox mechanisms repair the oxidised sites and again establish deactivation of K_V channels, recuperating contractility. In conclusion, our results demonstrate that K_V channels can be altered in a time-dependent manner by reversible redox-dependent intracellular alterations.

1. Introduction

Several studies have reported that hydrogen peroxide (H_2O_2) can mediate smooth muscle relaxation as an endothelium-derived hyperpolarising factor (EDHF) via activation of potassium (K^+) channels [1–4]. Activation of K^+ channels leads to hyperpolarisation and lowering of the calcium (Ca^{2+}) concentration, resulting in a smooth muscle relaxation. To date, several subtypes of K^+ channels have been identified in the rat uteri smooth muscle. The most abundant and most well studied include large conductance Ca^{2+}- and voltage-sensitive K^+ channels (BK_{Ca}), ATP-dependent K^+ channels (K_{ATP}), voltage-dependent K^+ channels (K_V), and small-conductance Ca^{2+}-sensitive K^+ channels (SK). Our previous study showed that H_2O_2 induces relaxation in the smooth muscle of rat uteri [5]. In an attempt to identify the signaling pathways used by H_2O_2 in this tissue, we then performed a variety of experiments using a range of inhibitors: Nw-nitro-L-arginine methyl ester (L-NAME; NOS inhibitor), methylene blue (MB; cGMP signalling pathway inhibitor), propranolol (non-selective β-adrenoceptor antagonist), tetraethylammonium (TEA; nonselective K^+ channel inhibitor), glibenclamide (selective ATP dependent K^+ channel inhibitor), and 4-aminopyridine (4-AP; voltage-dependent K^+ channel inhibitor). Our results indicated that H_2O_2-induced uterine relaxation is mediated predominantly through K^+ channels as in the presence of K^+ channel antagonists, higher doses of H_2O_2 were required to reduce uterine contractions compared with L-NAME, MB, and propranolol. The potency order of the K^+ channels inhibitor effect was 4-AP > TEA > glibenclamide (the latter being far less effective), indicating that K_V channels play the most significant role of K^+ channels in H_2O_2-induced smooth muscle relaxation of rat uteri [5]. These results were similar to those obtained by other investigators that employed arterial smooth muscles treated with H_2O_2 [6]. K_V channels are the biggest family

of potassium channels. They include about 40 members divided in 12 subfamilies, K_V1–K_V12, of which K_V5, K_V6, K_V8, and K_V9 are not independently functional, but are K_V2 channels modulators. Smith and coworkers showed expression of many $K_V\alpha$ subunits in nonpregnant and pregnant mouse myometrium [7]. Opening of K_V channels liberates positive charge leading to membrane repolarisation [8] and relaxation. Response to K_V channel inhibitor 4-AP disappeared in pregnant myometrium, what was correlated to loss of $K_V4.3$ α expression [9], what is probably oestrogen dependent [7].

H_2O_2 is uncharged oxidant that can diffuse easily trough cell membranes being an eligible signal molecule in many physiological responses. Role of H_2O_2 in the regulation of myometrium smooth muscle contractile activity is not fully resolved and is still under investigation. In intact single fibers, there is evidence of complex multifactorial effects in response to H_2O_2. For instance, myofibrillar Ca^{2+} sensitivity increases early during exposure to high H_2O_2 concentrations and then declines. Moreover, H_2O_2 has little immediate effect on intracellular Ca^{2+}, but prolonged exposure to H_2O_2 leads to decreased sarcoplasmic reticulum (SR) Ca^{2+} reuptake and increased resting $(Ca^{2+})_i$, suggestive of loss of Ca^{2+} homeostasis [10], also the opening probability of SR Ca^{2+} release channels increases after thiol oxidation [11]. $(Ca^{2+})_i$-increase is a constant feature of pathological states associated with oxidative stress [12]. Recent studies have underscored the notion that the Ca^{2+} and ROS signalling systems are intimately integrated such that Ca^{2+}-dependent regulation of components of ROS homeostasis might influence intracellular redox balance and vice versa [13].

In this study, we have further examined the mechanism of K_V channels-dependant H_2O_2-relaxing effect on rat smooth muscle contractility and correlated these effects with changes in endogenous antioxidative defence, with respect to two types of activation: spontaneous and calcium-induced.

2. Material and Methods

2.1. Experimental System. Isolated uteri from virgin Wistar rats (200–250 g) in estrous, determined by examination of a daily vaginal lavage [14], were used. All protocols for handling rats were approved by the local ethics committee for animal experimentation that strictly follows international regulations.

2.2. Isolated Organ Bath Study of Uterine K_V Channels Time-Dependent Inhibition. All rats were killed by cervical dislocation. The uterine horns were rapidly excised and carefully cleaned of surrounding connective tissue and mounted vertically in a 10 ml volume organ bath containing De Jalon's solution aerated with 95% oxygen and 5% carbon dioxide at 37°C.

The uteri, spontaneously active or Ca^{2+} (6 mM)-induced, were allowed to equilibrate at 1 g tension before addition of the experimental drugs. To inhibit K_V channels in uteri 1 mM 4-amino pyridine (4-AP) was applied for 15 min before adding H_2O_2. H_2O_2 was added cumulatively: 2 μM, 20 μM, 200 μM, 400 μM, and 3 mM. Myometrial tension was recorded isometrically with a TSZ-04-E isolated organ bath and transducer (Experimetria, Budapest, Hungary). Each H_2O_2 concentration was left to act for 15 min except 3 mM that was left for 30 min.

2.3. Determination of Uterine Antioxidative Enzyme Activities after High Impact of H_2O_2. To 7 h contracting uteri were applied 3.6 mM H_2O_2 (summed cumulative concentrations from previous experiment). Control group were uteri active equivalent time but untreated. After experiment, samples were immediately frozen, using liquid nitrogen, and then transferred to −80°C until enzyme analysis.

Thawed uteri were homogenised and sonicated in 0.25 M sucrose, 1 mM EDTA, and 0.05 M Tris-HCl buffer pH 7.4 before centrifugation for 90 min at 105 000 × g. The supernatant was used to determine enzyme activities (using a Shimadzu UV-160 spectrophotometer, Shimadzu Scientific Instruments, Shimadzu Corporation, Kyoto, Japan). Superoxide dismutase (SOD) activities were determined by the adrenaline method [15]. One unit of activity is defined as the amount of enzyme necessary to decrease by 50% the rate of adrenalin autooxidation at pH 10.2. Manganese SOD (MnSOD) activity was determined by incubating the samples with 8 mM KCN. Copper-zinc SOD (CuZnSOD) activity was calculated as the difference between total SOD and MnSOD activities. The activity of catalase (CAT) was determined by the rate of H_2O_2 disappearance measured at 240 nm, according to Claiborne [16]. One unit of CAT activity is defined as the amount of enzyme that decomposes 1 mmol H_2O_2 per minute at 25°C and pH 7.0. The activity of glutathione peroxidase (GSHPx) was determined by the GSH-dependent reduction of t-butyl hydroperoxide, using a modification of the assay described by Paglia and Valentine [17]. One unit of GSHPx activity is defined as the amount needed to oxidize 1 nmol NADPH per min at 25°C and pH 7.0. Glutathione reductase (GR) activity was determined using the method of Glatzle et al. [18]. This assay is based on NADPH oxidation concomitant with GSH reduction. One unit of GR activity is defined as the oxidation of 1 nmol NADPH per min at 25°C and pH 7.4. All enzyme activities were expressed as units·mg^{-1} protein.

2.4. Statistical Analyses. Results were analysed with statistical GraphPad Prism (version 5.03), GraphPad Software, San Diego, CA, USA. Statistical significance was determined with *t*-test, one-way ANOVA, and post hoc tests: Dunett—comparison with a control group, Tukey—comparison among all groups and test for linearity trend; two-way ANOVA and post hoc Bonferroni test; regression analysis: Linear regression and nonlinear regression (dose-dependant inhibition model with variable Hill slope).

2.5. Reagents. The following reagents were used: H_2O_2 (ZORKA Pharma, Sabac, Serbia); 4-AP (Sigma Chemical Co, St Louis, MO, USA). All were dissolved in distilled water. De Jalon's solution was comprised of (in gl-1): NaCl 9.0, KCl 0.42, $NaHCO_3$ 0.5, $CaCl_2$ 0.06, and glucose 0.5.

3. Results

3.1. K_V Channels Dependent H_2O_2 Effect on Contractile Activity. Inhibition of K_V channels with 1 mM 4-amino pyridine (4-AP) increased the basal tonus of uteri contractions (results not shown), confirming the presence of K_V channels in the uteri and their role in contractions. After rat uterine K_V channels were blocked with 1 mM 4-AP, H_2O_2 (400 μM and 3 mM) induced effect on contractile activity, both spontaneous and calcium induced. H_2O_2 first induced relaxation, after which contractions were gradually recovering. Additionally, H_2O_2 3 mM had a prolonged effect and therefore was left to act for 30 min. Average time for other H_2O_2 concentrations (2, 20, 200, and 400) μM to reach its full effect was 15 min (Figure 1).

3.2. Time-Dependent Changes in K_V Channel Inhibition. One-way ANOVA time-dependence analysis of H_2O_2 (400 μM and 3 mM) induced effect in spontaneous rat uteri showed time significance (time resp.: $P = 0.0246$, $P < 0.0001$). Post hoc test for linear trend showed significant trend of linear regression (400 μM H_2O_2: $r^2 = 0.3031$, $P = 0.0102$; 3 mM: $r^2 = 0.8142$, $P < 0.0001$). In Ca^{2+}-induced rat, uteri were also shown significant time dependency and linear trend in the effect of H_2O_2 3 mM (time: $P < 0.0001$; $r^2 = 0.5800$, $P < 0.0001$) but not with 400 μM (time: $P = 0.6628$; linear trend: $r^2 = 0.2673$, $P = 0.3712$) (Figure 2).

Two-way ANOVA showed significant differences in time dependency between spontaneous and Ca^{2+}-induced rat uteri after applying 3 mM H_2O_2 (type of contraction, $P = 0.0280$), but not 400 μM H_2O_2 ($P = 0.9271$). Regression analysis of fitted lines also showed similar time dependency but different contraction intensity after applying 3 mM H_2O_2 (similar slope, but different intercepts) between spontaneous and Ca^{2+}-induced rat uteri, as well as no significant differences after applying 400 μM H_2O_2 (no differences in slope and intercepts between spontaneous and Ca^{2+}-induced).

3.3. Changes in Antioxidative Enzyme Activity in Rat Uteri after Impact of H_2O_2 High Concentration. t-test analysis for changes of antioxidative enzymes activity after applying 3.6 mM H_2O_2 (concentration that equals summed cumulative concentrations from previous experiment) comparing to Ca^{2+}-induced uteri active equal time but without H_2O_2 treatment showed statistically significant increase of CuZnSOD ($P = 0.0381$) and GSHPx ($P = 0.0344$) activity after impact of 3.6 mM (Figure 3).

4. Discussion

Earlier studies have shown that H_2O_2 can act as contractile and relaxing agent, tissue dependent [19–21], and in some cases, it exhibits a biphasic effect [2, 22]. Role of H_2O_2 in the regulation of myometrial contractile activity is not fully resolved and is still under investigation. In some states as in the thrombosis postpartum [23] or during powerful myometrial contractions that restrict blood flow to the uterus, reperfusion/ischemia injury can occur [21, 24] possibly producing high impact of H_2O_2 on the uteri. In our study, we have observed that in K_V channel inhibitor (4-AP) pretreated uteri, high concentrations of H_2O_2 caused relaxation and recovery independent of the type of contractile activity (spontaneous or calcium induced), just with differences in intensity. Characteristic of this effect was its significant linear time dependency. After the first relaxation effect, there was a time dependent recovery of contractile activity. Additionally at 3 mM H_2O_2, effect lasted longer (30 min) then the equilibration time for other H_2O_2 concentrations (15 min). It is known that proteins are H_2O_2 targets and that K^+ channels and proteins that regulate them are redox-sensitive elements [4]. Main protein modifications include direct oxidation, above all amino acids with thiol groups, as cysteine oxidative glication and carbonylation [25–28]. Several electrophysiological studies showed that H_2O_2 acts on cysteine of K_V modulatory subunits and found specific cysteins that determine the channel sensitivity. Mutation of these cysteins impeded H_2O_2-dependent channel activation [29]. After oxidation thiol groups may interact with nearby cysteins forming disulfide bonds [30]. Rogers and coworkers [4] showed that K_V channels are regulated in a redox-sensitive manner as H_2O_2 1–10 mM-induced K_V currents in coronary artery smooth muscle cells were being antagonized by DTT, a thiol reductant, and blocked by NEM, a thiol-alkylating agent; but they did not observe rapid reversibility (i.e., the effect of H_2O_2 to increase K_V current was sustained). As a plausible explanation for their result, they suggest that H_2O_2 may oxidize an extracellular target, rather than a cytoplasmic one that could be repaired by endogenous intracellular reductants. They based this on the assumption that: (1) H_2O_2 crosses membrane easily and has access to extracellular and intracellular thiol groups; (2) thiol groups of extracellular components are always present outside the cell (e.g., extracellular loops of K_V channels); (3) intracellular targets include K_V channels or proteins that regulate them and always remain inside of the cell; (4) intracellular reductants do not cross the membrane but remain inside the cell having access only to intracellular components. Thus, if an extracellular target was oxidized, no "repair" (i.e., reduction) would be possible. Conversely, if an intracellular target was oxidized, it might be repaired by intracellular reducing mechanisms [4]. We believe that this oxidation could take place intracellular as well, since we did observe time dependent recovery of contractile activity after H_2O_2-induced relaxation in the presence of 4-AP. 4-AP blocks K_V channels intracellular [31, 32] and has small association and dissociation time (100 ms). 4-AP binding on the intracellular side of the K_V channel may be temporarily altered due to high concentration of highly diffusible H_2O_2. It is possible that H_2O_2 oxidises channel intracellular thiol groups and activates the channel, inducing relaxation. However, 4-AP gradually reassociates to intracellular channel sites as intracellular redox mechanisms repair the binding site and again establishes deactivation of K_V channels, recuperating contractility. That way H_2O_2 could directly interact with K_V channels on the intracellular side, but this bond could be overcome with time and 4-AP bond reestablished, making the H_2O_2 effect transient. Aikawa et al. also observed the transient response to high

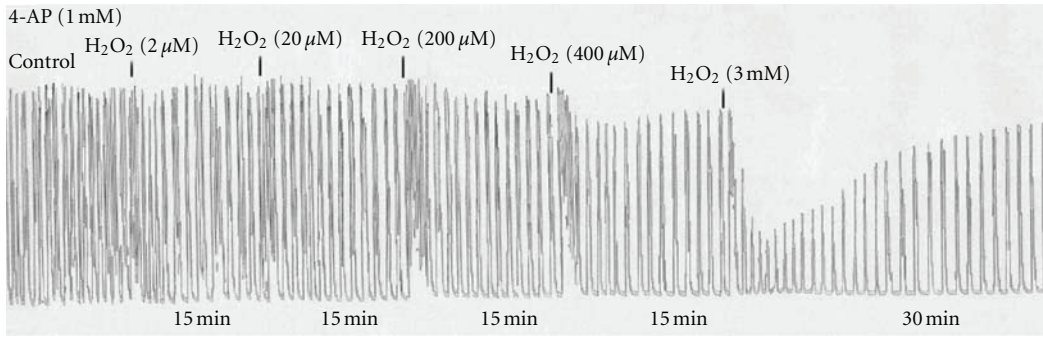

FIGURE 1: A representative original trace of contractions in rat uteri treated with H_2O_2 (2, 20, 200, 400 μM, and 3 mM) in the presence of 4-amino pyridine (4-AP). It can be noted the time-dependent recovery of contractions after applying H_2O_2 400 μM and 3 mM, as well as prolonged effect of 3 mM H_2O_2.

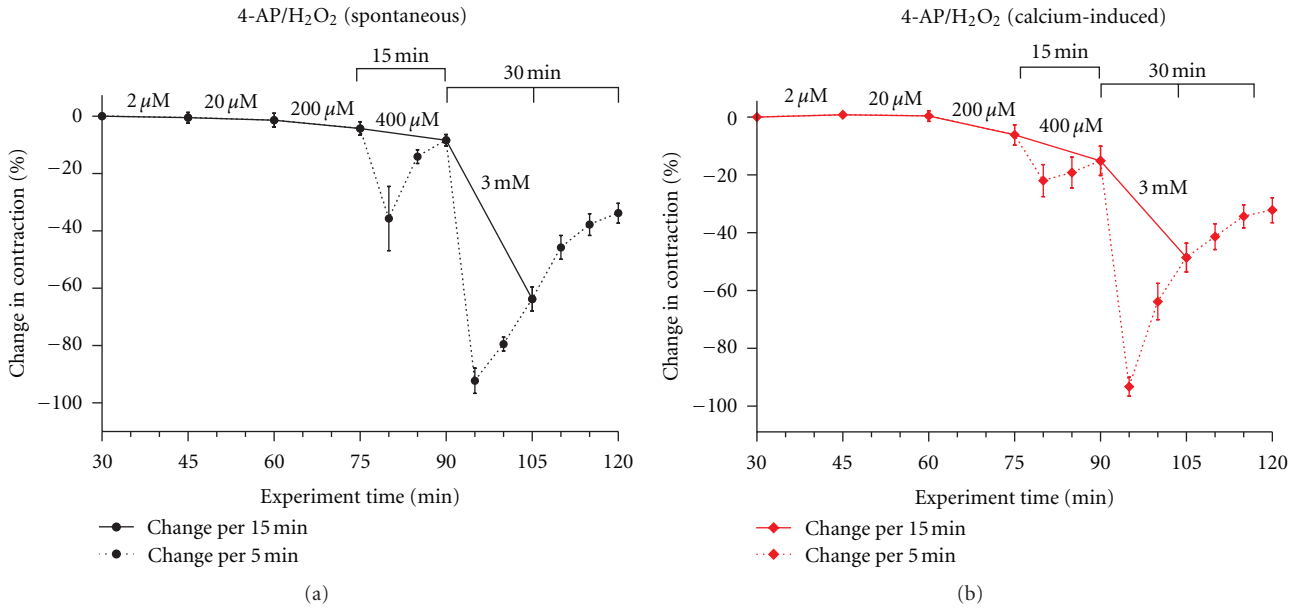

FIGURE 2: Time-dependent changes in effect of H_2O_2 (400 μM and 3 mM) in spontaneous (a) and Ca^{2+}-induced, (b) rat uteri contractile activity in the presence of K_V channel inhibitor, 4-AP 1 mM. Contractile activity is expressed as change in contraction amplitude (%) after applying H_2O_2. Full line represents amplitude changes on every 15 min and dotted line on every 5 min after applying H_2O_2 (400 μM and 3 mM). 3 mM H_2O_2 had prolonged effect (time for contractions to equilibrate: 30 min). Values are expressed as mean ± error. x-axis represents the full experiment time starting from 30 min, which is the uteri equilibration interval.

H_2O_2 concentrations in time. They showed that after 1 h of exposure to 6 different concentrations of H_2O_2 (0%, 0.0625%, 0.125%, 0.25%, 0.5%, and 1%), the contractile response of rat bladder smooth muscle decreased progressively to increase in H_2O_2 concentration [33]. In more recent study, Han at al. also found that increasing the duration of treatment with 3×10^{-4} g% H_2O_2 progressively decreased the contractile responses of the smooth muscle of bladder [34].

With an increase in H_2O_2, its toxic effect starts to appear. Therefore, timely elimination of messengers is important in cell signalisation. In our study after applying high concentration of H_2O_2 (3.6 mM) on a long-term activity, we observed increase in CuZnSOD and GSHPx activity. H_2O_2 is mainly scavenged by CAT and GSHPx. CuZnSOD, is a cytoplasmic $O_2^{\bullet-}$ scavenger, and as such implies $O_2^{\bullet-}$ increased production. Some researchers also showed that H_2O_2 causes increase in $O_2^{\bullet-}$ [35]. Others have observed decrease of reduced form of glutathione, a necessary factor in GSHPx activity [36]. As mentioned previously, we have observed increased activity of GSHPx, H_2O_2 scavenger. However, other important H_2O_2 scavenger, CAT, did not show any changes. It seems that cell antioxidative defence system quickly activates GSHPx defence mechanism but not CAT defence mechanism. CAT is mainly active in peroxisome though some of its activity is also present in mitochondria and endoplasmatic reticulum. CAT, comparing to GSHPx, has lesser affinity for H_2O_2 and as such is not effective in scavenging low concentrations of H_2O_2 [37, 38]. Therefore, GSHPx is considered as a H_2O_2 low concentration scavenger. However, it was shown that CAT is also active at H_2O_2 lower

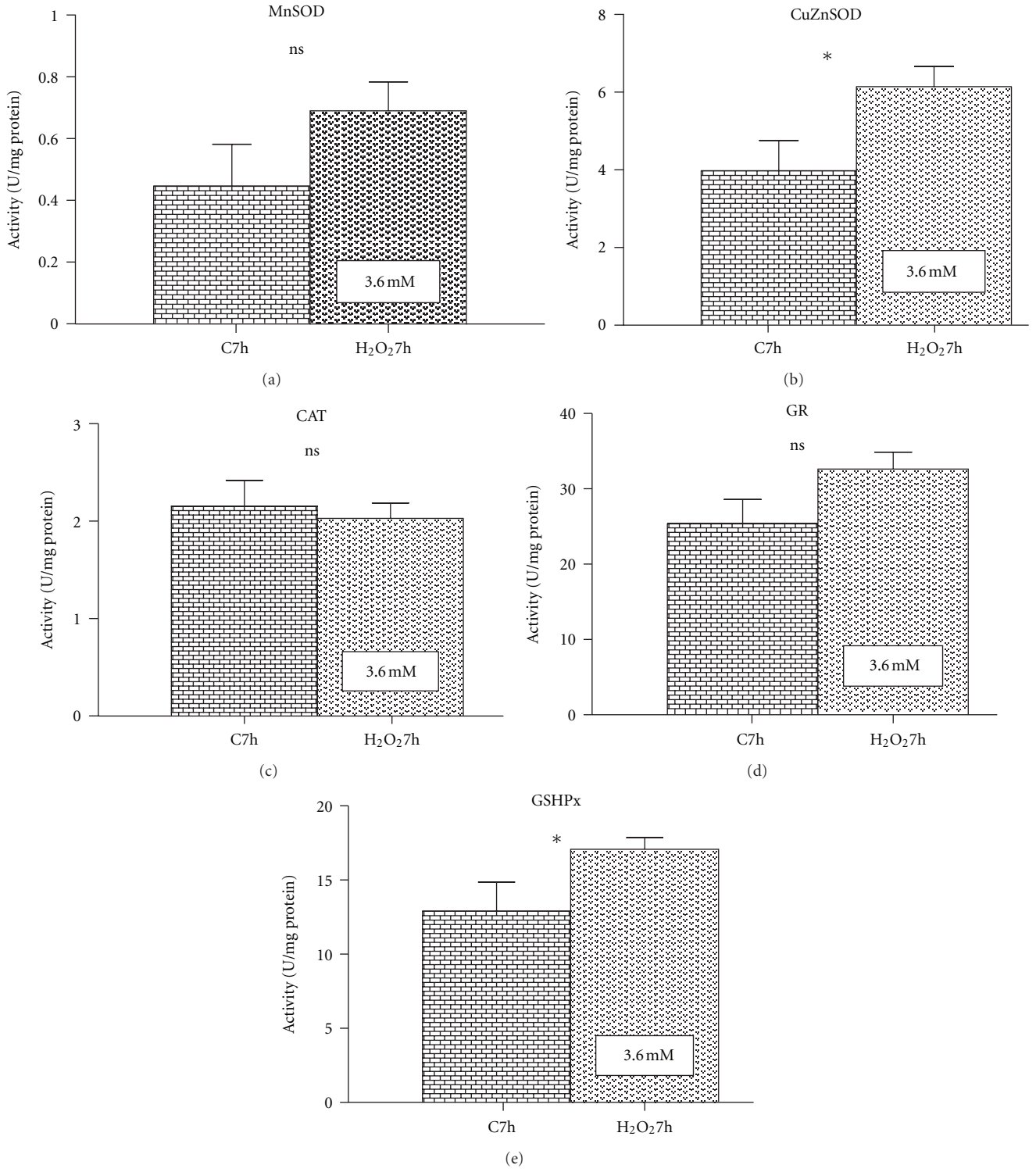

Figure 3: Change of AOS enzyme activity (MnSOD (a), CuZnSOD (b), CAT (c), GR (d), and GSHPx (e)) in Ca^{2+}-induced rat uteri after applying 3.6 mM H_2O_2. Control group were uteri active equivalent time interval without H_2O_2 treatment. Data are expressed as mean ± error. Groups were compared with t-test ($P < 0.05$, significant). Ns: non significant; $^*P < 0.05$.

concentrations and that it loses its activity at H_2O_2 higher concentrations [39, 40], what is implying to a possible cause of the absence in its activity in our study.

In conclusion, our results demonstrate that K_V channels can be altered in a time-dependent manner by possible time and redox-dependent alterations of K_V channels intracellular binding sites or proteins that regulate them and that GSHPx mechanism is the primary scavenging mechanism in this H_2O_2 conditions. Further studies may help to find the possible solutions in protecting myometrium in pathological states including strong redox impact on the cell, as reperfusion ischemia or thrombosis postpartum.

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Review Article

Mitochondrial Hormesis in Pancreatic β Cells: Does Uncoupling Protein 2 Play a Role?

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In pancreatic β cells, mitochondrial metabolism translates glucose sensing into signals regulating insulin secretion. Chronic exposure of β cells to excessive nutrients, namely, glucolipotoxicity, impairs β-cell function. This is associated with elevated ROS production from overstimulated mitochondria. Mitochondria are not only the major source of cellular ROS, they are also the primary target of ROS attacks. The mitochondrial uncoupling protein UCP2, even though its uncoupling properties are debated, has been associated with protective functions against ROS toxicity. Hormesis, an adaptive response to cellular stresses, might contribute to the protection against β-cell death, possibly limiting the development of type 2 diabetes. Mitochondrial hormesis, or mitohormesis, is a defense mechanism observed in ROS-induced stress-responses by mitochondria. In β cells, mitochondrial damages induced by sublethal exogenous H_2O_2 can induce secondary repair and defense mechanisms. In this context, UCP2 is a marker of mitohormesis, being upregulated following stress conditions. When overexpressed in nonstressed naïve cells, UCP2 confers resistance to oxidative stress. Whether treatment with mitohormetic inducers is sufficient to restore or ameliorate secretory function of β cells remains to be determined.

1. Introduction

Type 2 diabetes (T2D) is characterized by insufficient insulin release from pancreatic β cells that should compensate for peripheral insulin resistance [1]. Pancreatic β cell dysfunction and, eventually, death are considered to occur in response to metabolic stresses, which trigger mitochondrial oxidative damages, consequently interfering with glucose metabolism responsible for induction of insulin exocytosis. Accordingly, β cell should be equipped with efficient defense and adaptive mechanisms against chronic over stimulation of mitochondria, counteracting the adverse effects of oxidative stress. Emerging evidence indicate a hormetic nature of mitochondrial defensive response, that is, a cellular defense adaptation promoted by ROS-triggered signaling. Here, we discuss the putative mitohormetic role of mitochondrial uncoupling protein 2 (UCP2) in β cells and protection from mitochondrial oxidative damages.

2. Mitochondrial Metabolism, Glucose Sensing, and the Secretory Response

Pancreatic β cells function as glucose sensors to adjust insulin secretion to blood glucose levels, thereby maintaining glucose homeostasis. Translating nutrient signals into regulated insulin exocytosis relies on optimally tuned mitochondrial function [4]. Although glucose is the chief secretagogue for the β cell, metabolic profile of mitochondria is modulated by the relative contribution of glucose and lipid products for oxidative catabolism [5]. In the mitochondrion, substrates derived from glucose and fatty acids are oxidized and converted to ATP by the mitochondrial electron transport chain located in the inner mitochondrial membrane. Synthesized ATP is subsequently translocated to the cytosol, triggering insulin exocytosis thanks to calcium elevation secondary to ATP-mediated plasma membrane depolarization [6]. One byproduct of mitochondrial electron

transportation is the generation of reactive oxygen species (ROS) [5].

3. Impact of Mitochondrial ROS on β Cell Function

Physiological levels of glucose and fatty acids are essential to normal β cell function. However, continuous overstimulation of β cells by these nutrients may be deleterious to β cell function, a phenomenon referred to as glucolipotoxicity. Accordingly, pancreatic β cells chronically exposed to hyperglycemic and hyperlipidemic conditions steadily undergo deterioration and ultimately failure of insulin secreting capacity [7]. This loss of β cell function has been attributed to a variety of mechanisms, most of which having in common the formation of ROS [8–10]. Elevated ROS affect the function and survival of β cells through a direct oxidation of cellular macromolecules [11, 12] and activation of cellular stress-sensitive signaling pathway [13]. Glucose infusion in rats for 48 h to achieve chronic hyperglycaemia increases mitochondrial islet superoxide and reduces glucose-stimulated insulin secretion [14]. However, it should be noticed that physiological glucose stimulation prevents formation and accumulation of ROS [15, 16]. Increasing glucose usage by pharmacological activation of glucokinase reduces ROS toxicity in insulin-secreting cells [17].

4. Mitochondria Generate ROS

ROS refer to a diverse range of species, such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical. The biological consequences of ROS rely on the specific species being involved and the physiological or pathological context. Superoxide can be converted to less reactive H_2O_2 by superoxide dismutase (SOD) and then to O_2 and H_2O by catalase, glutathione peroxidase (GPX), and peroxiredoxin, which constitute antioxidant defenses [18].

Mitochondrial electron transport chain is a potent producer of $O_2^{\bullet -}$ within cell. Electrons from sugar, fatty acids, and amino acid catabolism accumulate in the electron carriers of the respiratory chain. $O_2^{\bullet -}$ formation is coupled to this electron transportation as a byproduct of normal mitochondrial respiration through one electron reduction of molecular $O_2^{\bullet -}$. Complexes I and III of the respiratory chain are the major sites for $O_2^{\bullet -}$ generation [19]. In complex I, electrons carried by NADH are accepted by flavin mononucleotide (FMN) and transferred to mobile electron carrier Coenzyme Q (CoQ), with $O_2^{\bullet -}$ formation at FMN. This formation of $O_2^{\bullet -}$ requires FMN in a fully reduced form, which is determined by the NADH/NAD^+ ratio [20]. Accumulation of NADH and enhanced ROS formation are favored by slowdown of mitochondrial respiration caused by complex I inhibition (e.g., rotenone effect), secondary to damages of respiratory chain, or because of low cellular ATP demand [21, 22].

Reverse electron transport (RET) from complex II to complex I also generates large amounts of $O_2^{\bullet -}$. Under conditions of substantial proton motive force, RET occurs when electron supply induces CoQ reduction driving electrons back to complex I, thereby reducing NAD^+ to NADH at the FMN site [23]. Succinate and fatty acid oxidation promote high proton motive force along with electron supply to CoQ, giving rise to $O_2^{\bullet -}$ formation under RET. Generation of $O_2^{\bullet -}$ by complex I, in particular through RET, is very sensitive to mitochondrial uncoupling because of the required proton motive force [24]. When inhibited by antimycin, complex III can produce significant amounts of $O_2^{\bullet -}$, although its production under physiological conditions is only marginal compared to that of complex I [22].

5. Mitochondrial ROS, Friends or Foes?

ROS impact differently on cell function depending on specific reactive oxygen species, their concentrations, and effectiveness of detoxifying systems; thereby, defining signaling or toxic effects of ROS [25]. In insulin-secreting cells, low concentrations of H_2O_2 have been reported to contribute to the stimulation of insulin secretion [26]. However, exposure to robust concentrations of H_2O_2 impairs β cell function [27]. When β cells are continuously overstimulated by nutrients, accumulation of ROS can overwhelm detoxification systems and induce deleterious effects [8]. This is particularly relevant for pancreatic islets because of the shifted redox balance favored by high metabolic rate, in particular under glucolipotoxic conditions, and relatively weak detoxifying systems [28]. Being a major source of ROS, mitochondria are in the eye of the storm. Specifically, H_2O_2 exposure to insulin-secreting cells inactivates mitochondria, thereby interrupting mitochondrial signals normally linking glucose metabolism to insulin exocytosis [27]. One single oxidative stress applied for just 10 minutes induces β cell dysfunction lasting over days, explained by persistent damages in mitochondrial components and accompanied by subsequent generation of endogenous ROS of mitochondrial origin [29]. In the close vicinity of free radical production, mitochondrial inner membrane components are particularly prone to oxidative injuries, such as subunits of electron transport chain complexes and the adenine nucleotide translocase (ANT) [29, 30]. Moreover, some iron-sulfur centers of mitochondrial matrix proteins, among them aconitase, are susceptible to damages induced by direct reaction with $O_2^{\bullet -}$ [31] or nitric oxide [32], leading to impaired mitochondria and β cell dysfunction.

Because of relatively low antioxidant-enzyme activities in pancreatic islets, enhancing expression of corresponding genes in insulin-secreting cells has been foreseen as potential protective intervention. However, contradictory findings were reported in studies testing this promising approach. The concept is favored by some studies, among them: (i) β cell-specific overexpression of cytosolic SOD (SOD1) enhances mouse resistance to alloxan-induced diabetogenesis [33]; (ii) adenovirus-mediated overexpression of mitochondrial SOD (SOD2) in isolated islets extends islet function following

transplantation into streptozotocin (STZ)-treated nonobese diabetic (NOD) mice [34]; (iii) β cell-specific overexpression of SOD2 and catalase protects islets from STZ-induced oxidative stress [35]; (iv) β cell-specific overexpression of glutathione peroxidase in *db/db* mice improves β cell volume and granulation [36]. Tempering these promising results, overexpression of antioxidant enzymes, such as catalase and metallothionein, specifically in β cells of NOD mice increases β cell death and sensitizes islets to cytokine-induced injuries [37]. The latter results suggest that a mild dominance of host ROS over detoxifying systems might exhibit beneficial effects.

6. Mitochondrial Protection against ROS: Role of UCP2

Mitochondrial uncoupling refers to the dissociation of electron-dependent oxygen consumption to ATP generation on the respiratory chain. The most efficient way to induce mitochondrial uncoupling is to allow protons to circulate freely across the inner mitochondrial membrane, in other words to create a proton leak. In this regard, UCP1 is a professional mitochondrial uncoupler by inducing proton leakage. As a result, the energy contributed by electron flow is dissipated as heat instead of ATP generation. UCP1 expression in brown adipose tissue confers to these fat depots highly thermogenic properties [38]. UCP2 was named after its loose homology (59%) with UCP1 and exhibits heterogeneous tissue expression, including in pancreatic islets [39]. Similarly to UCP1, UCP2 was proposed to induce proton leakage and to dissipate proton motive force [40], consequently limiting ATP production and glucose-stimulated insulin secretion in pancreatic β cells [41, 42].

Due to tight dependency of complex I on proton motive force for ROS formation, putative uncoupling effects of UCP2 were suggested to compromise mitochondrial ROS generation and associated cell damages [43, 44]. In insulin-secreting cells, a series of *in vitro* studies have shown that increasing UCP2 expression attenuates ATP synthesis and insulin secretion in response to glucose [41, 42, 45, 46]. Conversely, UCP2 deficiency enhances glucose-stimulated insulin secretion, as shown in islets isolated from both global [47, 48] and β cell-specific [49] UCP2 knockout mice. In diet-induced T2D mouse model, the lack of UCP2 improves blood glucose levels and insulin secretory capacity [47]. Chronic exposure of INS-1 β cells to fatty acid decreases the secretory response to glucose, along with *UCP2* gene induction and partial mitochondrial uncoupling [50].

Over the last decade, UCP2 effects in β cells have been tightly correlated with its presumed uncoupling properties and consequences on ATP synthesis from oxidative phosphorylation [41, 42, 45–48]. However, in β cell-specific UCP2-null mice, the potentiated glucose-stimulated insulin secretion correlates with higher intracellular ROS levels, without any changes on mitochondrial coupling and ATP generation [49]. Moreover, pancreatic islets form global UCP2 knockout mice studied on three congenic backgrounds, as opposed to mixed genetic background [48], exhibit impaired glucose response accompanied by increased ROS production and persistent oxidative stress [51]. Finally, cytokine-induced ROS production is reduced in insulin-secreting cells over-expressing UCP2, independently of uncoupling effects [3]. Collectively, these observations are contradictory regarding putative uncoupling properties of UCP2 and its effects on the secretory function of β cells. Instead, they suggest a role in defense mechanisms against oxidative stress [52], as shown by induction of UCP2 that prevents cytokine-induced β cell death through suppression of ROS production [3, 53].

Because UCP2 might play a protective role against ROS, one can hypothesize cooperation and feedback mechanisms with dedicated antioxidant enzymes. In β cells overexpressing UCP2, the associated sheltering effect against oxidative injuries is not associated with changes in antioxidant enzymes (personal communication from Françoise Assimacopoulos-Jeannet, University of Geneva). Ablation of UCP2 in β cells favors both ROS formation and induction of H_2O_2-scavenging GPX, but not of superoxide scavenger SODs [49]. In islets lacking SOD1 or GPX1, UCP2 is upregulated as a protective response against excessive cellular ROS [54]. Conversely, UCP2 is downregulated upon induction of GPX1 in mouse islets [55]. Collectively, these data indicate crosstalk between UCP2 and antioxidant enzymes through unidentified mechanisms.

7. Hormesis, a Stress-Induced Protective Response

Theophrastus Bombastus von Hohenheim, a Swiss pharmacist born in 1493 also named Paracelsus, developed this revolutionary idea at the Renaissance period: "*Alle Ding' sind Gift, und nichts ohn' Gift; allein die Dosis macht, daß ein Ding kein Gift ist*" freely translated to "the dose makes the poison." Five centuries later, this notion has been extended to the so-called hormesis. Hormesis is a phenomenon whereby exposure of cells or organs to low levels of a given toxin confers resistance to subsequent contacts to higher concentrations [56]. Accordingly, hormesis describes an adaptive response to continuous cellular stresses. Hormesis is well illustrated by ischemic preconditioning, a situation where short ischemic episodes protect brain and heart from prolonged lack of oxygen and nutrients [57, 58]. Regarding pancreatic β cells, emerging concepts suggest that efficiency of hormetic responses to detrimental lifestyle factors might set the level of protection, impacting on the progression of T2D [59].

8. Adaptation and Hormesis in β cells

Obesity is a strong risk factor for T2D, appearing in subjects developing β cell dysfunction and death in response to metabolic and inflammatory stresses [60, 61]. However, about half of obese individuals do not develop diabetes, due to efficient long-term adaptation to insulin resistance by increasing β cell mass and insulin secretion. In these resistant individuals, β cells may develop adaptive stress responses to prevent their loss, at least transiently. Peroxisome proliferator-activated receptor alpha (PPARα) is a

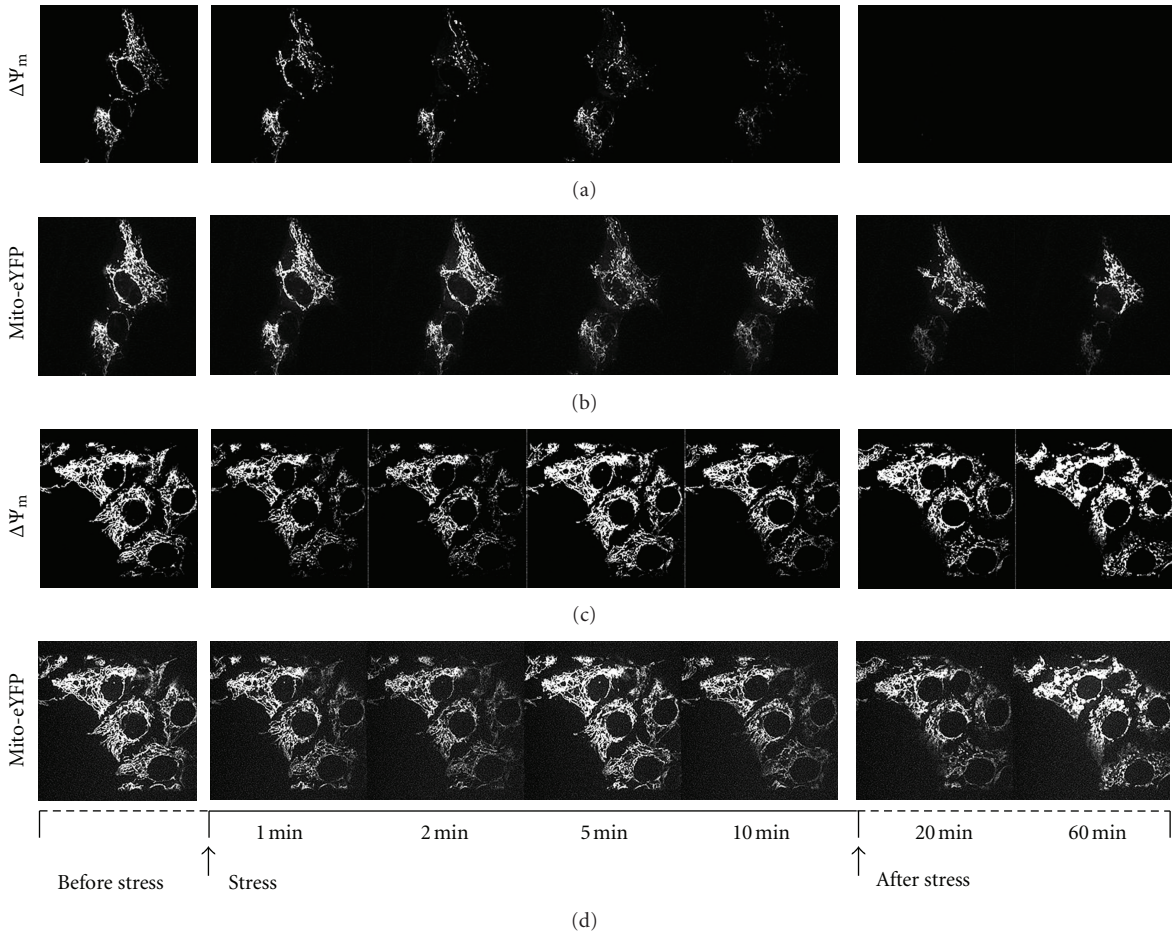

Figure 1: Simultaneous monitoring of mitochondrial membrane potential and morphology in INS-1E β cell under transient oxidative stress. Real-time imaging of INS-1E cells by simultaneous fluorescence recordings of mitochondrial potential ($\Delta\Psi_m$) by TMRE (a and c) and mitochondrial morphology by $\Delta\Psi_m$-independent mito-eYFP (b and d) as described [2]. (a), Signals recorded before oxidant exposure (before-stress), during the 10 min 200 μM H_2O_2 exposure (stress), and after neutralization of extracellular H_2O_2 by the addition of 100 U/mL catalase (after-stress). (b), Corresponding mitochondrial morphology monitored simultaneously with $\Delta\Psi_m$ shown in (a). (c) and (d) show control nonstressed cells.

transcription factor controlling lipid and glucose homeostasis. PPARα-deficient mice on an obese (*ob/ob*) background develop β cell dysfunction characterized by reduced islet area and glucose response [62]. Human islets treated with PPARα agonist are protected against fatty acid-induced impairment of glucose-induced insulin secretion and apoptosis [62]. This indicates that PPARα could be an adaptive candidate in β cells under pathological conditions, such as lipid-induced dysfunction [63].

Converging evidence suggest that stresses can induce specific responses rendering β cells more resistant to the stress-molecule, or even to other toxins. Pre-exposure to low dose IL-1β renders β cells less susceptible to toxin-induced cell necrosis and to radical-induced damages, though with a loss of normal phenotype [64]. Moreover, islets from pancreatectomized hyperglycemic rats exhibit reduced sensitivity to STZ, an effect associated with induction of protective antioxidant and antiapoptotic genes during chronic hyperglycemia [65]. Finally, islets from GK/Par rat (non-obese model of T2D) also show strong resistance to toxic effects of exogenous ROS, secondary to an adaptive response to the diabetic milieu [66]. Thus, β cells possess hormetic mechanisms in response to inflammatory and metabolic stresses. Stressors are not merely toxic; they can also prime the stressed cell to future pathogenic challenges by rendering them more resistant.

9. Mitochondrial Adaptation and Hormesis, or Mitohormesis, in β Cells

Mitochondrial adaptation and hormesis, or mitohormesis, originally referred to the hypothetical model of cell preservation in response to ROS-induced stresses originating from mitochondria [67]. The concept was substantiated by findings in *C. elegans* revealing that glucose restriction activates mitochondria and ROS formation, promoting hormetic extension of life span [68]. In conflict with Harman's

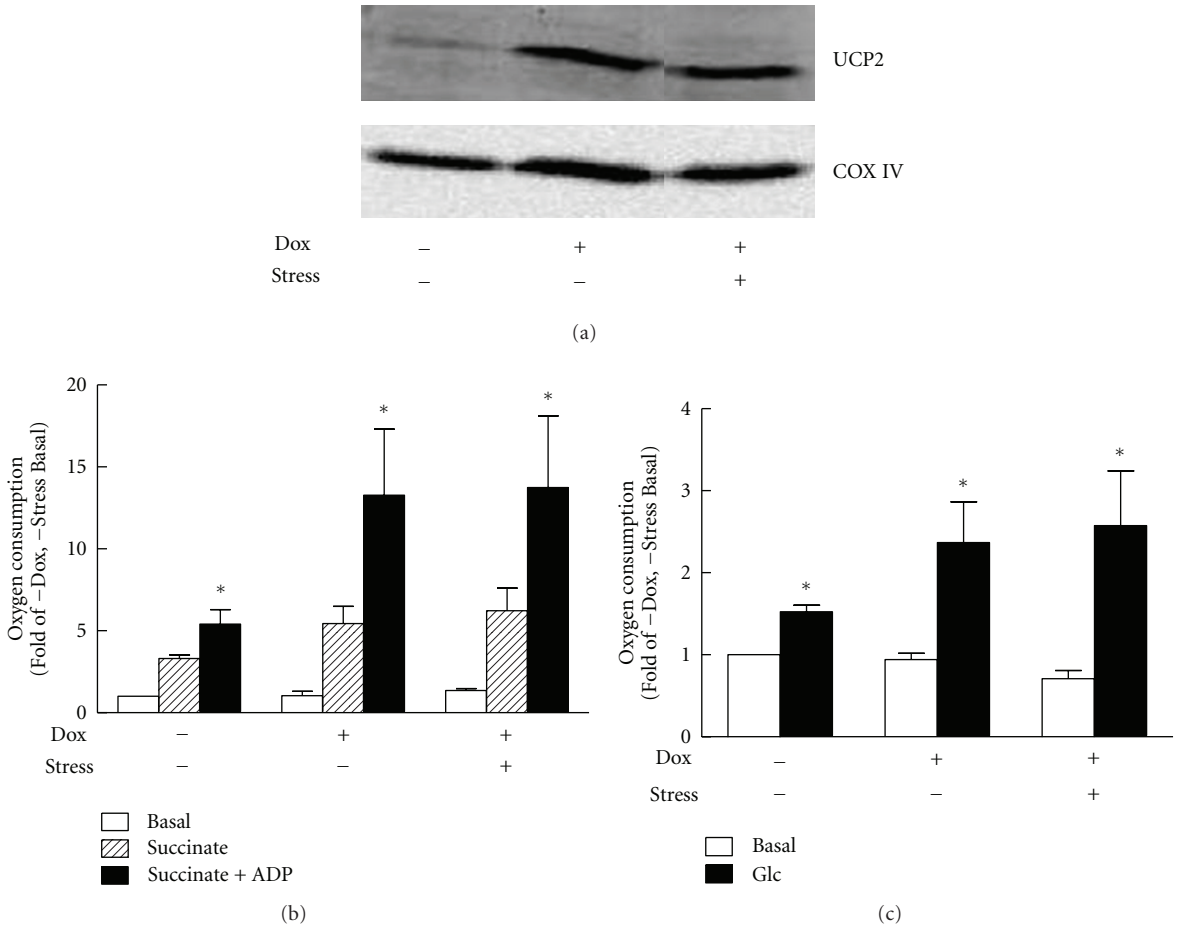

Figure 2: Effects of UCP2 overexpression and oxidative stress on mitochondrial respiration in INS-1 cells. UCP2 was induced in INS-1 cells (hUCP2 INS-1-r9, [3]) by 250 ng/mL doxycycline (+Dox) 2 days before oxidative stress and during stress period. (a) Immunoblotting showing UCP2 protein levels in noninduced (−Dox) *versus* induced (+Dox) INS-1 cells and nonstressed (−stress) *versus* stressed cells (+stress, 200 μM H_2O_2 for 10 min 3 days before analysis). Cytochrome oxidase (COX IV) is shown as control for inner mitochondrial protein. (b) O_2 consumption measured on mitochondria isolated from INS-1 cells 3 days after-stress. Respiration was induced by 5 mM succinate (Succinate) followed by addition of 150 μM ADP (Succinate + ADP). (c) O_2 consumption measured on intact INS-1 cells stimulated by 15 mM glucose (Glc), compared to basal respiration at 2.5 mM glucose (Basal). Data are means ± SE of 3 independent experiments expressed as nmol O_2/min per 100 μg mitochondrial protein (b) or nmol O_2/min per 10^6 cells (c) normalized to basal respiration of controls (no Dox, no stress). $^*P < 0.05$ *versus* basal O_2 consumption of corresponding condition.

free radical theory of aging [69], protective effects depend on mitochondrial ROS formation inducing an adaptive response, in turn conferring increased stress-resistance. This might ultimately give rise to long-term cell preservation. In agreement with this model, calorie restriction extend life span in different organisms by increasing mitochondrial ROS production [70]. In pancreatic β cells, mitohormetic response is suggested by adaptation to dietary fat-induced insulin resistance attributed to increased mitochondrial function [71], an effect correlating with elevated ROS levels secondary to fatty acid treatment of insulin-secreting cells [10].

The nature of mitohormesis in insulin-secreting cells can be studied when cells recover from a single transient exposure to sublethal H_2O_2. We previously reported that INS-1E β cells and rat islets subjected to a 10 min H_2O_2 exposure exhibit impaired secretory response associated with interrupted mitochondrial signals measured right after stress [27]. Loss of mitochondrial function occurs within the first minutes of oxidative stress [27], as revealed by collapse of mitochondrial membrane potential (Figure 1(a)). Monitored concomitantly on the same cells, gradual discontinuous mitochondrial network is observed, eventually exhibiting some globular patterns 60 min after stress (Figure 1(b)). These phenomena were not observed in control nonstressed cells (Figures 1(c) and 1(d)). Then, the question is whether such oxidative stress results in prolonged mitochondrial damages, recovery of cell function, or improved resistance to stress. After a 3-day recovery period following the 10 min stress, we observed increased mitochondrial H_2O_2 formation and persistence of mitochondrial dysfunction altering metabolism-secretion coupling [29]. The ROS-induced endogenous H_2O_2 generation contributes to prolongation of oxidative attacks days after exposure to exogenous H_2O_2.

This is accompanied by increased expression of genes participating to recovery of mitochondrial function, detoxification, and cell survival; such as subunits of mitochondrial electron transport chain complexes and antioxidant enzymes [29]. Mitochondrial defects induced by acute 10 min oxidant exposure are carried on to daughter cells. These cells ultimately achieve gradual turnover of mitochondrial components enabling recovery of their function in the following weeks of culture period [29]. Three weeks after transient oxidant exposure, those insulin-secreting cells respond normally to physiological stimuli. Remarkably, the recovered cells are more resistant than naïve cells to a new exogenous oxidative stress. This beneficial "memory" of mitochondrial oxidative injury represents mitohormetic property and is associated with a higher *UCP2* gene expression 3 weeks post-stress [29], suggesting a protective role for UCP2.

10. Does UCP2 Participate to Mitohormesis in β cell?

As described above, some studies have highlighted UCP2 as a protective element under stress conditions [3, 29, 49, 52], possibly implicated in β cell mitohormetic response. To address this question, INS-1 β cells with doxycycline-inducible overexpression of human UCP2 [3] were challenged with an oxidative stress by exposure to 200 μM H_2O_2 for 10 min as described [27, 29]. Consistent with previous report [3], increased expression of UCP2 (Figure 2(a)) did not alter mitochondrial coupling (Figures 2(b) and 2(c)). Indeed, INS-1 cells with induced UCP2 overexpression exhibited similar respiration upon glucose stimulation compared to non induced cells (Figure 2(c)). Moreover, state 3 respiration measured on isolated mitochondria stimulated with succinate plus ADP was even slightly higher versus controls (Figure 2(b)). INS-1E cells with basal UCP2 expression are highly sensitive to oxidative stress regarding mitochondrial respiration, exhibiting marked reduction of state 3 (−59% versus control nonstressed cells) 3 days after oxidative stress [29]. On the contrary, cells overexpressing UCP2 did not show any impairment of oxygen consumption at day 3 after stress, as shown both on isolated mitochondria and intact cells (Figures 2(b) and 2(c), resp.). Acute oxidant exposure did not further elevate UCP2 protein levels 3 days after stress in UCP2-induced cells (Figure 2(a)). Collectively, these observations support the concept that UCP2 upregulation observed previously as a mitohormetic response [29] can serve as defense mechanism against mitochondrial oxidative damages (Figure 2).

11. Conclusions

In pancreatic β cells, stress-response hormesis can develop under different metabolic insults, such as lipotoxicity, cytokines, or ROS. In particular, oxidative stress induces mitohormesis, rendering mitochondria more resistant to oxidative attacks. Various studies in this field reported conflicting results. However, converging evidence points to UCP2 as a marker of mitohormesis, this protein being upregulated following stress conditions. Moreover, overexpression of UCP2 in naïve cells lacking hormesis adaptation is sufficient to confer resistance to oxidative stress (Figure 2). The exact function of UCP2 is still unknown, although its partial homology with uncoupling UCP1 protein suggests a functional link with the electron transport chain. Whether treatment with UCP2 inducers, such as glutamine [72], could promote mitohormesis and protect β cells under metabolic stress remains to be determined.

Abbreviations

ANT: Adenine nucleotide translocase
CoQ: Coenzyme Q
ER: endoplasmic reticulum
FMN: Flavin mononucleotide
GPX: Glutathione peroxide
GSIS: Glucose-stimulated insulin secretion
NADH: Nicotinamide adenine dinucleotide
NOD: Nonobese diabetic
PPAR: Peroxisome proliferator-activated receptor
RET: Reverse nucleotide transport
ROS: Reactive oxygen species
SOD: Superoxide dismutase
STZ: Streptozotocin
UCP: Uncoupling protein.

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Review Article

Formation and Regulation of Adaptive Response in Nematode *Caenorhabditis elegans*

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All organisms respond to environmental stresses (e.g., heavy metal, heat, UV irradiation, hyperoxia, food limitation, etc.) with coordinated adjustments in order to deal with the consequences and/or injuries caused by the severe stress. The nematode *Caenorhabditis elegans* often exerts adaptive responses if preconditioned with low concentrations of agents or stressor. In *C. elegans*, three types of adaptive responses can be formed: hormesis, cross-adaptation, and dietary restriction. Several factors influence the formation of adaptive responses in nematodes, and some mechanisms can explain their response formation. In particular, antioxidation system, heat-shock proteins, metallothioneins, glutathione, signaling transduction, and metabolic signals may play important roles in regulating the formation of adaptive responses. In this paper, we summarize the published evidence demonstrating that several types of adaptive responses have converged in *C. elegans* and discussed some possible alternative theories explaining the adaptive response control.

1. Introduction

In many organisms, a mild (usually sublethal and/or nonlethal) exposure to a stressor increases resistance to subsequently higher doses of the same or different stressors. This adaptive response phenomenon was put forward in the 1970s–80s [1] and has been well characterized in manifold combinations of stress and model organisms [2–4]. Such adaptive responses can be broadly categorized as hormesis, cross-adaptation, and special adaptive responses. In addition to hormesis, which renders protection against higher doses or concentrations of the same substance, cross-adaptation, which is defined as the capacity of cells or organisms to become resistant to a different lethal agent, usually occurs as well [5, 6]. Dose-response relationship research has been based on the low dose-expose adaptive response leading to adjustments of the series of management or regulations related to environment and poisons, and this research has garnered increasing attention from toxicologists. Presently, it is postulated that oxidative stress is at least the conserved mechanism of adaptive response because of the reactive oxygen species (ROS) pretreatment or mild oxidative stress could increase resistance when organisms are challenged with higher doses or concentrations of that particular agent or stressor [6, 7].

The nematode *Caenorhabditis elegans* has emerged as an important model animal and has been widely used in environmental stress or toxicity assessments because of the features of easy maintenance, short lifespan, and small body size, and the fact that results of trials on *C. elegans* can be predictive of outcomes in higher organisms [8, 9]. In 1995, the adaptive response phenomenon to oxidative stress was discovered in nematode [10]. Since then, three types of adaptation to the response have been found, and related research on its mechanism of formation has been conducted [1, 11, 12].

We describe some of the researches that have been conducted in the areas of adaptive response in *C. elegans*, including the types, formation, mechanisms, and influencing factors responsible for the effects of the phenomenon. We

also suggest further research into *C. elegans* that will complement studies on adaptive response that are being conducted on other model systems.

2. Three Types of Adaptive Response in *C. elegans*

2.1. Hormesis Effect in C. elegans. C. elegans presents a clear adaptive response following exposure to a stressor. Hormesis occurs when a low-level stress elicits adaptive beneficial responses that protect against subsequent severe exposure to the same stress. In 2002, Cypser and Johnson found that pretreatment with heat as well as hyperoxia or juglone (a chemical that generates ROS) pretreatment could significantly increase subsequent resistance to the same challenge [13]. *C. elegans* preexposed to 2.5 μM of metals (Pb, Hg, Cu, and Cr) showed a moderate but significant reduction in the locomotive behavioral (assessed by endpoints of head thrash and body bend) defects that were induced by subsequent exposure to 50 and 100 μM of the same metals [11]. *C. elegans* treated under control conditions (0 mM MeHg) were significantly ($P < 0.05$) more sensitive to subsequent exposure to MeHg than those pretreated with 0.3 or 0.6 mM MeHg [14]. Prolonged exposure to low doses of alcohol induced a multifaceted "withdrawal syndrome," and the observed decreased reversal frequency was most likely result from an adaptation to alcohol caused by inhibition of feeding and a food-deprived behavioral state in *C. elegans* [15]. Shot-term exposure to antipsychotic drugs altered the frequency of turns/reversals off food, whereas drug withdrawal after 24 hr treatment was accompanied by a rebound in the number of turns/reversals in *C. elegans* [16]. Exposure of adult *C. elegans* to hypersmotic conditions protected their offspring from the same, which may be correlated with the changes in the sugar content of adults and embryos [17]. More interestingly, it was reported that nematodes can even adapt to high salt swell and then return to their initial body volume when exposed to low-salt agar [18].

2.2. Cross-Adaptation in C. elegans. Cross adaptation was first observed in *C. elegans* during preexposure of *C. elegans* to oxygen which conferred a protective effect against the lethality imposed by subsequent X-irradiation [1]. Cross-tolerance between juglone and oxygen was further observed by Cypser and Johnson in *C. elegans* [13]. Pretreatment with mild UV irradiation prevented the formation of locomotive behavioral defects and activated a remarkable reduction of the stress response and oxidative damage in 50 and 100 μM metal (Hg, Pb, and Cr) exposed nematodes [5]. Similarly, pre-treatment with UV irradiation suppressed the reproductive toxicity, as assessed by endpoints of blood size and generation time, that is normally induced by the subsequent cadmium exposure in nematodes [4]. Nonlethal stress such as mild heat shock had the beneficial effects on stress resistance and prevented the formation of the neurobehavioral defects and the activation of severe stress responses in lead and mercury exposed nematodes at concentrations of 50 and 100 μM [12].

2.3. Special Adaptive Response in C. elegans. It is proposed that lifespan extension by dietary restriction is an example of hormesis. Animals restricted to a balanced diet, containing as little as 60% of the calories they would consume *ad libitum*, have life expectancies up to 50% greater than controls [19]. Complete caloric deprivation—that is, starvation—can also mimic a hormetic treatment to increase lifespan, and young adult worms deprived of all food for 1–3 days display an extension in mean lifespan of 30–40% [20]. Similarly, glucose restriction extended lifespan by inducing mitochondrial respiration and increasing oxidative stress [21]. Moreover, it was observed that pre-exposure to low doses of plumbagin with toxicity to *C. elegans* by generating free radicals extended the lifespan of animals [22]. Pretreatment with low concentrations of the polyphenol tannic acid (TA) also induced a potent life-prolonging phenotype in *C. elegans* [23]. More interestingly, it was reported that the intermittent fasting (IF), another form of dietary restriction, also effectively entended the lifespan of *C. elegans*, and RHEB-1 regulated the IF in part by the insulin signaling effector DAF-16 [24].

3. Factors Influencing the Response Formation

3.1. Developmental Stage of Nematodes. Adaptation to stress has been observed in young *C. elegans* but not in mature or old individuals [10]. Young nematodes survived by increasing their content of superoxide dismutase (SOD); however, older nematodes did not and hence suffered loss of viability when treated with the quinone plumbagin or with hyperoxia, both of which are expected to increase production of ROS [10]. When exposed to a low concentration of the xenobiotic juglone, young nematodes mounted a robust hormetic stress response and survived a subsequent exposure to a higher concentration of juglone that is normally lethal to naïve animals [25]. Old nematodes, in contrast, were unable to mount this adaptive response [22].

3.2. Length of Pretreatment Duration. Lengths of time of pretreatment have important influences on adaptive response. Oxidative stress pre-treatment conferred a protective effect against the subsequent severe X-irradiation [1]. While the major protection effect was seen at 1 hr, the survival rate behind X-ray irradiation with pre-exposure to 90% of oxygen for 3 hr after oxygen pre-exposure was significantly lower than 2 hr [1]. There were significant decreases in head thrashes and body bend in heat-shock pre-treated nematodes for 1.5 and 2 hr compared to controls, but no changes were formed in 0.5 hr heat-shock pre-treated nematodes [12].

3.3. Pretreated Doses of the Agents. Pre-treatment with UV irradiation prevented the formation of locomotive behavioral defects (assessed by endpoints of head thrash and body bend) in 50 and 100 μM metal (Hg, Pb, and Cr) exposed nematodes [5]. The significant decrease in head thrashes and body bends could be observed in UV irradiated nematodes at 15, 20, and 30 J/m^2/min compared to controls, but no noticeable changes were recorded when pretreated with UV less than 5 J/m^2/min [5]. Similarly, the suppression

of reproductive toxicity could be observed in UV-irradiated nematodes at 15, 20, and 30 J/m^2/min compared to controls, but no obvious alterations were recorded in nematodes treated with UV irradiation less than 5 J/m^2/min [4]. Mild metal (Pb, Hg, Cu, Cr) exposure conferred an increased resistance when nematodes were challenged with higher concentrations of the same metal, but pre-treatment with several metals enhanced the stress response induced by subsequent mental exposure in *C. elegans* [11]. Pre-exposure to 2.5 μM of metals caused a reduction of locomotive behavioral defects and an increase of *hsp-16.2::gfp* expression that was induced by the subsequent 50 and 100 μM of metal exposure; in contrast, the hormesis was further decreased in nematodes that were examined following 50 μM metal pre-treatment [11].

3.4. Length of the Subsequent Treatment Duration. Sensitivities of nematodes to 400 Gy of X-rays at intervals between 0 and 5 hr after pre-exposure to 90% of oxygen for 1 hr were statistically significant [1]. The major beneficial effect was seen at intervals of 1 hr, although a residual protective effect was still apparent even 3 hr after oxygen pre-exposure [1]. The survival rate of nematodes incubated with oxygen pre-exposure for 5 hr before irradiation was even lower than control [1].

3.5. Dose of the Subsequent Treated Agents. Pre-treatment with mild heat shock effectively prevented the formation of the neurobehavioral defects and the activation of severe stress response in metal exposed nematodes at concentrations of 50 and 100 μM, but the heat pre-treatment could not prevent the formation of neurobehavioral defects in 200 μM of metal exposed nematodes [12]. Pre-treatment with UV irradiation prevented the reproductive toxicity (assessed by endpoints of blood size and generation time) caused by Cd in concentration of 50 and 100 μM compared to controls, but no obvious alterations were recorded in the concentration of 150 μM [4]. Similarly, the locomotive behavioral defects induced by 50 and 100 μM of metal (Hg, Pb and Cr) in UV preirradiated nematodes could be suppressed, but no obvious alterations were recorded in nematodes treated with 200 μM of metal [5]. Combined, this information suggests that the adaptive response in *C. elegans* can only be imposed within a certain range of stress or toxicant and is ineffectual when the extent of the challenge is too great.

4. Regulation Mechanisms of Adaptive Response

Hormesis is the induction of beneficial effects by exposure to low doses of harmful chemical or physical agents. We indicate here that the nematode, *C. elegans,* displays broad adaptive response abilities, but the biological mechanisms underlying these abilities have not yet been fully elucidated. Nevertheless, the studies conducted so far show that they may at least involve antioxidant defense system enhancement, stress protein (hsps) induction, signaling pathways modulation, and metabolic regulation.

4.1. Oxidative Stress and Antioxidant Defense System. The resistance to high amounts of ROS is explained by the prominent adaptive responses of the antioxidant defense system, which is of vital importance in the protection against oxidative stress. These effects may be due to increased formation of ROS within the mitochondria, causing an adaptive response that culminates in the subsequently increased stress resistance, which is assumed to ultimately cause a long-term reduction of oxidative stress.

Naphthalene quinone or high oxygen treatment can induce nematodes to produce ROS, and these ROS may cause the activity of peroxidase to increase, thus conferring resistance to nematodes facing subsequent stress treatments [10]. It was further observed that there was a noticeable elevation of SOD and CAT activities and an obvious decrease of oxidative damage in metal-exposed nematodes at high concentrations after mild UV or metal pre-treatment [5, 12]. At the molecular level, the adaptive response was thought to be mediated by the induction of a constellation of genes whose products confer resistance to the damaging agent. *age-1* mutant was characterized by resistance to paraquat, heat and oxygen resistance for superoxide dismutase genes (*sod-1* through *4*), and catalase genes (*clt-1* and *ctl-2)*, known to encode antioxidant enzymes, and expression levels of these genes were elevated in *age-1* young adults during the lifespan [26]. In *C. elegans*, SKN-1 mediates protective responses to oxidative stress, and genetic analysis indicated that *skn-1* activity was required for lifespan extension by low-dose plumbagin [22]. So far, it is believed that the adaptive response is mediated by events that suppress the mitochondrial $O_2^{\cdot -}$ production, and the oxidative stress-inducible hormesis is associated with a reduction of the mitochondrial $O_2^{\cdot -}$ production by activation of the antioxidant system in *C. elegans* [27].

However, some researchers argue the opposite. Yanase et al. observed that the expression of two superoxide dismutase genes—*sod-1* and *sod-3*—was relatively unaffected in hyperoxia pre-exposed *C. elegans*, which suggests that the SOD activity may not play a role in the adaptive response against a specific oxidative stress [1].

4.2. Heat-Shock Proteins (HSPs). Small heat-shock proteins (HSPs) appear of general importance for adaptations because their expression correlates well with the presence of various stressors. Pre-exposure of *C. elegans* to oxygen conferred a protective effect against the lethally imposed X-radiation, probably due to dramatically increased expression of the heat shock protein genes, *hsp-16.1* and *hsp-16.48* [1]. Previous studies in *C. elegans* have demonstrated that HSPs, of the HSP70 family, could be upregulated following exposure to heavy metals [11, 14]. After exposure to 2.5 μM of metals, the induction of *hsp-16.2::gfp* expression, caused by the subsequent 50 and 100 μM of metal exposure, was significantly suppressed [11]. A sublethal exposure to MeHg rendered *C. elegans* resistant to the subsequent exposure to the organometal, showing a potential role of HSP-4 in MeHg-induced hormesis [14]. In *C. elegans,* HSP-16.2 expression was identified as a valuable predictor of the ability to withstand a lethal thermal stress, and its levels correlate well with

the hormetic effects in response to heat treatment [28]. HSP-16 could affect lifespan by reducing oxidative stress through raising the pool of reduced glutathione (GSH) in nematodes [29]. Lifespan extension under moderate oxidative stress was also associated with the increased expression of HSP-16.2 [30, 31].

4.3. Metallothioneins. Metallothioneins (MTs) are small, cysteine-rich metal-binding proteins involved in metal detoxification, homeostasis, and protection from oxidative stress. In *C. elegans, mtl* knockout animals displayed increased sensitivity to MeHg exposure; a slight decrease in baseline activity of *mtl-1* and *mtl-2* was observed when nematodes were further exposed to MeHg [14]. The normal formation of cross-adaptation responses to metal toxicity may need sufficient MTs protein to be available in tissues of nematodes. During the formation of cross-adaptation responses, the induction of *mtl-1* and *mtl-2* promoter activity was sharply increased in 50 or 100 mM of metal exposed nematodes after mild heat-shock treatment compared with those treated with mild heat-shock or metal exposure alone [12]. Moreover, after pre-treatment with mild heat shock, no noticeable increase of locomotive behaviors could be noted in metal (Pb, Hg) exposed *mtl-1* or *mtl-2* mutant strains, and overexpression of MTL-1 and MTL-2 at the L2-larval stage could significantly suppress the adverse effects on locomotive behaviors following metal exposure [12].

4.4. Glutathione (GSH). Sublethal exposure to MeHg rendered *C. elegans* resistant to subsequent exposure to the organometal, and an increase in expressionof *gst-4* gene indicated that *gst-4* gene may be involved in this response: the increase in *gst-4* catalyzes the conjugation of MeHg to GSH, causing GSH and total glutathione levels to decrease [14]. MeHg exposure induced significant decreases in GSH, GSH/GSSG ratio, and total glutathione levels which suggests an increased conjugation of GSH to MeHg, facilitating its elimination from the system [14].

4.5. Signaling Pathways. In *C. elegans*, the insulin/insulin-like growth factor-like signaling (ILS) pathway mediates both intrinsic stress resistance and lifespan [13]. Lifespan extension from stress hormesis by thermal stress and juglone-induced oxidative stress require *daf-16*, a downstream target of the insulin/IGF-1 receptor [22, 30]. Both DAF-16 and SKN-1 signals were required for the adaptation to low concentrations of juglone and plumbagin [22, 25]. Mean lifespan extension by plumbagin was dependent on the activated expression of a *skn-1* target, a transcription factor that promotes antioxidant gene expression in response to oxidative stress [22]. Reduced insulin/IGF-1-like signaling from the DAF-2/ILS receptor increased the nuclear accumulation of SKN-1 and activated a subset of *skn-1* dependent genes (including *gst-4*) independently of DAF-16 [22]. When exposed to a low concentration of the xenobiotic juglone, young nematodes mounted a robust hormetic stress response; however, old nematodes were unable to mount this adaptive response because DAF-16 and SKN-1 were reduced [25]. Extreme hypertonic stress response was also linked to the transcriptional targets of DAF-16-mediated insulin signaling pathway [32]. Moreover, Kim et al. found DAF-16 nuclear accumulation in cells throughout the body and accumulated excess fat after exposure to hypergravity for 3 hr [33].

The protection in oxidative stress resistance appears to be accomplished by small HSPs through a glucose-6-phosphate dehydrogenase-dependent increase in NADPH generation needed to maintain GSH in its reduced form via the GSSG-reductase and by using this redox modulator as an essential cofactor of their in vivo chaperone activity against oxidized proteins [28]. This function in stress resistance and longevity is further embedded into the insulin/IGF-1 signaling pathway that has been shown in several studies to be a central determinant of lifespan in *C. elegans* [28].

In low concentration of polyphenol tannic acid (TA) exposed *C. elegans*, accompanied with the potent life-prolonging properties, enhanced thermal stress resistance, reduced growth, and slightly increased oxidative stress resistance, the mitogen-activated protein kinase kinase SEK-1 (SAPK/ERK kinase) played a key role in the formation of hormesis effect [23]. *hif-1* gene encoding a bHLH-PAS protein was required for adaptation to hypoxia because the majority of *hif-1*-defective nematodes died in the condition of 1% oxygen although the wild-type nematodes can survive and reproduce in these conditions [34]. *egl-3* gene encoding a neuropeptide was required for adaptive response to alcohol exposure because a mutation deficient in *egl-3* was resistant to the withdrawal behavior caused by alcohol exposure [15]. MDT-15 integrated several transcriptional regulatory pathways to monitor both the availability and quality of ingested materials and abrogates induction of specific detoxification genes in response to certain xenobiotics or heavy metals, thus rendering nematodes hypersensitive to toxin exposure [35].

For the adaptive response under the hyperosmotic environments, Solomon et al. suggest that OSR-1 plays a central role in integrating stress detection and adaptation responses by invoking multiple signaling pathways to promote survival under hyperosmotic environments [36]. Genetic epistasis analysis indicated that OSR-1 regulated survival under osmotic stress via a conserved p38 MAP kinase signaling cascade and regulates osmotic avoidance and resistance to acute dehydration likely by distinct mechanisms [36].

4.6. Metabolic Signals. Minois has proposed an alternative description wherein hormesis is seen as a consequence of metabolic regulation coupled to the expression of stress response proteins [37]. Nematodes are able to survive well on agar containing up to 500 mM NaCl after being grown on 200 mM NaCl for 2 weeks. The analysis demonstrated that expression levels of glycerol 3-phosphate dehydrogenase, an enzyme that is rate limiting for hypertonicity-induced glycerol synthesis, increased 15- to 20-fold when grown on 200 mM NaCl agar [18]. Schulz et al. found that the impaired glucose metabolism (glucose restriction) extended life expectancy by promoting mitochondrial metabolism, causing increased ROS formation [21]. The adaptation to hyperosmotic conditions was correlated to changes in the

sugar content of adults and embryo, and mutations in genes products which alter sugar homeostasis altered the ability of embryos to survive in hyperosmotic conditions in the adaptive parental effect [17].

4.7. RNA Interference. The recent research further indicated that elimination of RNA interference by silencing key enzymes in microRNA biogenesis, *dcr-1* or *pash-1*, restored the diminished intrinsic thermotolerance of aged and H_2O_2-elimination compromised (catalase-2 and peroxiredoxin-2 deficient) nematodes, which uncovers a novel posttranscriptional element in the regulation of heat stress adaptation under oxidative conditions [38].

5. Conclusions

From studies to date, three forms of adaptive response have been found in *C. elegans*: two basic forms response (hormesis and cross adaptation) and one special adaptive response. Compared with research on hormesis, only limited studies relating to cross-adaptation in nematodes have been conducted. Consequently, in-depth investigations are needed to decipher the widespread adaptive response to the same challenge, which will be an important prerequisite for cautious regulation mechanisms of corresponding research. For cross adaptation, it is recommended that joint exposure to the toxin be combined in order to fully analyze the system of possible interactions between coercion or toxins and stress or poison. And it is still controversial whether dietary restriction is an example of an adaptive response. Nevertheless, dietary restriction, or starvation, and aging factors have a central role in reducing the phenomenon of adaptive response, and lifespan extension does exist when exerted in *C. elegans* chronologically.

Factors influencing the formation of adaptive responses are focused on three aspects: the time lengths and doses of pre-exposure agents or stress; the time lengths and doses of subsequent challenges; the sensitivity of *C. elegans* to the challenge. All these data reveal that degree is the crucial impact factor, regardless of previous or subsequent coercion or poison exposure, as well as the sensitivity to the challenge.

Adaptive response formation could be suppressed because time of pre-exposure is too long, doses too high, or sensitivity to agents is too great. Conversely, stress or poison processing cannot produce toxic or animals for the corresponding stress or poison processing is not sensitive (such as mature adult larvae relative to stress or poison to deal with not sensitive) also difficult to form the response. Similarly, if the length of pre-exposure time is too long, doses too high or sensitivity to the subsequent exposure agents too great, the formation of adaptive response could be suppressed. In contrast to pre-treatment, more robust responses tend to be mounted if animals have corresponding moderate resistance to the subsequent stress agents. With continued research, more factors related to pre- or subsequent stress, poison processing, and animal sensitivity will be discovered. Additionally, the pre- and subsequent is just a relative processing, not on the strict development of larva or adult age, and the period of choice between laboratories is not consistent.

As for the illustration of the intricacies of regulatory mechanisms of environmental stresses, antioxidant stress systems, heat shock proteins, metallothionein and nucleoside of GSH, signaling pathways, and metabolic regulation have been shown to be involved in response formation, but in-depth investigations are needed to decipher the underlying mechanisms. In view of antioxidant stress systems, heat shock proteins, metallothionein and nucleoside of GSH corresponding to the self-protection system, a very important question arises: what function do the other genes serve in response formation? That is, in addition to the animal's self-protection system, are there other molecules or genetic mechanisms used to regulate the formation of adaptive responses? Characterized by a rich background of research in genetics and developmental biology, the *C. elegans* model provides the most detailed and comprehensive information about the biochemistry and molecular biology involved in adaptive response. The nematode model also allows for suggestions of additional mechanisms that could be fundamental principles of adaptive response and gives us many new roads of exploration to determine the common and disparate mechanisms that underlie the many forms of adaptive response.

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Review Article

Regulation of ROS Production and Vascular Function by Carbon Monoxide

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Carbon monoxide (CO) is a gaseous molecule produced from heme by heme oxygenase (HO). CO interacts with reduced iron of heme-containing proteins, leading to its involvement in various cellular events via its production of mitochondrial reactive oxygen species (ROS). CO-mediated ROS production initiates intracellular signal events, which regulate the expression of adaptive genes implicated in oxidative stress and functions as signaling molecule for promoting vascular functions, including angiogenesis and mitochondrial biogenesis. Therefore, CO generated either by exogenous delivery or by HO activity can be fundamentally involved in regulating mitochondria-mediated redox cascades for adaptive gene expression and improving blood circulation (i.e., O_2 delivery) via neovascularization, leading to the regulation of mitochondrial energy metabolism. This paper will highlight the biological effects of CO on ROS generation and cellular redox changes involved in mitochondrial metabolism and angiogenesis. Moreover, cellular mechanisms by which CO is exploited for disease prevention and therapeutic applications will also be discussed.

1. Overview

Similar to nitric oxide (NO), produced by a family of nitric oxide synthase (NOS), CO is also endogenously generated from heme by the catalytic reaction of HO. To date, three isoforms of HO (e.g., HO-1, HO-2 and HO-3) have been identified [1]. The isoforms are produced by different genes and show different tissue distributions and molecular properties. HO-1, first identified as a stress protein (32-kDa), may be induced in response to a broad spectrum of stimuli, including oxidants, heavy metals, NO, and immune stimulants [2, 3]. The second isoform HO-2, a 36-kDa protein, is constitutively expressed and present in high levels in the brain and testes [4]. Although not expressed in humans, the third isoform identified, HO-3 (33-kDa), is highly homologous to HO-2 but possesses significantly lower catalytic activity. HO-3 is considered as a pseudogene derived from HO-2. HO proteins can be localized in several subcellular compartments. Rat liver HO was first identified to be integrated into microsomes by embedding its C-terminal portion into membranes [5, 6]. HO-1 and HO-2 were also found in the endoplasmic reticulum (ER) with an insertion sequence at the C-terminal region, while the rest of the protein is cytoplasmic [7–9]. HO-1 can be proteolytically cleaved from the ER and translocated to the nucleus after exposure to hemin or hypoxia, and this localization is linked to the transcriptional upregulation of cytoprotective genes [10]. HO-1 can also be translocated into mitochondria and is attributed as a novel cytoprotective mechanism against mitochondrial oxidative stress [11]. Interestingly, HO-1 is localized in plasma membrane caveolae when endothelial cells were exposed to stimuli, such as hemin, LPS, or hypoxia, in which caveolin-1 interacts with and modulates HO-1 activity [12], suggesting that caveolin-1 may act as an important regulatory molecule in CO-mediated signaling in endothelial cells. In the human body, both HO-1 and HO-2 catalyze three successive monooxygenation steps to convert intracellular heme to CO, biliverdin, and free iron

(Figure 1(a)). Biliverdin is subsequently metabolized to bilirubin by biliverdin reductase, and the free iron induces ferritin synthesis. Thus, the predominant endogenous source of CO arises from oxidative degradation of heme by HO (Figure 1(a)). Although the majority of heme is derived from senescing red blood cells and ineffective erythropoiesis, a small fraction arises from the degradation of other heme proteins, such as myoglobin, catalase, peroxidases, and cytochromes [13]. Under pathological conditions, additional CO is produced via lipid peroxidation [14] and the metabolic activity of intestinal bacteria [15].

To adapt to challenges by unfavorable environmental conditions, living cells have evolved networks of different responses that control diverse forms of stimuli. One of the key adaptive responses is Nrf2-mediated transcriptional upregulation of HO-1. CO production by HO-1 not only influences O_2 consumption during mitochondrial respiration by acting as a reversible inhibitor of cytochrome *c* oxidase in the electron transfer chain and as a guardian of cellular energy homeostasis, but also enhances O_2 delivery by stimulating angiogenesis [16]. Moreover, CO produces superoxide and H_2O_2 from mitochondrial complex III by binding to cytochrome a, a_3 (complex IV) of the electron transport chain, thus, triggering the activation of redox-sensitive signaling pathways for cytoprotection and energy balance. Therefore, CO is increasingly accepted as a cytoprotective and homeostatic molecule with important signaling capabilities in physiological and pathological situations.

It has been proposed that cytoprotective roles of the HO-1/CO system are initiated by a series of molecular reactions or interactions, or both in response to changes in redox states of the cell [13]. The earliest event in cells and tissues exposed to a low concentration of CO is the rapid production of ROS (i.e., superoxide, H_2O_2, and hydroxyl radical). Accumulating evidence suggests that endogenous ROS stimulates cell proliferation, migration and tube formation, which are typical properties of angiogenesis. Moreover, these angiogenic effects can be regulated by endogenous antioxidant enzymes such as superoxide dismutase (SOD) and thioredoxin [17]. It is known that ROS modulates new blood vessel formation via regulation of various angiogenic factors such as hypoxia-inducible factor (HIF)-1α and vascular endothelial growth factor (VEGF) [18]. As described above, CO can promote ROS production from mitochondria by inhibiting complex IV. These results suggest that CO can stimulate angiogenesis by elevating intracellular ROS levels. In addition, CO synthesized from the catalytic reaction of HO-1 induces the production of proangiogenic factors, such as VEGF, interleukin (IL)-8, and stromal-derived factor (SDF)-1 and decreases antiangiogenic mediators such as soluble VEGF receptor-1 (sFlt-1) and soluble endoglin (sEng). Consequently, these events lead to the promotion of endothelial cell proliferation, migration, and antiapoptotic responses [19–21]. In addition to CO, other endogenous products can play an obligatory role in HO-mediated regulation of vascular function, including angiogenesis. Biliverdin stimulates the induction of proangiogenic factors, such as VEGF and IL-8 in human keratinocytes [22]. Although, ferrous iron has not been shown to induce protein levels of HIF-1α and VEGF, this metal ion increases the synthesis of ferritin synthesis, which promotes angiogenesis by inhibiting the antiangiogenic activity of cleaved high-molecular-weight kininogen (HKa) [23] (Figure 1(b)).

Cells require O_2 for energy metabolism, and modulators of mitochondrial oxidative metabolism can stimulate the supply of O_2 and nutrients by improving blood circulation via angiogenesis. CO stimulates angiogenesis via production of intracellular ROS and angiogenic factors, but also stimulates mitochondrial biogenesis via induction of metabolic modulators such as peroxisome-proliferator-activated receptor-γ (PPAR-γ) and PPAR-γ coactivator-1α (PGC-1α) [24, 25]. Inhibition of CO-induced ROS by antioxidant enzymes (SOD and catalase), antimycin A (an inhibitor of complex III of the electron transport chain), or other antioxidants abrogates CO-induced PPAR-γ expression [24]. In addition, CO elicits a mild oxidative stress response that stimulates mitochondrial energy metabolism, PGC-1α protein expression, and mitochondrial DNA copy number [26]. These observations support the idea that CO ameliorates cellular energetics and stimulates mitochondrial biogenesis via a modest mitochondrial oxidative stress.

The HO-1/CO system has received considerable attention as a target for the development of effective therapeutic interventions against mitochondrial energy metabolism and degenerative vascular diseases, which are directly associated with cellular ROS generation. This paper will highlight the biological effects of CO on energy metabolism and angiogenesis in terms of redox states and discuss mechanisms by which this gas is currently exploited for disease prevention and therapeutic applications.

2. Physiological Significance of CO on Redox Control

Proximal targets for CO include those on or near the cellular surface, such as soluble guanylyl cyclase (sGC), heme-containing potassium channels, NOS, and NADPH oxidase (Nox). More distal cellular targets include mitochondria and heme-containing transcription factors such as BACH1 or NPAS2. Although CO can activate sGC to produce the cellular second messenger cGMP [27], it predominantly inhibits the activity of hemeproteins, such as Nox, to modulate the production of superoxide in tumor-necrosis-factor-(TNF-)α-stimulated endothelial cells (ECs) [28]. CO is known to inhibit O_2 consumption by inhibiting mitochondrial cytochrome c oxidase (COX), which is a terminal electron acceptor of the electron transport chain [29, 30]. Inhibition of COX by CO suppresses oxidative phosphorylation and reduces ATP production. Simultaneously, suppression of oxidative phosphorylation alters the redox state of the electron transport chain and produces ROS (e.g., superoxide and H_2O_2). Although superoxide and H_2O_2 are generally considered to be toxic byproducts of respiration, recent evidence suggests that ROS is an important modulator of eukaryotic signal transduction regulating biological processes as diverse as adaptation for oxidative stress, immune activation, and vascular remodeling in mammals [13, 31].

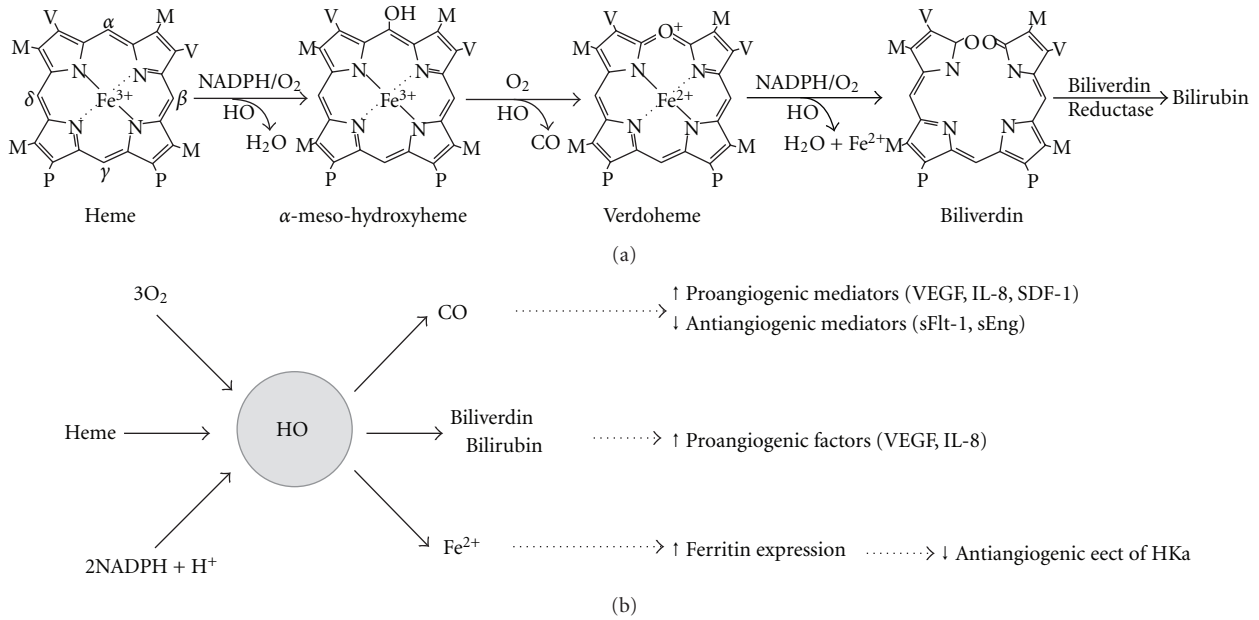

Figure 1: The heme degradation pathway and roles of its byproducts in the production of angiogenic modulators. (a) Reaction intermediates in the heme oxygenase-catalyzed oxidation of heme to biliverdin. The substituents on porphyrin are vinyl (V), methyl (M), and propionate (P). The α-, β-, γ-, and δ-meso positions are labeled. The HO reaction consists of three oxidation steps and initiates with the formation of the Fe^{3+} heme-HO complex. Next, Fe^{3+} heme is reduced to the Fe^{2+} state by the electron donated from NADPH, and this step produces CO by the region-specific cleavage of the porphyrin ring of heme at the *α-meso* carbon atom. The final step is O_2 binding to verdoheme, which produces Fe^{2+} and biliverdin. Biliverdin is converted by biliverdin reductase to bilirubin. (b) Potential proangiogenic effects of heme-degraded products such as CO, biliverdin/bilirubin and Fe^{2+}. These products possess potential proangiogenic effects by inducing proangiogenic mediators or by antagonizing antiangiogenic factors.

O_2-sensing enzyme systems in ECs, including COX, NADPH oxidase, and endothelial nitric oxide synthase (eNOS) are important targets of ROS generation in vessels (Figure 2).

2.1. Effects of CO on Mitochondrial ROS. ROS induced by HO-1-mediated CO production could be predominantly generated from mitochondria via inhibition of COX activity. Mitochondrial ROS production is modulated largely by the rate of electron flow through respiratory chain complexes. Mitochondria contain three classes of cytochromes, denoted a, b, and c, which are potential CO targets. CO binds to the reduced heme iron of COX, with a Ki value of 0.3 μM, resulting in the formation of cytochrome a, a_3-CO complex, and its binding to COX is reversible and competitive with O_2. CO derived from HO-1 inhibits mitochondrial respiration by 12% under 20% O_2, but by 70% under 1% O_2 (reviewed in [13]). The primary effect of CO on mitochondria is to retard the rate of electron transport, thus enabling electrons to accumulate in the cytochrome *bc1* region of complex III in the electron chain. In turn, electrons can be donated to molecular oxygen to generate superoxide and thereby increase the amount of the ROS oxidants in the cell to drive subsequent cellular redox signaling events (reviewed in [32]) (Figure 2).

Cells exposed to CO increase ROS generation, which is inhibited by antimycin A [33] and depletion of mitochondria [21], further confirming the implication of mitochondria as the source of CO-mediated ROS production. It has been also demonstrated that endogenous CO increases the reduction state of cytochrome *bc1* of the respiratory chain in an animal model [25]. Treatment with exogenous CO-releasing compound CORM-2 stimulates platelet-derived-growth-factor-(PDGF-) induced ROS generation and inhibits vascular smooth muscle cell (VSMC) proliferation, which are reversed by the antioxidant *N*-acetylcysteine [34]. Authentic CO gas protects against lung ischemia-reperfusion injury via an increase in the activity of antioxidant enzymes such as MnSOD (SOD2) and glutathione by mitochondrial ROS generation [21]. These results implicate CO in physiological and pathological consequences of mitochondria-dependent ROS generation and redox signal cascades. Furthermore, regulation of mitochondrial function is critical for the control of oxidative stress and regulation of gene expression, especially in view of the fact that HO-1-deficient individuals are afflicted with excessive and persistent oxidant-mediated damage [35, 36]. These observations suggest that CO regulates mitochondrial function to generate ROS, which is responsible for controlling cellular redox states and adaptive responses for oxidative stress.

2.2. Effects of CO on NADPH Oxidase. CO can also regulate ROS generation from cytoplasmic membrane-bound hemoproteins, including NADPH oxidase. In phagocytic cells, NADPH oxidases consist of membrane-bound cytochrome b_{558}, comprising the catalytic $gp91^{phox}$ and the $p22^{phox}$ subunits, cytosolic regulatory components including $p47^{phox}$,

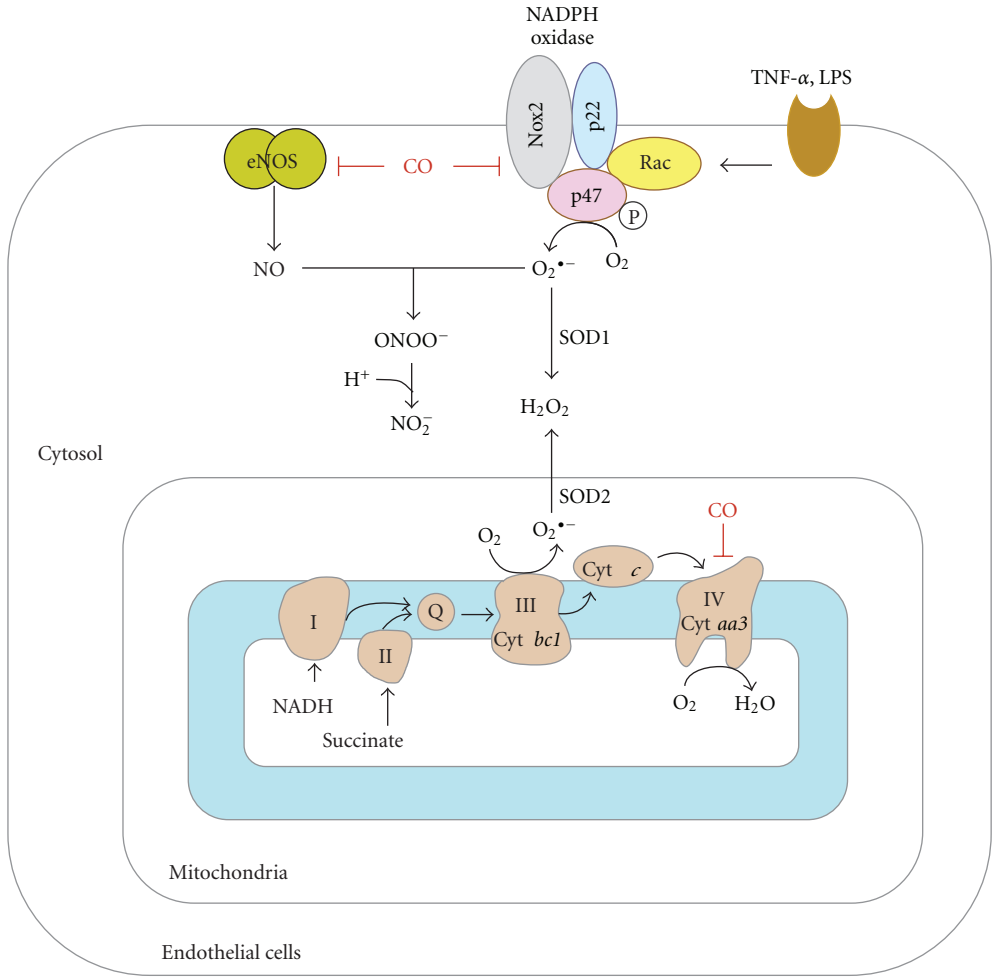

Figure 2: Effects of CO on targets of ROS generation in endothelial cells. CO binds to cytochrome c oxidase (COX), which is a terminal electron acceptor (complex IV) of the electron transport chain, which changes the redox state of the electron transport chain and produces ROS in mitochondria. CO-dependent mitochondrial superoxide is converted to H_2O_2 by SOD2 (Mn-SOD). Stimulation with TNF-α and LPS induces the recruitment of Nox2, $p47^{phox}$, and Rac1 into lipid rafts, thereby promoting Nox activation and ROS production. Superoxide interacts with eNOS-derived NO to produce peroxynitrite, which contributes to endothelial dysfunction. Binding of CO to the heme moiety of Nox and eNOS inhibits production of superoxide and NO, respectively.

$p67^{phox}$, and the small Rho GTPase Rac1 (reviewed in [37]). Several homologues along with $gp91^{phox}$ (also termed Nox2) are designated the Nox family of NADPH oxidases to include Nox1 to Nox5, Duox1 and Duox2, which differ in catalytic activity, response to stimuli, and type of ROS released [38]. The NADPH oxidase isoforms Nox1, Nox3, Nox4, Nox5 (only human), and Duox1/2 are expressed in nonphagocytic cells. Nox1, Nox2, Nox4, and Nox5 (only human) are expressed in ECs, whereas Nox2 and Nox4 are found in stem/progenitor cells. ECs express the entire phagocytic NADPH oxidase subunits such as Nox1, Nox2, Nox4, and Nox5, as well as $p22^{phox}$, Rac1, and $p47^{phox}$. These Nox enzymes are major source of ROS generation in ECs stimulated with angiogenic factors, including VEGF and angiopoietin-1. Subsequent increases in cell proliferation and migration suggest that Nox homologues expressed in EC play an important role in angiogenesis (reviewed in [37]). Binding of CO to the heme moiety of Nox decreases ROS accumulation, and recent reports demonstrate that the protective effects of CO seen in macrophages, ECs, and VSMCs are attributed to a decrease in ROS generation via inhibition of catalytic activity of activated Nox (reviewed in [32]). The plasma membrane-associated $gp91^{phox}$ subunit is a flavohemoprotein containing one FAD and two hemes that catalyze the NADPH-dependent reduction of oxygen to form superoxide in LPS-treated macrophages [39]. The toll-like receptor 4 (TLR4) signaling pathway, responsible for inflammatory gene expression, is negatively regulated by CO derived from HO-1. CO functions are to block the translocation of TLR4 to lipid rafts via suppression of Nox-mediated ROS production in LPS-stimulated macrophages [39]. Interestingly, CO produced by

the CO-releasing molecule CORM-A1 and endogenous HO-1 induction protects cerebral microvascular ECs from TNF-α-induced apoptosis by inhibiting Nox4-dependent ROS production [28]. In airway VSMCs, the CO donor CORM-2 decreases VSMC proliferation by inhibiting ERK1/2 MAPK phosphorylation and cyclin D1 expression via suppression of ROS generation from Nox [34]. Therefore, CO can play an important role in various cell functions, such as EC survival, VSMC proliferation, and angiogenesis by decreasing ROS generation via interaction with the heme iron of Nox.

Despite the differential effects of CO on mitochondria and Nox enzymes, crosstalk between mitochondrial and Nox-induced ROS has been suggested. Recent reports demonstrate that ROS production by hypoxic mitochondria induces Nox1 expression to amplify ROS generation [40, 41], thus, resulting in an elevation of cytosolic calcium to induce contraction in pulmonary artery VSMCs [41]. Does CO-induced ROS generation in mitochondria influence NADPH oxidase activity, resulting in the amplification of ROS? Lamon et al. [42] demonstrated that both authentic CO and the exogenous CO donor CORM-3 induces vasoconstriction in isolated interlobular arteries via mitochondrial production of superoxide, which is implicated in pathways associated with decreased NO bioavailability via direct interaction with NO as well as reduced cGMP production via oxidation of the heme moiety of sGC. These events were reversed by pharmacological inhibitors of multiple oxidases, including NADPH oxidase, resulting in the conversion of function of CO from constrictor to dilator [42]. Similarly, the antioxidants, biliverdin and bilirubin, can inhibit CO-induced superoxide production and vasoconstriction, allowing for a vasodilatory response to CO. These results suggest that CO can regulate vascular relaxation and constriction in a ROS-dependent manner.

2.3. Effects of CO on NOS. NOS, a heme-containing enzyme, predominantly produces not only NO from L-arginine and NADPH, but also superoxide from NADPH in the absence of L-arginine. NO rapidly reacts with O_2^- to produce peroxynitrite ($ONOO^-$), a highly reactive nitrogen species that might elicit harmful effects to cells and could be removed via glutathione [43]. The contribution of CO to the regulation of NO is Janus-faced since low levels of CO increase steady-state levels of NO, without altering NOS activity, by competing against heme moieties, which are alternative targets or scavenging molecules for NO [44]. However, higher levels of CO inhibit NO synthase [45], indicating that the relationship between CO and NO is complex, dynamic, and adaptable. Thus, a high concentration of CO can inhibit NO synthesis and/or actions, interfering with vasodilatory mechanisms mediated by NO. It suggests that there are several mechanisms by which the HO/CO system might modulate NO-dependent biological events (reviewed in [13]). A first feasible mechanism is that HO can downregulate catalytic activities of hemoproteins, including NOS, via oxidative degradation of heme [46, 47]. A second possibility is related to substrate availability. Since both HO and NOS enzymes use NADPH as a reducing equivalent and molecular O_2 as cosubstrates, both enzyme activities under normal conditions may depend on their competition for these substrates. Thus, both enzymes may regulate each other's activity through reciprocal competition with these substrates. A third potential mechanism is associated with a key observation which indicates that NOS activity is directly inhibited by CO *in vitro* [48]. This observation suggests that an inhibitory function of CO on NO generation appears to be mediated by CO binding to the prosthetic heme of NOS (Figure 2). A high concentration of CO, as much as 1 mM, should be available to generate this inhibition. However, CO concentrations in tissues have been reported to be only on the order of $\sim 1\,\mu$M, significantly lower than the required concentration of CO for the inhibition. Consequently, the reciprocal regulatory mechanism between HO/CO pathway and NOS/NO axis *in vivo* relies on local concentrations of CO, which may be significantly higher than global averages would suggest.

CO has been shown to function as both a vasoconstrictor and vasodilator. While the majority of data supports a prodilatory role for CO in the liver where endogenous NO production appears low [49], evidence for vasoconstrictor effects of CO under sufficient amounts of NO has also emerged [50]. High levels of CO can act as a negative regulator of eNOS activity by interacting with its catalytic heme moiety in ECs, leading to suppression of NO production and elevation of vasoconstriction [45, 51]. Specific overexpression of HO-1 in VSMCs exhibits systemic hypertension through attenuation of NO-induced vasodilation [50]. Lamon et al. [42] showed that inhibition of NOS with L-NAME reduces CO-induced elevation in superoxide levels, consequently inducing vasodilation. Since ROS is implicated in pathways associated with direct vasoconstriction as well as impairment of vasodilation, CO produced in the vasculature may promote vasoconstriction in a ROS-dependent manner. The result showing that antioxidants can convert a role of CO from a vasoconstrictor to vasodilator role [42] supports this idea.

Although CO can function as vasoconstrictor, as described above, exogenous CO was first shown to dilate rat coronary arteries and proposed to act in a manner similar to NO, that is, via activation of sGC to produce cGMP [52, 53]. Although CO binds sGC with 30–100-fold less potency than NO, this gas is able to efficiently activate sGC in physiological settings. Apparent discrepancy, as to whether or not CO functions as a vasodilator or vasoconstrictor, is thought to depend on intracellular redox potential of vascular ECs exposed to CO. There are two potential regulatory systems for controlling vascular tone in CO-exposed endothelium, which simultaneously produce both CO-mediated ROS and eNOS-dependent NO. Firstly, ECs containing high levels of redox potential decrease intracellular levels of ROS, particularly superoxide as a NO scavenger and increase cGMP production from sGC by NO and CO, leading to promotion of vasorelaxation. Secondly, cells with low antioxidant levels lead to an increase in intracellular ROS accumulation and a decrease in NO bioavailability, allowing for vasoconstriction. Therefore, differential effects of CO on the regulation of vascular tone depend on intracellular

levels of ROS from mitochondria and Nox. Furthermore, mitochondrial ROS generated by CO can be involved in the activation of other oxidases, further increasing intracellular ROS level and redox-mediated signaling pathways. Therefore, vasoregulatory mechanisms associated with CO rely on the relationship between CO and ROS generation.

NO and related reactive nitrogen species are among the most potent inducers of the HO-1 gene [54]. NO applied as a gas or released from chemical donor compounds markedly induces HO-1 activity through transcriptional and posttranscriptional regulation [54], resulting in CO-mediated cytoprotective effects. Interestingly, exposure of hepatocytes to exogenous CO resulted in a rapid induction of iNOS expression via nuclear factor-κB (NF-κB) activation and a subsequent increase in NO production, which is required for the protective effect of CO. Conversely, the protective effect of NO is required for CO production via up-regulation of HO-1 expression [55]. These results indicate that both NOS/NO and HO-1/CO pathways are involved in protective effects and vascular function via synergistic interaction between these two pathways.

To summarize, although NO acts as a principal vasodilator, NO alone does not solely dominate the control of vascular tone. CO derived from HO-1 may play dual functions in vasoregulation by activating sGC similar to NO (vasodilation) or by promoting direct inhibition of eNOS activity and ROS generation (vasoconstriction). This differential effect can be generated depending on local concentrations of CO [56]. In addition, reciprocal crosstalk between NOS/NO and HO-1/CO pathways are required for the cytoprotective effect showing an essential synergy between these two molecules in tandem. Organs and cells are specialized in their ability to produce different gases (i.e., CO and NO) and modulate their diverse signals via ROS generation at different rate. As such, this would imply a critical role for CO, NO, and ROS in maintaining vascular tone as well as cytoprotective activity.

3. Physiological Significance of CO on Angiogenesis

Oxygen homeostasis is of critical importance for maintaining the viability of all tissues. Lack of sufficient tissue oxygenation is predominantly caused by impaired blood flow. Hypoxia or ischemia results in the upregulation of HIF-1α, which is an important O_2 sensor. HIF-1α regulates several stages of vessel formation, ranging from EC fate decisions to vasculogenesis and angiogenesis [56]. Recently, we reported that overexpression of HO-1 stabilizes HIF-1α protein in astrocytes cells, leading to upregulation of VEGF expression [57]. HIF-1α can also activate the transcription of SDF-1 (also known as CXCL12) in ECs, resulting in increases in the adhesion, migration, and homing of circulating CXCR4- (a cognate receptor for SDF-1) positive progenitor cells to ischemic tissue [58]. Thus, the HO-1/CO pathway promotes angiogenesis by increasing HIF-1α-mediated proangiogenic factors.

Genetic and gene transfer studies have shed light on the distinct roles of HO-1 in angiogenesis. Mice lacking the functional HO-1 gene are embryonically lethal in >95% of all fertilizations, suggesting that the HO-1/CO axis may play an important role in prenatal angiogenesis [35, 59]. In fact, HO-1 knockout mice showed lower basal and H_2O_2-induced production of the strong proangiogenic factor VEGF [60]. VEGF can elevate HO-1 expression and activity in ECs and vice versa, and VEGF-induced angiogenesis is inhibited by the HO-1 antagonist [61], suggesting a positive-feedback loop between VEGF and the HO-1/CO pathway for synergistic promotion of angiogenesis. HO-derived CO contributes to angiogenesis by increasing the synthesis of VEGF, HO-1 and SDF-1 and potentiating their effects on ECs [62–64]. CO also induces VEGF synthesis in the EC-surrounding cells such as VSMCs, macrophages, and astrocytes [22, 57, 65]. The systemic effect of CO on VEGF expression was shown by Marti and Risau [66], who demonstrated that animals kept for 6 h in an atmosphere containing 0.1% CO exhibited significant induction of VEGF and VEGF receptors in various organs. These pieces of evidence implicate a link between HO-1/CO, HIF-1α, VEGF, and SDF-1 for the promotion of angiogenesis.

3.1. HO-1/CO-Induced VEGF Expression via HIF-1α. Angiogenesis is an adaptive response to hypoxia under the master command of HIF. HIF-1α is a potent inducer of VEGF, and VEGF is a pivotal mediator of vasculogenesis, angiogenesis, and vascular permeability. Mitochondrial ROS may diffuse to the cytosol where it activates a variety of signaling pathways, resulting in divergent biological responses such as the activation of HIF-α. All three isoforms, HIF-1, HIF-2, and HIF-3 are composed of two subunits: HIF-α and HIF-β. Since the first discovery of HIF-1 in 1992, a variety of O_2-sensing genes encoding proteins that mediate cell survival, metabolism, and angiogenesis have been identified to be regulated by HIF-1α [56]. A critical regulatory mechanism for HIF-1α stability is mainly associated with its rapid ubiquitin-mediated proteosomal degradation. In the presence of oxygen, proline hydroxylation of HIF-1α by prolyl hydroxylase (PHD) activity is a critical step for regulating its protein level. PHD belongs to an α-ketoglutarate (2-oxoglutarate)-dependent dioxygenase superfamily [67], which uses O_2 as a cosubstrate to add a hydroxyl group to specific proline residues at 402 and 564 within the oxygen-dependent degradation domain of HIF-1α [68] (Figure 3). This enzyme requires Fe^{2+} to assemble into its active conformation, and the oxidation of Fe^{2+} to Fe^{3+} abolishes the catalytic activity of PHD [69]. Therefore, oxidizing agents, including ROS, inhibit PHD activity via oxidation of iron, while reductants or antioxidants, such as ascorbate and cysteine, reduce the Fe^{3+} back to Fe^{2+} in order for the enzyme to be recycled [70, 71]. Therefore, cellular redox potential can affect PHD activity and HIF-1α-dependent VEGF expression (Figure 3).

Treatments of cells with exogenous H_2O_2 and growth factors, which induce H_2O_2 production are sufficient to stabilize HIF-1α under normoxic condition [71]. The cells deficient in junD, which is responsible for antioxidant gene

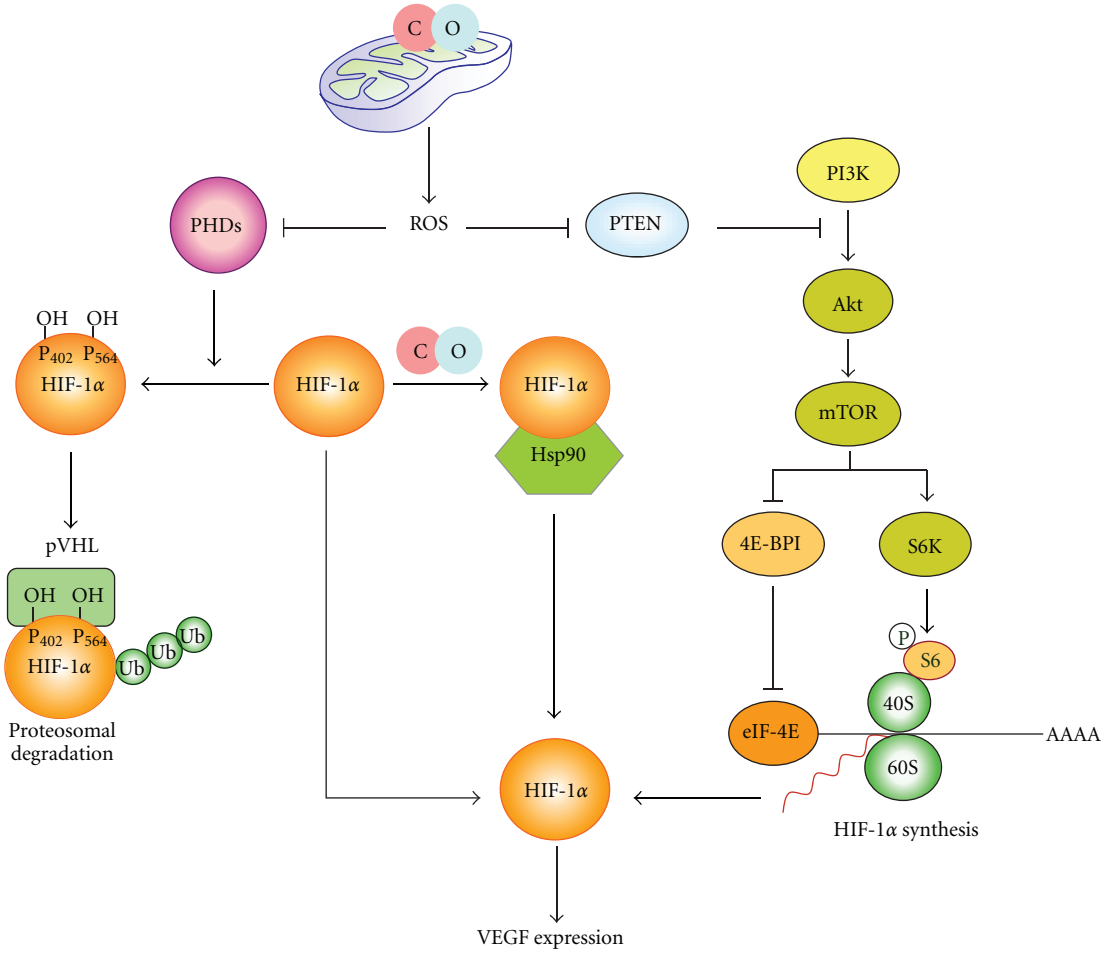

Figure 3: CO-induced HIF-1α stabilization via ROS-dependent and -independent manners. ROS produced from mitochondria by CO inhibit PHD activity, resulting in inhibition of pVHL-mediated proteasomal degradation of HIF-1α. CO-mediated ROS production can inhibit PTEN, consequently activating Akt/mTOR-mediated HIF-1α translation. In addition, CO directly promotes the binding of HIF-1α/Hsp90, which stabilizes the HIF-1α protein in a ROS-independent manner.

expression, accumulates intracellular ROS and reduces PHD activity [71]. A significant amount of supportive data has proposed a role of mitochondrial ROS in the regulation of HIF-1α stability [72], and the exogenous addition or endogenous expression of antioxidants is sufficient in some systems to reverse hypoxia-induced HIF-1α activation. Furthermore, Bell et al. [73] have provided strength to the possibility of a role for ROS in mitochondrial signaling to the HIF pathway in hypoxia, using the mitochondrial-targeted antioxidant Mito-Q. This group demonstrated that the Qo site of the mitochondrial complex III is necessary for the generation of mitochondrial ROS in hypoxia. Critical evidence suggesting a mitochondrial oxygen sensor was revealed with the discovery that ρ^0 Hep3B cells, which contain no mitochondrial DNA and thus no electron transport, are incapable of HIF-1 DNA binding activity and erythropoietin expression following hypoxia [74]. In addition, ROS derived from Nox can be also involved in the induction of HIF-1α under normoxia and hypoxia in vascular cells. Görlach et al. [75] reported that overexpression of Rac1 increased HIF-1α expression through ROS. Thus, the Rac1/Nox/ROS pathway plays an important role in the upregulation of HIF-1α and VEGF expression in response to VEGF and hypoxia.

CO has been demonstrated to increase HIF-1α stability in macrophages, leading to the protection of lungs from ischemia-reperfusion injury [21]. This effect is dependent on ROS production from mitochondria and leads to increased synthesis of TGF-β [21]. However, precise mechanisms by which CO promotes HIF-1α stability and VEGF expression in ECs remain unclear. Our previous results showed that conditioned media from astrocytes treated with CORM-2 significantly increased HUVEC proliferation, migration, and tube formation, which was inhibited by treatment with a VEGF-neutralizing antibody [57]. In addition, CO increases HIF-1α-dependent VEGF expression in astrocytes by dual mechanisms. Firstly, CO promotes *de novo* protein synthesis of HIF-1α through activation of the PI3-K/Akt/mTOR and MEK/ERK pathways responsible for activation of translational machinery. Secondly, CO stabilizes the HIF-1α protein through functional activation of Hsp90, which inhibits proteosomal degradation by interacting with HIF-1α [57]. Consistent with our data, recent studies demonstrated that

Hsp90α directly interacts with HIF-1α and protects it from oxygen- and PHD-independent ubiquitination and degradation [76, 77] (Figure 3), resulting in elevation of HIF-1α protein levels and VEGF expression. However, mechanisms by which CO regulates the interaction between HIF-1α and Hsp90α should be further investigated.

Although low concentrations of CO can rapidly stabilize HIF-1α by mitochondria-dependent ROS generation [21] or by the functional activation of Hsp90 [57], high levels of CO suppressed hypoxia-induced increases in HIF-1 stability and Epo mRNA expression. CO had no effect on the elevation of HIF-1 activity and Epo expression by the free iron chelator desferrioxamine [78], indicating that CO acts presumably as a heme ligand binding to the oxygen sensor. In fact, CO decreases the binding activity of HIF-1 to hypoxia-responsive *cis*-acting element of target genes without altering HIF-1 protein levels through the elevation of cGMP cellular levels [79]. The contribution of CO to the regulation of HIF-1α-dependent pathway is somewhat complicated since CO is reported to stabilize HIF-1α in normoxia, whereas this gaseous molecule can promote HIF-1α degradation under hypoxia [80, 81]. These pieces of evidence indicate that the effect of CO on HIF-1 activity is dependent on multiple factors, as described above. Therefore, a delicate balance between CO, ROS, and O_2 appears to determine the collective outcome of HIF-dependent physiological events.

3.2. HO-1/CO-Induced Angiogenesis via SDF-1. HO-1/CO is closely linked to the proangiogenic effects of SDF-1. Mice exposed to CO gas (250 ppm) for 2 hours per day enhance reendothelialization after vascular injury by increasing circulating endothelial progenitor cells (EPCs) and elevating the serum level of SDF-1 [63]. SDF-1 also promotes angiogenesis via a HO-1-dependent mechanism in EPCs and aortic ECs isolated from wild-type, but not from HO-$1^{-/-}$ mice [82]. HO-$1^{-/-}$ ECs and EPCs show defective angiogenic responses in SDF-1-induced proliferation and migration [82], indicating that SDF-1 promotes angiogenesis via HO-1 induction. Local gene transfer of SDF-1 enhances ischemia-induced vasculogenesis and angiogenesis *in vivo* through elevation of VEGF expression and NO production [83], suggesting that the VEGF/eNOS pathway is critically involved in SDF-1-induced vascular remodeling. In addition, both endogenous and exogenous NO increase angiogenesis through the elevation of VEGF and SDF-1 production and steady-state levels of NO [44]. These findings indicate that a positively coordinated link among HO-1/CO, eNOS, VEGF, and SDF-1 can provide new avenues for ischemia-induced neovascularization in adults. Moreover, these pieces of evidence support a vital role of HO-1 and its reaction byproduct, CO, in vascular repair by enhancing endothelial progenitor cell mobilization.

Regulation of vascular function by SDF-1 is most likely linked to ROS generation and relative O_2 availability. SDF-1 did not induce chemotaxis and invasion in Nox-deficient stem and progenitor cells by blunting SDF-1-mediated phosphorylation of Akt [84]. Ceradini et al. [58] reported that recruitment of CXCR4-positive progenitor cells to regenerating tissues is mediated by hypoxic gradients via HIF-1α-induced expression of SDF-1, resulting in increases in adhesion, migration, and homing of circulating CXCR4-positive progenitor cells to ischemic tissue. Therefore, we speculate that coordinated crosstalk among CO, ROS, and HIF-1α appears to determine the collective outcome of SDF1-dependent neovascularization.

3.3. CO-Mediated Angiogenesis and Mitochondrial Metabolism. Inhibition of the respiratory chain by CO results in hypoxia, leading to the retardation of ATP turnover and accumulation of mitochondrial biogenic sensors. Recently, the crosslink between angiogenic factors and mitochondrial biogenic factors has become of great interest. Therefore, it would be worthwhile to describe the interrelationship between mitochondrial biogenesis and angiogenesis, which are regulated by the HO-1/CO pathway. Binding of CO to a heme moiety of COX increases mitochondrial ROS production [85], the prosurvival PI3-K/Akt pathway [86], and stimulation of mitochondrial DNA (mtDNA) replication by oxidant-dependent phosphorylation of nuclear respiratory factor 1 (NRF1) and expression of the mitochondrial transcription factor A (Tfam) [87]. Suppression of CO-mediated mitochondrial ROS production and Akt activation by catalase and a PI3K inhibitor has been shown to prevent binding of NRF1 to the Tfam promoter, suggesting that CO promotes mtDNA replication by elevating ROS production from mitochondria [25]. The findings indicate that CO and ROS play a complementary role in mitochondrial biogenesis.

An example of metabolic regulators regulated by CO is the PPARs, which are ligand-activated transcription factors that govern the expression of genes involved in the regulation of lipid and glucose metabolism. Three isoforms, PPAR-α, PPAR-β/δ, and PPAR-γ, are expressed in vascular cells and regulate vascular functions via regulation of vascular inflammation, EC function, and VSMC proliferation [88]. PPAR-α can inhibit VSMC proliferation, while PPAR-β activators upregulate the expression of VEGF, resulting in enhanced EC proliferation, sprouting, and tube formation [89, 90]. PPAR-γ agonists increase VEGF expression in human VSMC cells [91] and induce EC tube formation and neovascularization in the murine corneal angiogenic model [92]. The effects of PPAR-γ are very similar to those of CO when examined in identical experimental models, regarding anti-inflammatory and antiproliferative effects [93–95]. PPAR-γ is upregulated by CO in macrophages *in vitro* and *in vivo* [24]. The suppression of CO-mediated ROS generation or accumulation by antimycin A and antioxidant (SOD and catalase) abrogated CO-induced PPAR-γ expression, suggesting that mitochondrial ROS generation by CO is required for PPAR-γ induction [24]. PPAR-γ reduces superoxide formation by upregulating Cu/Zn-SOD (SOD1) and downregulating the $p22^{phox}$ component of the Nox system [96] and enhances NO production by increasing eNOS phosphorylation and its interaction with Hsp90 [97]. Akin to NO, the beneficial effects of PPAR-α and PPAR-γ on the vascular wall may also stem from HO-1 induction [98].

Another metabolic regulator is adenosine monophosphate protein kinase (AMPK), which is activated upon energy deprivation, stimulates glucose uptake, and attenuates energy-consuming processes. AMPK leads to increases in NO production via phosphorylation-dependent activation of eNOS and upregulation of VEGF levels, resulting in improved revascularization of ischemic limbs [99]. Recent studies show that a HO-1 activator, hemin, improves insulin sensitivity and glucose metabolism by enhancing adiponectin levels, AMPK activation, and translocation of glucose transporter-4 in diabetes models [100, 101]. AMPK can also enhance SIRT1 (NAD^+-dependent class III histone deacetylase) activity by increasing cellular NAD^+ levels, resulting in the deacetylation and modulation of downstream SIRT1 targets, including PGC-1α [102]. Modest increases in cellular CO concentrations (150 pmol/mg) activate mitochondrial biogenesis via upregulation of PGC-1α by increasing mitochondrial ROS production and activating sGC and Akt in mouse hearts [25].

Therefore, the HO-1/CO pathway controls vascular homeostasis and mitochondrial biogenesis by regulating ROS generation and metabolic modulators (PPARs, AMPK, SIRT1, and PGC-1α). These lines of evidence suggest that CO acts as an extraordinary signaling molecule by regulating not only angiogenesis but also mitochondrial energy metabolism in a ROS-dependent manner.

4. Role of CO in Vasculopathy and Stroke

HO-1 is one of the most redox-sensitive genes described in nature so far; its expression is regulated by an array of conditions that are associated with a moderate or persistent imbalance of the intracellular redox state, which is the common denominator in the development of most human pathologies. Elevation of intracellular ROS levels activates the Nrf2 pathway and induces HO-1, leading to CO production [103]. In addition, CO produced by HO-1 or exogenous delivery also increases the transcriptional induction of HO-1, probably by mitochondrial ROS generation [104]. These results indicate that there is positive circuit between ROS/Nrf2 and HO-1/CO pathways, leading to continuous production of CO. Endogenous and exogenous CO protects cells from cytotoxicity induced by pathological levels of ROS, which is an important factor in the pathogenesis of human diseases, including atherosclerosis, restenosis, neointimal hyperplasia, and stroke.

4.1. CO in Vascular Disorders. Atherosclerosis, restenosis, and neointimal hyperplasia are the most common vascular disorders and result in significant long-term morbidity and mortality. Their pathogenesis is multifactorial and the common initiating event appears to be endothelial dysfunction and subsequent overproliferation of the underlying SMCs. CO has emerged as a potently protective, homeostatic molecule that prevents the development of vascular disorders when administered prophylactically. Growing evidence reveals a close relationship between oxidative stress and endothelial dysfunction [105]. Enhanced ROS production and decreased antioxidant levels potentially contribute to endothelial dysfunction by regulating NO production, vascular inflammation, and apoptosis. In addition, ROS promotes vascular remodeling via stimulation of VSMC proliferation. However, CO improves function, proliferation, and survival of ECs, but inhibits VSMC proliferation [106]. These observations suggest that the HO-1/CO pathway prevents vascular disorders via improvement of endothelial function and suppression of VSMC proliferation.

Aortic transplantation in HO-1-deficient mice results in 100% mortality within 4 days as a consequence of severe arterial thrombosis. Notably, treatment of these mice with CORM-2 considerably improved survival (62% survival at >56 days) [107]. HO-1 gene transfer into the vessel wall or systemic CO delivery resulted in a significant reduction in intimal hyperplasia in a rat model of allogenic aorta transplantation compared to controls by decreasing leukocyte activation, adhesion molecule expression, and VSMC accumulation in the intima [108]. HO-1 gene transfer and CO delivery showed a reduction in the number of leukocytes and expression of adhesion molecules and cytokines, with gene transfer treatment displaying a more pronounced effect than CO treatment. Conversely, CO exerts a greater inhibitory effect on VSMC accumulation in the intima than HO-1 gene delivery. These studies indicate that the beneficial effects of HO-1 gene expression on vascular inflammation and VSMC proliferation are associated with CO production, but possibly also with other antioxidant end products (biliverdin and bilirubin) from heme degradation by HO-1.

Higher levels of HO-1 expression exhibit a lower restenosis and cardiac vasculopathy after balloon angioplasty and heart transplantation compared with low HO-1 response to stress stimuli [109, 110]. In addition, direct exposure to CO suppresses restenosis after carotid balloon injury in rats and mice, which is closely associated with a strong inhibition of VSMC proliferation, constrictive neointimal hyperplasia, and recurrent lumen narrowing [111]. These effects probably elicit the generation of cGMP, activation of p38 MAPK, and upregulation of the cyclin-dependent protein kinase inhibitor p21 [111]. It has been also shown that probucol, a drug used to prevent restenosis, inhibits intimal thickening by inhibiting the proliferation of VSMCs via induction of HO-1 [112]. These evidences indicate that the HO-1/CO pathway plays an important role in the suppressive effect on VSMC proliferation, subsequently preventing neointimal hyperplasia and restenosis.

Inhalation of authentic CO gas suppresses established pulmonary arterial hypertension by upregulating eNOS expression and NO production, as well as elicits a simultaneous increase in apoptosis and decrease in VSMC proliferation [113], supporting the use of CO clinically to treat pulmonary hypertension and vasculopathy. CO gas activates the Akt-eNOS-retinoblastoma axis, which is responsible for survival, proliferation, and migration of ECs, leading to the promotion of reendothelialization [106]. CO accelerates reendothelialization following balloon angioplasty in rats and wire injury in mice, and neovascularization does not occur in mice lacking eNOS, suggesting that CO requires NO to enhance reendothelialization of denuded vessels

[106]. CORM-3 causes a rapid and prompt vasorelaxation and hypotension via a cGMP-dependent and endothelium-dependent manner [51]. In concert with NO, CO-releasing compounds exert therapeutically beneficial effects on vascular damage and dysfunction, which cause hypotension, intimal hyperplasia, and restenosis, by promoting vasorelaxation and reendothelialization.

It has been shown that HO-1 downregulates expression of vascular inflammatory molecules, such as ICAM-1, VCAM-1, and E-selectin in TNF-α-stimulated ECs [114], consequently blocking attachment of monocytes/leukocytes to endothelium and inhibiting subsequent transmigration across the subendothelial basal lamina. CORM-2-liberated CO significantly reduced LPS-induced ROS generation, NF-κB activation, and ICAM-1 expression in primary cultured HUVECs (Figure 4(a)), resulting in a decrease in interaction between neutrophils and LPS-stimulated HUVECs (Figure 4(b)) [115]. We have recently found that the HO-1/CO pathway suppresses expression of the adhesion molecules, ICAM-1 and VCAM-1, in TNF-1α-stimulated human ECs by inhibiting intracellular ROS levels and NF-κB activation [116] (Figure 4). Moreover, *in vivo* induction of HO-1 by specific Keap-1 knockdown reduced the expression of ICAM-1 and VCAM-1 in endotoxemic mice, leading to the protection from liver and lung injuries and lethality [117]. NF-κB plays a crucial function in the expression of various inflammatory genes, such as cytokine genes and adhesion molecules. This transcription factor is activated by two distinct signaling pathways, receptor-mediated phosphorylation-dependent signal cascade [118] and ROS-mediated redox cascade [119]. CO downregulates phosphorylation-dependent NF-κB (canonical pathway) activation by increasing interaction between caveolin-1 and TLR4 in LPS-stimulated macrophages (Figure 4), preventing TLR4 association with MyD88. The association between TLR4 and MyD88 activates IκB kinase activity, leading to NF-κB activation via p65 phosphorylation and nuclear translocation [120, 121]. On the other hand, CO inhibits NADPH oxidase activity and ROS generation in LPS-simulated macrophages [51]. Similarly, we also found that HO-1/CO inhibited cellular levels of ROS, which play a role of intracellular signal molecules in activating NF-κB activation for adhesion molecule expression, in TNF-α-stimulated HUVECs [117, 122]. These observations indicate that HO-1 induction or CO delivery can regulate inflammation-mediated vascular disorders, including atherosclerosis, via suppression of NF-κB activation by inhibiting both NADPH oxidase-mediated ROS generation and phosphorylation-dependent cascade.

Although HO-1/CO can accelerate pathologic conditions (tumor and retinopathy) via persistent angiogenic vascular activation, HO-1 induction or exogenous CO delivery imparts potent cytoprotective and homeostatic actions in preclinical models. The beneficial activities mediated by HO-1/CO play a key role in regulating EC function, VSMC proliferation, and inflammation responsible for pathogenesis of vascular diseases, such as hypertension, restenosis, and atherosclerosis in part by regulating ROS-mediated multiple signal pathways.

4.2. CO in Stroke. The involvement of the HO/CO system has received a considerable amount of attention as a target for therapeutic interventions against degenerative and inflammatory diseases in the central nervous system, including stroke. Stroke is a clinical condition in which the blood supply to the brain is limited or severed by embolism and blood clotting or by the rupture of blood vessels and subsequent cerebral hemorrhage. Brain ECs closely interact with other types of cells, such as astrocytes, microglia, and neurons, to form a functional "neurovascular unit," which maintains proper brain homeostasis. Free heme released from hemoglobin of erythrocytes following cerebral hemorrhage may contribute to oxidative toxicity [123]. Heme toxicity has been shown to occur in ECs [124] and brain cells [125].

Although protective in pathological conditions, HO is not suggested to be as beneficial for neurons as it is for other cell types. Free heme levels are thought to increase with ischemic insults due to the hydrolysis of heme proteins. Astrocytes isolated from HO-1-deficient animals were more vulnerable to the toxicity of hemoglobin and to hemin than wild-type cells [126, 127]. HO-1 is essential for the resistance of astrocytes to hemoglobin but has no protective effect in neurons. As a consequence of the differential effects of HO-1 in neurons and in astrocytes, it can be important to induce HO-1 overexpression selectively in astrocytes. An interesting approach has been utilized to express HO-1 in astrocytes using the glial fibrillary acidic protein (GFAP) promoter. Ad-GFAP-HO-1 gene transfer resulted in exclusive overexpression of HO-1 in astrocytes, but not in neurons and was able to reduce hemin-induced cell death [128]. In contrast to HO-1, HO-2 gene deletion attenuated oxidative stress of neurons exposed to heme, thus suggesting that HO-2/CO exacerbates oxidative injury of neuronal cells by cerebral hemorrhage [129–131].

However, many recent studies using stroke models suggest cytoprotective effects of HO-1 and HO-2, both in neurons and astrocytes. These conflicting results may arise since HO products can reduce or induce oxidative damage, depending on the concentration and environment. HO-1 induction is generally considered an adaptive cytoprotective response against the toxicity of oxidative stress. Overexpression of HO-1 in the mouse brain reveals a reduction in infarct volumes induced by middle cerebral artery occlusion [132]. When challenged acutely with the excitotoxic amino acid glutamate, the viability of primary neuronal cells from HO-1-knockout mice was lowered, compared with those of wild type [133]. HO-1 overexpression also significantly protected neurons from glutamate- and H_2O_2-induced cell death [134]. Inducible HO-1 is not normally detectable in an unaffected brain, whereas constitutive HO-2 is highly expressed in brain cells, neurons, astrocytes, and cerebral ECs [135, 136] and appears to play an important role in the protection of the brain from stroke. HO-2 appears to protect against lipid peroxidation-mediated cell loss and impaired motor recovery after traumatic brain injury [137], whereas its deletion exacerbated cerebral hemorrhage-induced brain edema [138]. These lines of evidence indicate that

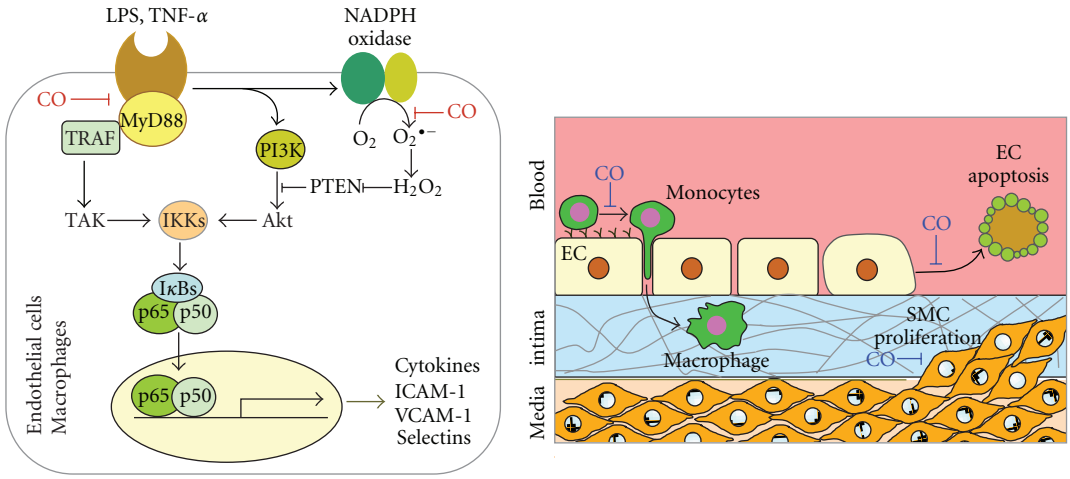

FIGURE 4: CO prevents inflammatory responses via inhibition of NF-κB signaling pathway. (a) Inflammatory stimuli such as TNF-α and LPS lead to activation of ECs, which in turn activate inflammatory signaling cascades. The association between TLR4 and MyD88 inhibits IκB kinase activity, leading to NF-κB activation via p65/p50 nuclear translocation in TNF-α-stimulated ECs. CO significantly reduces TNF-α-induced Nox-mediated ROS generation, NF-κB activation and the expression of adhesion molecules such as ICAM-1, VCAM-1, and selectins. (b) Inflammatory stimuli induce (i) the recruitment of monocytes to the endothelium, thereby promoting their transmigration into the arterial intima, (ii) ECs apoptosis and (iii) VSMC proliferation. CO diminishes this inflammatory activation by reducing the expression of adhesion molecules, stimulating EC survival and inhibiting VSMC proliferation.

both HO-1 and HO-2 protect neuronal cells and cerebral ECs from glutamate- and ROS-mediated cell death.

Ischemic stroke resulting from vascular disorders induces several biochemical and cellular reactions such as increased ROS production, inflammatory response, impairment of blood-brain barrier, and calcium overload. During reperfusion, ROS concentration is further raised to a peak point, which causes cytotoxicity through lipid peroxidation, oxidation of proteins, and DNA fragmentation [139, 140]. Increasing evidence has considered HO-1, Hsp70, antiapoptotic Bcl-2 family, and antioxidant enzymes as a therapeutic funnel in a number of pathophysiological situations and has labeled these several protective genes as the "vitagene system" [141]. Exogenous treatment of CO increases Hsp70 expression by elevating p38 MPAK activity and subsequent nuclear translocation of HSF-1 and protects ECs from TNF-α-induced apoptosis [142] and retinal cells form ischemia/reperfusion injury [143]. In addition, exogenous CO increases the expression of anti-apoptotic Bcl-2 and Bcl-X_L in an animal model and cultured ECs and protects mice from ischemia/reperfusion injury [144]. HO-1 overexpression has been also shown to be neuroprotective in a model of permanent middle cerebral artery by increasing Bcl-2 levels in neurons [132]. A recent study demonstrates that CO protects astrocytes against oxidative stress-induced apoptosis by improving metabolic functions, namely: (i) inhibition of apoptosis, (ii) stimulation of COX activity, and (iii) mitochondrial biogenesis, particularly enhancing Bcl-2 expression [145]. Treatment of mice with the water-soluble CO-releasing molecule CORM-3 significantly increases expression of extracellular SOD and $cFLIP_{L/s}$, compared with control mice. Moreover, CORM-3 induced a delayed protection against myocardial infarction similar to that observed in the late phase of ischemic preconditioning [146]. Therefore, HO-1/CO can protect against ischemic stroke and other oxidative neuronal cytotoxicity by upregulating the expression of vitagenes.

5. Therapeutic Effects of CO

There is an abundance of preclinical evidence in large and small animals demonstrating the beneficial effects of CO, administered as an authentic CO gas or as a CO-releasing molecule CORM, in cardiovascular disease, stroke, sepsis, transplanted organ rejection, and acute organ injury [35, 147]. The first single, blinded, randomized, placebo-controlled Phase I trial to evaluate the clinical safety and pharmacokinetics of inhaled CO in healthy humans has recently been completed (reviewed in [35]). Authentic CO gas has been evaluated for safety in Phase I, testing in healthy humans with rigorous dose-escalation studies and was acceptable for Phase II testing, which is currently ongoing. Since a larger number of people are studied, further information is gained on safety during this study. In addition, the Phase III study is currently evaluating the safety and potential efficacy of inhaled CO in subjects with severe pulmonary arterial hypertension.

The technology is now in place to bring CO to clinical applications in the form of inhaled gaseous therapy or through the use of potentially parenteral and orally active CORMs. CO gas is nonreactive, inexpensive, and straightforward to produce and thus is easily administrated as an

inhaled gas similar to NO, which is already used clinically [35]. CO gas is not metabolized and reversibly binds to cellular targets. Tissue levels of CO were transient and followed similar elimination kinetics. Under normoxic conditions, CO has a half-life of 3–7 hours in human volunteers, and elimination of CO is strictly through exhalation in the lungs with no further metabolism [35]. However, clinicians must consider that the critical concentration of upper limit of carboxyhaemoglobin is 14.3% in human blood, which can be reached following inhalation of 3 mg of CO per kg for a 1 hour.

By contrast, all CORMs possess a backbone carrier moiety that will need to be stringently characterized from a metabolic and toxicological standpoint. Several CORMs have been synthesized for therapeutic agents aimed at delivering controlled amounts of CO to tissues and organs [148]. CORM-1 is soluble and stable in water giving an approximate pH of 11 and decomposes rapidly releasing CO under physiological condition with slow kinetics ($t_{1/2}$ = 21 min) [149]. CORM-2 is soluble in DMSO and olive oil and releases CO by photodissociation [148]. On the other hand, CORM-3 is a water-soluble CO-releasing agent with half-life of 1 min [150]. These compounds release CO and promote vasorelaxation and hypotension via activation of cGC and potassium channel, resulting in the improvement of vascular function. In addition, CO released from CORMs inhibits NF-κB-mediated expression of inflammatory genes and upregulates expression of adaptive genes for oxidative stress. Therefore, CO-releasing compounds can be used for therapeutic treatment of vascular diseases, inflammation-associated diseases, and oxidative stress-induced disorders.

6. Summary

The significant actions of biological gaseous molecules, such as CO and NO, generated by HO and NOS, respectively, have attracted substantial interest. The reciprocal interactions between CO and NO are complex, dynamic, and adaptable. Numerous experiments demonstrate that CO is involved in cellular adaptation to oxidative stress and vascular dysfunction, leading to the maintenance of cellular and vascular homeostasis. CO produced by HO can stimulate a prooxidant milieu in aerobic cells by promoting ROS-dependent signaling, resulting in the activation of redox-sensitive transcription factors such as HIF-1α. CO signaling through the regulation of ROS production has been implicated in a number of cellular regulatory processes, including cell proliferation, angiogenesis, mitochondrial biogenesis, and adaptation to oxidative stress. In addition, CO elicits a beneficial effect in animal models of oxidative injury, vascular diseases, and stroke. Although CORMs have been developed for tissue-selective agents for disease-specific treatments, CORMs and CO gas are still in the early stages of clinical treatment. As a consequence, understanding the mechanism by which CO regulates oxidative stress and vascular function will assist in the medical battle against vascular diseases and oxidative stress.

Abbreviations

CO: Carbon monoxide
HO: Heme oxygenase
ROS: Reactive oxygen species
NOS: Nitric oxide synthase
COX: Cytochrome c oxidase
SOD: Superoxide dismutase
HIF: Hypoxia-inducible factor
VEGF: Vascular endothelial growth factor
IL-18: Interleukin-18
SDF-1: Stromal-derived factor-1
sEng: Soluble endoglin
PPAR-γ: Peroxisome-proliferator-activated receptor-γ
PGC-1α: PPAR-γ coactivator-1α
HKa: High-molecular-weight kininogen
Nox: NADPH oxidase
EC: Endothelial cell
HUVEC: Human umbilical vein endothelial cell
VSMC: Vascular smooth muscle cell
EPC: Endothelial progenitor cell
sGC: Soluble guanylyl cyclase
NF-κB: Nuclear factor-κB
TLR4: Toll-like receptor 4
TNF-α: Tumor necrosis factor-α.

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Review Article

The Role of Hydrogen Peroxide in Environmental Adaptation of Oral Microbial Communities

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Oral streptococci are able to produce growth-inhibiting amounts of hydrogen peroxide (H_2O_2) as byproduct of aerobic metabolism. Several recent studies showed that the produced H_2O_2 is not a simple byproduct of metabolism but functions in several aspects of oral bacterial biofilm ecology. First, the release of DNA from cells is closely associated to the production of H_2O_2 in *Streptococcus sanguinis* and *Streptococcus gordonii*. Extracellular DNA is crucial for biofilm development and stabilization and can also serve as source for horizontal gene transfer between oral streptococci. Second, due to the growth inhibiting nature of H_2O_2, H_2O_2 compatible species associate with the producers. H_2O_2 production therefore might help in structuring the initial biofilm development. On the other hand, the oral environment harbors salivary peroxidases that are potent in H_2O_2 scavenging. Therefore, the effects of biofilm intrinsic H_2O_2 production might be locally confined. However, taking into account that 80% of initial oral biofilm constituents are streptococci, the influence of H_2O_2 on biofilm development and environmental adaptation might be under appreciated in current research.

1. The Oral Biofilm: A Highly Adapted Microbial Consortium

Oral bacteria residing in the supragingival biofilm have a remarkable degree of structural organization [1, 2]. This organization is the result of a successive buildup and continuous integration of new species into the developing biofilm. Starting with a cleaned or recently emerged tooth, initial oral streptococcal colonizers adhere via specific surface proteins to salivary proteins covering the tooth surface [1]. Oral streptococci by themselves provide surface proteins for the attachment and integration of other oral bacteria [3]. Initial binding of oral streptococci therefore sets the stage for the development of a mature biofilm community. Beside the physical contact, biofilm development involves several layers of interactions among the biofilm community members. This includes efficient nutrient usage by metabolic cooperativity, communication by small signal molecules, and genetic exchange [4, 5].

The crucial steps in initial attachment and biofilm development have been well documented in the past years. Using specific removable appliances harboring dental enamel chips, Diaz et al. were able to trace the spatiotemporal pattern of oral biofilm formation in the human host [6]. Oral streptococci were the predominant species in the initial colonization stage after 4 and 8 hours. Up to 80% of the detected initial colonizers belonged to the genus *Streptococcus* with some species discussed as constant members presenting a core group of initial biofilm formation [6, 7]. The biofilm developmental process starts with small microcolonies consisting mainly of streptococci and few non-streptococci [6]. This developmental process has implications on other species efforts to join the biofilm community or attach in close proximity. Oral streptococci are known for their production and secretion of antimicrobial substances, one of them is hydrogen peroxide (H_2O_2) [8, 9]. The production of antimicrobial substances like H_2O_2 could therefore be regarded as an important protection mechanism of the initial colonizers of the resident biofilm community against invading and competing species. More importantly, it might also be a mechanism to shape the colonization process toward a specific

species composition. Only species coevolved with oral streptococci and therefore adapted to withstand H_2O_2 can integrate or colonize in close proximity to the initial colonizers and extend the developing biofilm community.

After initial attachment of streptococci, the biofilm builds up and several other species join the biofilm community [1, 6]. This also leads to an increase in biofilm thickness and subsequent anaerobic conditions [10–12], which in turn can attract anaerobic bacteria. H_2O_2 production inside the oral biofilm most likely declines under these conditions due to insufficient oxygen availability. The role of H_2O_2 becomes less important and other factors might influence biofilm maturation. From the perspective of the oral streptococci, H_2O_2 fulfills its purpose exactly when it is needed, during initial biofilm formation, when oxygen for H_2O_2 production is readily available [13]. The ecological niche of oral streptococci is freely accessible for competing species during initial biofilm formation, and this competition is counteracted either by the direct bactericidal effect of H_2O_2 or the preferred integration of compatible species into the growing community. Once the streptococci are established and have built up an association of compatible neighboring biofilm inhabitants, they already occupy their favorite ecological niche and the antimicrobial activity of H_2O_2 is no longer required.

The multispecies oral biofilm community provides a protective function to prevent invasion of foreign (pathogenic) bacteria [14]. Unfortunately, some of the bacterial species commonly found in the human oral biofilm consortium have the ability to cause diseases like tooth decay (caries). Under healthy conditions, these species would not cause any harm. Disease development is the result of a disturbed biofilm homeostasis leading to an overgrowth of conditional pathogenic bacteria and a general reduction of the species composition normally found in healthy supragingival plaque [15, 16]. Interestingly, clinical evidence emerges that some of the H_2O_2 producing oral streptococci seem to be reduced in their abundance in subjects having oral diseases like caries or periodontal disease [17–19].

The available *in vivo* and *in vitro* studies point to H_2O_2 as an important metabolic product generated in the early cycles of oral biofilm formation. In the following sections, specific examples important in biofilm development and in the adaptation to the oral biofilm environment are discussed.

2. Sources of H_2O_2

H_2O_2 in the oral cavity originates from bacteria and from the host [20]. At the present time, it is not clear how both sources influence each other and if at all the production of H_2O_2 by the host directly impacts the biofilm and *vice versa*. H_2O_2 has not been detected directly in saliva [21, 22]. The transient concentration has been calculated to be around 10 μM based on known concentrations of thiocyanate and hypothiocyanite in saliva [22]. One potential reason is the presence of a salivary scavenging system for H_2O_2 to protect the host from H_2O_2 toxicity [23, 24]. Two host-derived peroxidases are present in the human oral cavity, salivary peroxidase, and myeloperoxidase [23]. Both are able to use H_2O_2 as an oxidant and thiocyanate as a substrate to produce hypothiocyanite [23]. Interestingly, hypothiocyanite is not only a detoxification product, but also a general antimicrobial substance, and the combination of H_2O_2, hypothiocyanite, and salivary peroxidase seems to be most potent in inhibiting bacterial metabolism [25, 26]. Salivary peroxidase, a noninducible component of saliva originates in the parotid and submandibular glands [27]. Myeloperoxidase is an offensive component of polymorphonuclear leukocytes [28], which are present in saliva with elevated levels during inflammatory diseases like periodontal disease [29].

2.1. Sources of H_2O_2 in the Oral Biofilm. Oral streptococci have long been known to produce H_2O_2, mainly due to their ability to inhibit various other species in *in vitro* tests. Early reports already indicate that H_2O_2 production might be widely distributed among oral streptococci. Thompson and Shibuya tested 55 *alpha*-hemolytic oral streptococci and found that 48 were able to inhibit the growth of *Corynebacterium diphtheria* [30]. Tests with identified streptococcal species showed that *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus sanguinis,* and *Streptococcus sobrinus* all were able to produce significant amounts of H_2O_2 during growth *in vitro*, which can be detected in the supernatants of the growth medium [31]. These oral streptococci are commonly isolated and present in a relatively high abundance in the human oral biofilm [32]. Variations in H_2O_2 production among streptococci were shown to be growth medium and carbohydrate dependent [31], indicating environmental influences on regulatory mechanisms of H_2O_2 production.

The enzyme responsible for the production of H_2O_2 in *S. sanguinis* and *S. gordonii* was identified as pyruvate oxidase, encoded by gene *spxB* (also referred to as *pox*) [33–35]. The pyruvate oxidase is an oxidoreductase that catalyzes the conversion of pyruvate, inorganic phosphate (P_i), and molecular oxygen (O_2) to H_2O_2, carbon dioxide (CO_2), and the high-energy phosphoryl group donor acetyl phosphate in an aerobic environment. Genetic inactivation of the respective open reading frames encoding for putative pyruvate oxidase orthologs in *S. sanguinis* and *S. gordonii* confirmed the pyruvate oxidase as the enzyme responsible for significant H_2O_2 production [35]. The production of growth inhibiting amounts of H_2O_2 is not exclusive to the pyruvate oxidase in oral streptococci. Detailed genetic inactivation studies in *Streptococcus oligofermentans* showed that at least two other enzymes in addition to the pyruvate oxidase are able of producing growth-inhibiting amounts of H_2O_2 [36, 37]. The lactate oxidase, gene *lctO* (also referred to as *lox*), catalyzes the formation of pyruvate and H_2O_2 from L-lactate and oxygen and an L-amino acid oxidase generates H_2O_2 from amino acids and peptones. Dual species biofilm antagonism assays with *S. oligofermentans* and *S. mutans* demonstrated that the H_2O_2 produced by LctO activity is still able to antagonize *S. mutans* in an *spxB* background. The role of the L-amino acid oxidase in interspecies competition is not clear since its H_2O_2 producing activity is low, and only visible in a *lctO/spxB* double knockout mutant [36, 38]. Nonetheless, the L-amino acid oxidase seems to be important as suggested

Table 1: Distribution and nucleotide identity of *spxB* and *lctO* among sequenced oral streptococcal isolates.

Species	Strain	*spxB* identity (%)	*lctO* identity (%)
S. mitis	B6	100	100
	NCTC 12261	97	95
	SK564	97	95
	SK321	97	94
	SK597	96	94
	F0392	96	93
	SK95	96	—
	ATCC 6249	96	90
S. sanguinis	SK36	94	—
	SK49	95	—
	AATCC 49296	96	91
S. gordonii	CH1	96	—
S. oralis	Uo5	96	91
	ATCC 35037	96	91
S. parasanguinis	SK236	95	—
S. vestibularis	FO396	95	—
	ATCC 49124	95	—
S. peroris	ATCC 700780	95	90
S. cristatus	ATCC 51100	—	87
S. oligofermentans	AS 1.3089	95	88

by a recent study, Boggs et al. showed that the L-amino acid oxidase gene *aao* from *S. oligofermentans* was probably acquired via horizontal gene transfer from a source closely related to *S. sanguinis* and *S. gordonii*, while evolutionary *S. oligofermentans* seems to be more closely related to *S. oralis*, *S. mitis,* and *S. pneumoniae* [39]. The authors speculate that the *aao* gene is important for *S. oligofermentans* to occupy a specific ecological niche in the oral biofilm [39]. The regulation of *aao* gene expression is not known, and the gene might be induced under specific conditions *in vivo*.

Using the available genome sequence data from the Human Oral Microbiome Database (http://www.homd.org/), the distribution of *spxB* and *lctO* among oral streptococci was determined using *spxB* and *lctO* from *S. mitis* B6 as a template. As shown in Table 1, several important oral streptococci encode open reading frames with a high homology to *spxB* and *lctO*. All species listed in Table 1 are commonly isolated from subjects suggesting a wide distribution of *spxB* and *lctO* in oral streptococci. Interestingly, *spxB* seemed to be more conserved among species when compared to *lctO*. The relatively wide distribution of *spxB* and *lctO* and the high degree of conservation suggest that both genes play an important role in the H_2O_2 production capabilities of the oral biofilm and might be considered as oral streptococcal community genes. Interestingly, inactivation of *spxB* in *S. sanguinis* diminishes competitive H_2O_2 production, suggesting that LctO plays no role in interspecies competition under the tested conditions in *S. sanguinis* [35].

2.2. Sources of H_2O_2 from the Host. H_2O_2 originates from several sources in the human body. Mitochondria are well-known producers of reactive oxygen species (ROS) as a byproduct of respiration [40]. Effective intracellular scavenging systems are in place to avoid ROS inflicted damage [41] and the H_2O_2 might not leave the oral mucosa in sufficient amounts to play a role in oral microbial biofilm ecology. A regulated production of ROS is observed as part of the oxidative burst from phagocytic cells [42]. The ROS production is directed towards the outsides of the phagocytic cell to defend the host from microbial pathogens and might therefore freely diffuse to nearby locations. Polymorphonuclear leukocytes seem to be the predominant phagocytic cells in saliva originating from the gingival crevice fluid and are constantly replenished [29]. However, one study with healthy individuals observed a high intraindividual day-to-day variability of salivary polymorphonuclear leukocyte content [43], making it difficult to judge how much H_2O_2 is being released as a consequence of phagocytic cell activity.

A more constant source of H_2O_2 supplied into saliva could originate from salivary gland cells expressing the dual oxidase 2 gene (Duox2) as shown by Geiszt et al. [44]. The same study also suggests that that ROS production occurs in the last step of saliva formation for direct delivery of ROS into the oral cavity [42, 44] and could therefore be the major source for salivary H_2O_2 originating from the host.

3. Hydrogen Peroxide in Oral Bacterial Ecology

3.1. Where Does It Matter: The Importance of Bacterial Proximity. The fact that H_2O_2 was never detected in saliva so far and the existence of a major scavenging system comprised of salivary peroxidases raise an important question: how likely does H_2O_2 affect oral bacterial ecology or aid in biofilm community adaptation? This question might be addressed by the fact that a bacterial biofilm comprises its own microcosm with intrinsic biofilm H_2O_2 production and most likely has a localized effect due to diffusion restrictions. By measuring the H_2O_2 concentration produced by single species, *S. gordonii* biofilms, Liu et al. were able to show that a steady state level of 1.4 mM H_2O_2 was produced at a distance of 100 μm above the biofilm surface [45]. Only 0.4 mM H_2O_2 is produced when measured 200 μm above the biofilm. This localized production of 1.4 mM is a concentration able to inhibit H_2O_2 susceptible bacteria, which have to be in close proximity. Remarkably, the same study also measured higher concentrations of H_2O_2 close to the surface of the biofilm as compared to planktonic grown cells [45]. This is in contrast to an earlier study by Nguyen et al. showing that *S. sanguinis* and *S. gordonii* had lower H_2O_2 production rates in biofilms when compared to planktonic cells [46]. This discrepancy might be partially explainable by the advanced method used in the study by Liu et al., allowing realtime detection with an H_2O_2 specific probe measuring directly above the biofilm surface [45]. Also, the study by Nguyen et al. used a higher concentration of glucose in the growth media, which might have repressed the H_2O_2 production rate [46] (see below: regulatory studies on H_2O_2 production). The difference in H_2O_2 concentration as a function of biofilm surface distance

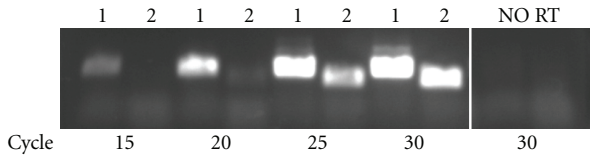

Figure 1: Expression of *spxB* in freshly isolated human plaque. To detect the expression of *spxB* among streptococcal species in the oral biofilm, plaque samples were collected from a healthy subject without active caries. Bacterial RNA was isolated and cDNA synthesized after standard protocols [59]. The *spxB* gene was PCR amplified from the synthesized cDNA with primers described by us earlier specific for *spxB* and 16S rRNA [59]. Samples were removed during the PCR run after 15, 20, 25, and 30 PCR cycles and loaded on an agarose gel for visualization. 1 = 16S rRNA; 2 = spxB; no RT = control for chromosomal DNA contamination.

supports the suggestion that H_2O_2 producing species most likely have an effect on close neighboring species. When the cells dislodge and enter a planktonic state, H_2O_2 production becomes irrelevant. Taking into account that the oral biofilm is a diffusion barrier for larger proteins and molecules [47], the intrinsic H_2O_2 production of biofilm would also be more protected against the action of salivary lactoperoxidases, which might not penetrate preformed biofilms [48].

Detection of actual *spxB* expression in the human oral biofilm would support the importance of *spxB*-dependent H_2O_2 production. If *spxB* plays a vital role in oral biofilm ecology, one would expect that cells residing in the human oral biofilm express the *spxB* gene. Using freshly isolated plaque samples from a subject with no active caries, *spxB* specific cDNA was synthesized from RNA isolated from human oral biofilm bacteria and *spxB* expression confirmed (Figure 1; unpublished results). This observation not only shows for the first time the expression of an oral biofilm relevant gene *in vivo* but also strongly supports *spxB* relevance in the human dental plaque and suggests that *spxB* plays a role in biofilm specific processes.

3.2. Adaptation to a Competitive Environment-Genetic Exchange. Adaptation to the constantly changing oral environment requires some kind of genetic flexibility. This can be achieved by specific gene expression regulation and the adjustment of the transcriptome to sudden perturbations in the environment or by the acquisition of new genetic traits to cope with long-term environmental changes. Oral streptococci are known for their natural ability to take up extracellular DNA, a physiological state called competence [49]. Bacterial competence has long been recognized as the ability to take up DNA, but recent studies show that competence is part of a larger stress response, which enables competent bacteria to cope with a stressful environment [50]. Competent oral streptococci are able to take up homologous and heterologous DNA [51–53]. This increases the available DNA pool and allows for acquisition of new genetic traits from other species. Expression of newly acquired genetic traits depends on the homologous recombination of the incorporated DNA into the host chromosome [54, 55]. The mechanisms and genetic regulation of natural competence leading to the uptake and integration of DNA via homologous recombination are documented in numerous studies, and the basic blueprint of competence seems to be similar among oral streptococci [49, 56]. What is less known is how the biofilm community generates the extracellular DNA for DNA uptake by competent bacteria. A general mechanism of bacteria to produce extracellular DNA is an autolytic event leading to bacterial disintegration. Recent studies show that autolysis is a regulated process.

The release of DNA into the environment by *S. gordonii* and *S. sanguinis* is closely associated with the production of H_2O_2 [35]. The wild type organisms release high molecular weight DNA during aerobic growth, which was shown to be of chromosomal origin [57]. A deletion of the pyruvate oxidase gene affected this release process dramatically [57]. In addition, a significant reduced concentration of extracellular DNA was detected under oxygen limited growth conditions [58], correlating with a reduced expression of *spxB* and a lower amount of SpxB [59, 60]. Further studies showed that H_2O_2 is the only requirement to induce the DNA release process. Addition of H_2O_2 to anaerobically grown cells does induces DNA release. Although mechanistic studies are still in progress and the release process is not fully understood, our group has demonstrated a correlation between H_2O_2 induced DNA damage and extracellular DNA generation. Treatment with DNA damaging agents like UV light and mitomycin C also triggered the release of DNA under anaerobic conditions [58].

Initial evidence of an autolytic activity involved in the DNA release process comes from Robert A. Burne's group, showing that the major autolysin AtlS is involved in DNA release [61]. A deletion of AtlS in *S. gordonii* prevented autolysis under aerobic conditions, and as a consequence, a decreased production of extracellular DNA was observed [61]. Their observation, however, is in contrast to an observation by our group, showing that under anaerobic conditions, extracellular DNA release can be induced by H_2O_2 addition without any obvious bacterial cell lysis [58]. A possible explanation for these observations is that streptococci may have several mechanisms to trigger lysis responding to different internal and/or external stimuli. Autolysis may also not necessarily mean complete lysis of the bacterial cell or might only affect a small portion of the population. A recent report showed that *S. gordonii* expresses a murein hydrolase, *LytF*, involved in competence dependent bacterial lysis [62]. In fact, *lytF* is only expressed during competence because its expression is under the control of the competence stimulating peptide CSP, a small secreted peptide which accumulates in the environment after reaching a critical threshold concentration initiating the competence signaling cascade (see [50] for a detailed overview of competence in bacteria). DNA transfers experiments relying on *LytF* dependent cell lysis, and subsequent DNA uptake by *S. gordonii* showed that most cells are protected from the muralytic activity of *LytF* [62]. This is in agreement with our observation of a lysis resistant population [57, 58]. A close association, however, of H_2O_2 induced release of DNA and competence development is evident since cells

grown under H_2O_2 producing conditions are also induced for competence development [58]. Interestingly, competence development in *S. pneumoniae* can be initiated by mitomycin C induced DNA damage, which also leads to the release of DNA [63]. This is reminiscent of our observation that DNA damaging agents induce DNA release [58], which is associated with the ecological advantage of H_2O_2 induced DNA release and the adaptation of oral streptococci to stress. *S. gordonii* and probably other H_2O_2-producing oral streptococci release DNA into the environment as a consequence of DNA damage. This pool of released DNA likely contains mutations in various genes because of the DNA damage. If such mutated DNA is taken up and integrated into the chromosome, the transformation event would lead to a bacterium able to grow and outcompete bacteria without the respective mutation under selective conditions. Even nonmutated extracellular DNA or genes would be useful as a template for the repair of stress-induced DNA damage [58]. The extracellular DNA is precisely produced at a time when it is biologically meaningful, under aerobic conditions during initial biofilm formation with its fierce interspecies competition and environmental stress, hence, when the cells are most competent for DNA transformation. Finally, H_2O_2 can also cause the release of DNA from streptococci not producing H_2O_2, but the mechanism for this is not known (unpublished results).

3.3. The Other Role of Extracellular DNA. Besides providing genetic information for transformation of competent oral streptococci, the DNA released as a consequence of H_2O_2-production might aid in initial biofilm development [64]. Although not directly shown for H_2O_2 producing oral streptococci, studies with *S. mutans* demonstrate the importance of extracellular DNA in initial adhesion. Das et al. showed that adhesion kinetics in the presence and absence of naturally occurring extracellular DNA were different. *S. mutans* cells adhered better and in greater numbers to the provided test surface when extracellular DNA was present [65, 66].

Initial biofilm formation involves the adhesion of pioneer colonizers to the tooth surface [3]. Another important event in early biofilm formation is bacteria-bacteria aggregation: (1) aggregation of bacteria before the actual attachment event in saliva increases the cluster size of bacteria able to adhere; (2) bacterial aggregation will also aid in the recruitment of other bacteria into the developing biofilm. Although aggregation of bacteria is well described with the identification of several surface proteins involved in the process [3], the role of extracellular DNA in oral bacterial aggregation is not well investigated. Studies with fresh water bacteria show that the released DNA functions in a netlike manner able to trap bacteria [67]. Initial evidence shows that extracellular DNA plays a role in the intraspecies aggregation of *S. sanguinis*. When grown as a planktonic culture, addition of extracellular DNA degrading DNase inhibits partially the aggregation [57]. Further studies are required to fully understand the role of extracellular DNA in multispecies biofilm formation and bacterial aggregation.

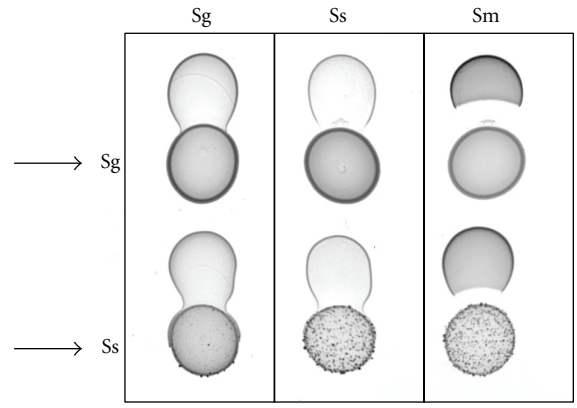

Figure 2: Oral streptococcal antagonism assay with *S. sanguinis*, *S. gordonii,* and *S. mutans*. The lower row in the plate dual-species antagonism assay were inoculated first (indicated by an arrow) and allowed to grow for 16 h. Subsequently, the to be tested species was inoculated in close proximity. Diffusible H_2O_2 produced by *S. sanguinis* (Ss) and *S. gordonii* (Sg) during growth caused inhibition of *S. mutans* (Sm), while no obvious growth inhibition was observed when *S. sanguinis* or *S. gordonii* was tested against themselves or against each other.

3.4. Biofilm Community Development. Earlier clinical studies have demonstrated the inverse relationship between *S. sanguinis* and cariogenic *S. mutans* [17, 19]. A recent study showed that *S. oligofermentans* is also frequently isolated from healthy human subjects [68]. The clinical evidence suggests that the initial colonization by H_2O_2-producing bacteria has a beneficial aspect for the human host with regard to caries development, possibly through the influential role of H_2O_2 on biofilm community development. Detailed *in vitro* experiments and relevant biofilm studies confirmed that *S. sanguinis*, *S. gordonii,* and *S. oligofermentans* produce H_2O_2 to inhibit *S. mutans* [8, 35, 37]. Although, the produced H_2O_2 has a slight self-inhibitory effect on the producing species in batch cultures, no obvious inhibition occurs when H_2O_2 producers are tested against each other in an antagonistic plate diffusion assay (Figure 2). As a consequence, community development favors integration of species that are compatible with the production of H_2O_2. Jakubovics et al. showed an interesting relationship between *S. gordonii* and *Actinomyces naeslundii*. Although *A. naeslundii* is severely inhibited in the aforementioned antagonistic plate diffusion assay, coaggregation cultures showed that both species could grow together in close proximity [69, 70]. *S. gordonii* is, however, the dominant species in this consortium, leading to a ratio of about 9 to 1. *S. gordonii* might benefit from this relationship by the fact that the H_2O_2 degrading catalase produced by *A. naeslundii* can reduce oxidative damage to *S. gordonii* proteins inflicted by its own H_2O_2 [70]. The low ratio of *A. naeslundii* to *S. gordonii* would still allow for sufficient inhibition of H_2O_2 susceptible species, but a clear ecological niche is necessary to support growth of both species, which could lead to the formation of more stable plaque communities. Another common oral isolate found in close association with *S. gordonii* is *Veillonella* ssp. [71].

Both species interact at the physiologic and metabolic level as shown by several studies [72–74]. Some strains of *Veillonella* also produce catalase, indicating that a similar effect as described for *A. naeslundii* might exist in the relationship between *S. gordonii* and *Veillonella ssp*. The biological relevance of the interactions between *Streptococci*, *Veillonella,* and *Actinomyces* has recently been demonstrated *in vivo* by confirming the spatial association of the three species in human plaque samples [2]. Further studies are required to determine the exact role of catalase production in the dual species relationship between H_2O_2-producing streptococci and catalase-expressing species.

The production of H_2O_2 seems to select for a close association with compatible bacteria during biofilm community development. Therefore, H_2O_2 might shape the colonization pattern during initial biofilm formation and provide an ecological advantage for the producer and the accompanying H_2O_2 resistant species.

4. Regulatory Studies on H_2O_2 Production

S. gordonii and S. sanguinis. A detailed analysis of environmental influences on *S. gordonii*'s H_2O_2 production showed two important behaviors. (1) During growth under limited glucose and sucrose availability, *S. gordonii* produces only H_2O_2, while H_2O_2 and L-lactic acid are produced in equal amounts when concentrations of carbohydrates were higher than 0.1 mM. Since lower carbohydrate availability means increased competition among the biofilm microflora, a switch to only H_2O_2 production might increase the ecological competitiveness. (2) High glucose and sucrose concentrations inhibit the production of H_2O_2 [75]. This observation prompted us to further investigate the mechanism of H_2O_2 production control by determining *spxB* expression and SpxB abundance in *S. sanguinis* and *S. gordonii* under different environmental conditions. We could confirm the influence of carbohydrate concentration on *spxB* expression and abundance showing glucose repression in *S. gordonii* [59]. The carbohydrate dependent repression of *spxB* expression was also confirmed for galactose, maltose, and lactose, while sucrose and fructose seemed to have no effect in our strain [59]. This indicates that strain variability among *S. gordonii* might exist in the regulation of *spxB* expression. A detailed analysis of the promoter region of *spxB* from *S. gordonii* showed the existence of two putative binding sites for the catabolite control protein A (CcpA). CcpA is the main regulator of carbon catabolite repression in Gram-positive bacteria [76]. Mutational analysis of the promoter sequence confirmed the role of the CcpA binding sites and purified CcpA was able to bind to the respective regions in *in vitro* electromobility shift assays [59]. Surprisingly, the *spxB* expression in *S. sanguinis* is not influenced by carbohydrate availability, despite a high degree of promoter homology between both species and the presence of respective CcpA binding sites. However, a deletion of CcpA in *S. sanguinis* increased expression of *spxB* several folds [77]. This suggests that *S. sanguinis* constantly represses the expression of *spxB* or only lifts the repression due to a yet unknown environmental signal. One reason for this alternative *spxB* expression control could be *S. sanguinis* increased susceptibility to H_2O_2 when compared to *S. gordonii* (unpublished results). By keeping the production of H_2O_2 low, *S. sanguinis* might prevent self-damage of cellular components like surface adhesins, making it less competitive in the oral environment. The observation that monospecies biofilms of a *S. sanguinis* CcpA mutant had a higher proportion of dead cells when compared to the wild type further supports this hypothesis [77].

Both species do not produce competitive H_2O_2 under anaerobic growth conditions. Accordingly, *spxB* expression and SpxB abundance is greatly reduced under anaerobic growth conditions, but the protein is still detectable [59, 60]. This finding suggests that both streptococci keep a low level of SpxB present to remain competitive once they encounter aerobic conditions. The mechanisms of oxygen-dependent *spxB* expression control are not known at this time.

The *spxB* expression control involves additional regulators and proteins. Most notable is the identification of an SpxR homolog in *S. sanguinis* [78]. SpxR was originally identified in *S. pneumoniae* and it was hypothesized that SpxR in *S. pneumoniae* regulates *spxB* transcription in response to the energy and metabolic state of the cell [79]. Although not confirmed experimentally, this regulatory function might well be active in *S. sanguinis*, since no carbohydrate-dependent regulation was detected. Future research might address this question and identify what actual signal is involved in *spxB* regulation in *S. sanguinis*.

S. oligofermentans. S. oligofermentans developed an interesting mechanism to produce antagonistic H_2O_2 and maximize its competitiveness. SpxB produces the majority of H_2O_2 during active growth [36] leading to the generation of an extra ATP through the *spxB* pathway. This ATP provides a metabolic growth advantage in addition to the ecological advantage of H_2O_2 production. The *lctO*-dependent H_2O_2 generation on the other hand is more prominent in the early stationary phase, due to an increased availability of lactate [36]. Several other oral streptococci encode genes for both H_2O_2 forming enzymes suggesting a similar role in H_2O_2 production. This dual SpxB/LctO presence indicates that even under starving conditions, oral streptococci might still produce competitive amounts of H_2O_2 to shape biofilm development towards a health compatible composition.

5. H_2O_2 in Oral Bacterial-Host Interactions

Oral streptococcal interactions occur in the mouth and therefore in close proximity to human host cells and the mucosal surface. Interactions with human innate immunity components are inevitable. Marvin Whiteley's group showed that the production of H_2O_2 has an unexpected effect on the recognition of pathogenic species by the immune response [80]. Using the recognized periodontal pathogen *Aggregatibacter actinomycetemcomitans* and *S. gordonii* as model organisms to study a combined effect on the host innate immune response, they described an interesting relationship between both species. Not only is *A. actinomycetemcomitans* able to effectively use the lactic acid produced by *S. gordonii*

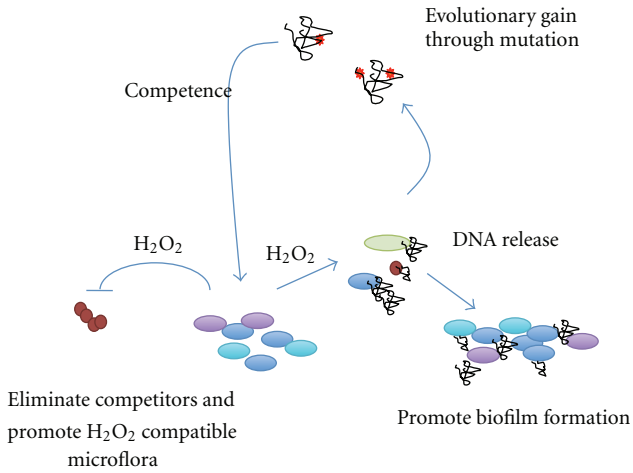

Figure 3: Overview of the effects of H_2O_2 production on oral biofilm development. Initially, the antagonistic effect of streptococcal H_2O_2 production was described. As a consequence, competitors are eliminated, and the integration of H_2O_2 compatible species into the developing biofilm is promoted. H_2O_2 production also causes the release of DNA into the environment. The extracellular DNA promotes biofilm formation and cell-cell aggregation. In addition, H_2O_2 causes DNA damage, which in turn could lead to beneficial mutations in competent oral streptococci uptake of extracellular DNA. Extracellular DNA could therefore support adaptational processes to changing environmental conditions and promote evolution of oral biofilm development.

for growth [81] but it also responded to H_2O_2 as a signal to induce the expression of an immune evasion gene, *apiA*. This gene encodes an outer membrane protein able to bind factor H, conferring protection against killing by the alternative complement component of the innate immunity. In addition, the *katA* gene encoding cytoplasmic catalase is also induced, conferring resistance to the destructive action of H_2O_2 on *A. actinomycetemcomitans* cellular components [80].

This observation demonstrates that biofilm community development is capable of remarkable evolutionary adaptations and that H_2O_2 plays a prominent role in the process of oral biofilm development (Figure 3).

6. Concluding Remarks

One of the most important problems in current oral microbial research is to confirm biological relevance of *in vitro* experimental results. Accepted animal models to simulate oral biofilm ecology are generally rodent models. Although these models increase complexity, the transplanted human oral flora faces a rodent oral microbial consortium and a distinct oral environment. It therefore competes with species and conditions not encountered under normal conditions. It is not known if this complexity affects competition studies. A recent rodent study actually questions the validity of the importance of H_2O_2 production in *S. gordonii* competitiveness. Performing coinoculation studies in rats, Tanzer et al. showed that *S. mutans* is always able to outcompete *S. gordonii* under all experimental conditions [82]. Unfortunately, it was not determined whether the *S. gordonii* strain in their study produced competitive amounts of H_2O_2 or if the *spxB* gene was expressed in the rat oral biofilm. It is also unclear if the respective *S. mutans* strain was H_2O_2 susceptible. It is therefore important that animal studies about ecological questions actually demonstrate that the respective competitive gene set(s) are expressed under animal test conditions. It is also important to verify the expression of the gene(s) of interest in the human oral biofilm. Our initial data for *spxB* gene expression in the human oral biofilm are promising and warrant further research regarding the ecological role of H_2O_2 production in human oral biofilm.

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Review Article

Reactive Oxygen Species in the Signaling and Adaptation of Multicellular Microbial Communities

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One of the universal traits of microorganisms is their ability to form multicellular structures, the cells of which differentiate and communicate via various signaling molecules. Reactive oxygen species (ROS), and hydrogen peroxide in particular, have recently become well-established signaling molecules in higher eukaryotes, but still little is known about the regulatory functions of ROS in microbial structures. Here we summarize current knowledge on the possible roles of ROS during the development of colonies and biofilms, representatives of microbial multicellularity. In *Saccharomyces cerevisiae* colonies, ROS are predicted to participate in regulatory events involved in the induction of ammonia signaling and later on in programmed cell death in the colony center. While the latter process seems to be induced by the total ROS, the former event is likely to be regulated by ROS-homeostasis, possibly H_2O_2-homeostasis between the cytosol and mitochondria. In *Candida albicans* biofilms, the predicted signaling role of ROS is linked with quorum sensing molecule farnesol that significantly affects biofilm formation. In bacterial biofilms, ROS induce genetic variability, promote cell death in specific biofilm regions, and possibly regulate biofilm development. Thus, the number of examples suggesting ROS as signaling molecules and effectors in the development of microbial multicellularity is rapidly increasing.

1. Introduction

Since the first observations of microorganisms by Antonie van Leeuwenhoek and their isolation and cultivation by Robert Koch, microorganisms have been traditionally viewed as simple unicellular organisms. As a result of this presumption, all microbial studies have been conducted using shaken liquid cultivations. However, during the last few decades, it has become obvious that microorganisms are able to form multicellular structures such as colonies and biofilms. More and more examples of multicellularity have been described, and it has become evident that multicellular behavior, although initially considered an exception, is instead the rule for microbes. Species of the most distant clades of both Archea and Bacteria form biofilms, as did their ancestors some 3.3 billion years ago, indicating that biofilm formation is a universal and ancient bacterial trait [1]. Biofilms and colonies are also formed by eukaryotic microbes (yeasts and molds) in different environments. Multicellular communities are thus prevalent forms of microbial existence in natural settings.

The structural complexity and degree of organization of microbial multicellular structures vary from a simple single-layer biofilm and simple aggregates to complicated structures like the fruiting bodies of myxobacteria and slime molds [2, 3], complex natural biofilms [4] and the colonies of various microbes [5]. Importantly, cells within these structures differentiate and use various signaling molecules to coordinate and regulate the metabolism and development of the community. All these characteristics, that is, structural complexity, cellular differentiation, intercellular signaling and coordinated development, are basic attributes of true multicellularity. However, unlike conventional multicellular organisms, microorganisms retained the ability to survive and divide in their unicellular state.

The biofilm community gains a number of unique properties, especially in terms of resistance to various stresses and toxins. This is of particular importance, because

the resistance of biofilms to medical treatment is an important problem in current infection control. For this reason, biofilms have become the subject of intensive research in recent years [1, 6–10].

The production of reactive oxygen species (ROS) is an inevitable consequence of an aerobic lifestyle. Because of their reactive nature, ROS can cause oxidative damage to DNA, proteins, lipids and other cellular components, and an excess of them leads to extensive cell damage and eventually cell death. To protect themselves from the deleterious effect of ROS, cells have evolved many defensive mechanisms including, for example, enzymes capable of ROS removal, such as catalase, and various peroxidases for the removal of hydrogen peroxide or superoxide dismutase, eliminating superoxide radicals. Although ROS have been traditionally viewed as purely harmful, a more complex picture of their role in cellular physiology has been gradually emerging over the last decade. Recent data have suggested that a certain level of ROS is in fact beneficial to longevity through the adaptive mechanism called hormesis [11, 12]. During hormesis, low doses of stress or toxin induce mechanisms that protect the organism against this stressor and evoke crossadaptation to other stresses. In addition, a growing number of data suggest that ROS participate in signaling pathways in plants, animals, and fungi [13–15] and even in interspecies communication [16], and it has also been proposed that they play a role in the development of multicellularity [17]. Despite being widely accepted as signaling molecules in higher organisms, little is known about the role of ROS in microbial populations. However, the last few years have produced intriguing new data indicating that ROS-induced processes are involved in differentiation and signaling in yeast and bacterial communities.

Here, we focus on the functions of ROS in multicellular communities of unicellular yeast and bacteria. We summarize current knowledge on the possible roles of ROS and stress defense in the development of *S. cerevisiae* colonies. In the second part of this review, we summarize current knowledge on the role of oxidative stress defense and endogenous ROS production in other well-studied microbial multicellular structures—*Candida albicans* and bacterial biofilms.

2. *Saccharomyces cerevisiae* Colonies

The yeast *S. cerevisiae* is one of the most studied model organisms in genetics and molecular biology. It is also widely used for studies on the mechanisms of cellular aging, longevity, stress resistance, and adaptation. However, little is known about yeast life within multicellular communities and only a few groups (including ours) have performed pilot studies that regard development, ageing, adaptation, and differentiation of both laboratory strain colonies (e.g., [18–22]; see also below) and biofilm colonies resembling natural biofilms in various aspects (e.g., [23, 24]).

2.1. Signaling, Stress Defense, and Colony Differentiation. Yeast colonies growing on a complex agar medium with a nonfermentable carbon source undergo several developmental stages characterized by changes in the pH of the surrounding medium, shifting from acidic to alkali and vice versa [25] (Figure 1). Alkalization of the medium is accompanied by the release of volatile ammonia that can act as a signaling molecule that is able to induce alkalization and ammonia production in neighboring colonies. Thus the development of colonies is synchronized [26]. After several days, the ammonia production declines and colonies enter the second acidic phase. Extensive transcriptional changes occur during the transition from the first acidic to the alkali phase (which occurs between day 7 and 11 of colony development) [27] indicating metabolic reprogramming from a typical respirative metabolism to a different, not yet fully characterized metabolic program. Among others, genes involved in the mitochondrial TCA cycle and oxidative phosphorylation are repressed while other metabolic genes (e.g., peroxisomal β-oxidation, amino acid metabolic genes, methyl glyoxylate cycle) are induced. Interestingly, in parallel to metabolic reprogramming, the repression of a group of genes that belong to environmental stress response (ESR) genes [28] was observed. These genes also include important players in oxidative stress defense *CTT1*, *SOD1,* and *CCP1* encoding for cytosolic catalase, cytosolic superoxide dismutase, and mitochondrial cytochrome c peroxidase, respectively, and a master regulator of ESR genes, *MSN4* [27]. Later on during the alkali-to-2nd-acidic-phase transition, some ESR genes, for example, *CTT1,* are derepressed, while the expression of others such as *SOD1* is kept lower than in the 1st acidic phase. Expression changes agreed with the levels of the enzyme activities. Ctt1p and Sod1p activities decrease during the alkali phase and increase again after the alkali phase has turned into the second acidic phase [19, 22].

In parallel with metabolic reprogramming, the cells of a colony population significantly diversify. Until the beginning of the alkali phase and ammonia production, the colony population is relatively homogeneous. Upon entering the alkali phase, nondividing cells in the colony, which account for the vast majority of the colony population, start to differentiate both in a horizontal and vertical direction. This horizontal diversification leads to the emergence of a cell population of nondividing or slowly dividing chronologically aged cells in the colony center and to the cell population of the colony periphery, where colony accrual occurs and a significant number of cells still divide. Notable physiological differences were found between central and marginal cells in terms of both their metabolism and stress-related features. First, central cells produce levels of ROS several times higher than cells from the colony margin and exhibit some features of programmed cell death, also in contrast to cells from the colony margin [19, 29]. Secondly, central cells maintain a relatively high activity of oxidative stress defense enzymes Sod1p, Sod2p, and Ctt1p during the alkali phase, while a significant decrease in these activities was observed in marginal cells [19]. Nevertheless, their increased antioxidant capacity obviously does not protect some of the central cells from high ROS production and cell death. Thirdly, changes in carbon metabolism typical for the acidic-to-alkali transition are mainly induced in the marginal cells [19].

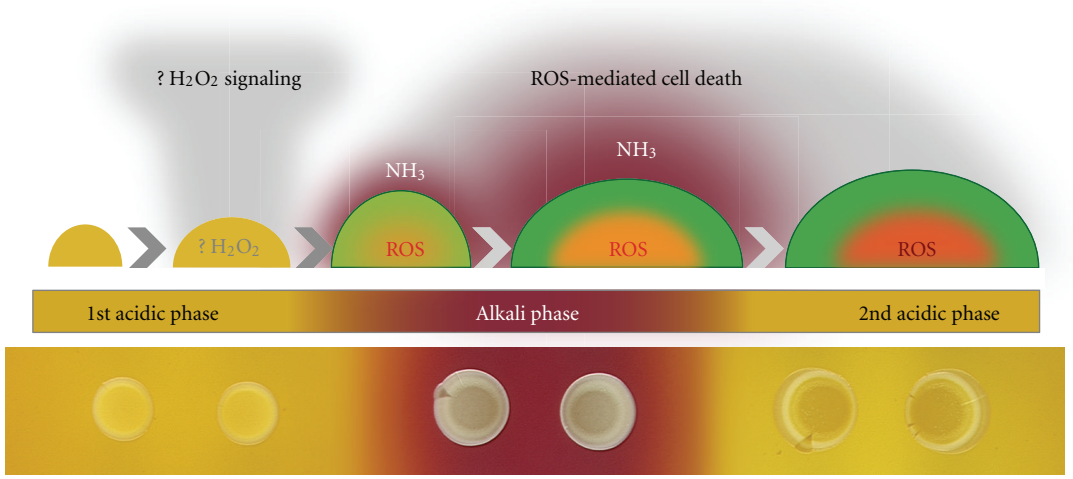

Figure 1: Regulatory role of H_2O_2/ROS in the development of yeast colony. Below: development of *S. cerevisiae* colony, which passes through 1st acidic, alkali and 2nd acidic. Above: periods of hypothesized H_2O_2 and ROS involvement in the regulation of colony development. Colonies were photographed with a Hitachi HV-C20 color camera with Cosmicar lenses, Kaiser Prolite illumination system, and NIS Elements software (Laboratory Imaging).

Ammonia signaling seems to be important for this differentiation. A detailed study of *sok2*Δ strain, Sok2p being a transcription factor involved in various signaling events regulated by the Ras-cAMP-PKA pathway, showed that the center-margin differences described in wild type colonies are diminished or absent in colonies of this ammonia-signaling-deficient strain [30]. Surprisingly, a very similar colony phenotype in terms of differentiation was observed in strains lacking mitochondrial superoxide dismutase Sod2p and cytosolic catalase Ctt1p, both of which diminish ammonia signaling [19]. However, the absence of cytosolic superoxide dismutase Sod1p has a different phenotype. Colonies of the *sod1*Δ strain produce ammonia at the same time and even in slightly larger quantities than the wild type with colony differentiation even more pronounced than in the wild type [19].

The vertical differentiation observed in the central part of the colony results in two cell layers, the upper and the lower, composed of cells possessing completely different physiologies [18]. Cells on the upper layer are multiple-stress resistant and long-living, while the cells of the lower layer produce more ROS, despite activating the expression of some stress-defense genes, and slowly die.

2.2. Role of Stress-Defense Mechanisms in Colonies: Direct Defense or Regulation? Some of the results obtained using colonial populations are in stark contrast to studies on yeast liquid cultivations under starvation conditions, where the indispensability of ESR genes (including those encoding stress defense enzymes) and genes involved in mitochondrial respiration for the long-term survival was shown [31–33]. For example, strains deficient in cytosolic superoxide dismutase Sod1p are known to have a severe oxygen-dependent growth defects, including lysine and methionine auxotrophies as a consequence of oxidative damage to the metabolic pathways synthesizing these amino acids [34]. The deletion of *SOD1* also dramatically decreases survival during aging [35]. These defects put selective pressure on the emergence of suppressor mutations compensating for these defects [36, 37]. On the other hand, the role of mitochondrial Sod2p and Ctt1p in the survival and longevity of liquid yeast populations is less evident. The absence of Sod2p has little effect during fermentative growth but its importance increases when growing on respiratory substrates, consistent with the presumed role of Sod2p in removing the superoxide radicals resulting from mitochondrial respiration [35]. The role of cytosolic catalase seems ambiguous, since its deletion leads to lower stress protection and a decreased ability to adapt to stress conditions on the one hand [38, 39], but also to increased survival during chronological aging on the other [40]. On the whole, antioxidant protection, or at least some of its components, seems to be important for the long-term survival of yeast populations in shaken liquid cultures.

The situation in colonies is almost the opposite. While *sod2*Δ and *ctt1*Δ colonies are incapable of ammonia signaling, sufficient metabolic reprogramming and differentiation and, consequently, their marginal cell population exhibit decreased survival, *sod1*Δ colonies produce ammonia, differentiate and survive in the same manner as wild-type colonies. In addition, *sod1*Δ colonies were almost free of cells with mutations suppressing the stress-sensitivity of *sod1*Δ in liquid cultivations. Altogether, *sod1*Δ, *sod2*Δ, and *ctt1*Δ behavior as well as the observed drop in some stress-defense enzyme activities during the alkali phase suggests that it is alkalization, ammonia production, and metabolic reprogramming, not stress defense and direct removal of radicals, that matter in colony differentiation and the survival of part of the population [19, 41]. In addition, from day 5 onwards, the ROS level in the colony is considerably lower

than that in liquid cultivations ([19] and unpublished data) in both wild-type colonies and colonies of the *sod1*Δ, *sod2*Δ and *ctt1*Δ strains.

Why do *sod1*Δ colonies develop normally with even more pronounced differentiation than wild-type colonies, while the deletion of some other genes involved in antioxidative defense (*SOD2*, *CTT1*) is deleterious to the colony's ability to produce ammonia and differentiate? One possibility is that a ROS signaling pathway exists that regulates colony development (Figure 2). This pathway would be activated in wild-type colonies at a particular point in their development and could regulate the beginning of ammonia production (Figure 1). As hypothesized in Figure 2, the signal would be stronger in *sod1*Δ colonies, but weaker in *ctt1*Δ colonies and even weaker or absent in *sod2*Δ colonies. The observed phenotypes cannot be simply explained by an increase or decrease in a particular ROS concentration and some more complex mechanism is likely to be involved. Superoxide dismutases catalyze the dismutation of superoxide to hydrogen peroxide and oxygen and so participate in the interconversion of various ROS types. Thus the absence of either of cytosolic or mitochondrial SOD would lead to an increase in superoxide concentration and, simultaneously, to a decrease in H_2O_2 concentration in the respective compartment. This leads to alternation in the homeostasis and/or ratio of H_2O_2 concentration between the mitochondrial matrix and cytosol. In contrast to superoxide, H_2O_2 is relatively stable and can penetrate into other cellular compartments through the membranes. Changes in its production in different compartments thus lead to changes in the H_2O_2 gradients and homeostasis and/or H_2O_2 concentration ratio between the mitochondrial matrix and cytosol. We propose that this ratio, rather than the absolute concentration of any of the ROS, is the signal that leads to some of the initial changes resulting in ammonia production, alkalization and to the physiological changes connected with it. This model is consistent with the phenotype of *ctt1*Δ colonies, since the absence of this enzyme lowers the mitochondrial-to-cytosolic H_2O_2 ratio, similarly to the *sod2*Δ strain. A possible mode of action of H_2O_2 includes the regulation of protein function through peroxiredoxins and thioredoxins, protein modifications by S-glutathiolation and direct inactivation by H_2O_2 [13, 42, 43].

Since it has been shown that different ROS trigger different adaptive responses [44], there clearly must be multiple ROS sensing pathways. That different ROS have different effects was shown in studies of the hormetic effect of superoxide and hydrogen peroxide on liquid cultivations. Both oxidants increase the longevity of the population when applied at moderate concentrations. While superoxide only induces longevity when applied during the logarithmic phase of growth, in contrast H_2O_2 only induces longevity when applied to stationary cultures [40, 45]. This would be consistent with its role in chronologically aging colonies, as proposed above. Moreover, evidence from plant research indicates that the ROS signal is often generated in short pulses [14]. This indicates that not only the amount and type of ROS, but also the precise timing of the ROS signal could be important.

Alternatively, ROS-scavenging enzymes could possess other regulatory functions, possibly independent of their antioxidant properties. For example, the function of the voltage-dependent anion channel (VDAC), a porin of the mitochondrial outer membrane, is diminished in the absence of Sod1p [46]. VDAC plays an important role in regulating mitochondrial activity and apoptosis [47] and its closing leads to a decrease in metabolite exchange and communication between the mitochondrial intermembrane space and the cytosol [48, 49]. The absence of Sod1p also affects metabolic regulation in cells [50]. Deletion of the *SOD1* gene causes a lessening of glucose repression, an important regulatory mechanism affecting nearly all aspects of *S. cerevisiae* metabolism. Moreover, *sod1*Δ cells have an increased level of mitochondrial biomass when growing in both media, with a repressing or nonrepressing carbon source. Both superoxide dismutases, Sod1p and Sod2p, were identified in a large-scale protein-protein interaction study as potential regulators of DNA repair and chromatin remodeling [51].

2.3. ROS in Programmed Cell Death in Colony. Beside their possible role in signaling leading to ammonia production and the start of colony differentiation, ROS seem to play an important role in the further development of differentiated cells (Figure 1). Programmed cell death in yeast can be induced by various signals, but their common factor is an increased ROS concentration (for reviews on yeast apoptosis, see [52–55]). ROS therefore seem to be the executioners of programmed cell death in yeast. As described above, cells in the center of a differentiated colony undergo programmed cell death, while cells at the colony margin are healthy and free of ROS [19, 29]. In contrast, the decreased center-margin differentiation observed in the colonies of non-ammonia-producing strains results in an increased cell death rate at the colony margin [19, 29]. A relatively high production of ROS occurs in the center of differentiated ammonia-producing wild-type and *sod1*Δ colonies. Notably, oxidative-stress-defense-deficient mutants with a defect in ammonia production (*sod2*Δ and *ctt1*Δ) do not exhibit increased ROS production in the colony center [19]. We propose that ROS are produced by the central cells in response to ammonia and/or alkalization and their production leads to cell death. ROS production in these cells is probably not a consequence of a low antioxidant capacity of the central cells, but rather part of a developmental program of the colony.

The dead cells in the colony center are likely to release nutrients that are then used by the cells at the edge of the colony to grow and survive [29, 56]. In addition, dead cells not only release nutrients from their biomass, but also stop consuming from the common nutrient pool. Since cells in the margin of the colony are younger and have better prospects of colonizing other localities, it makes sense if the colony invests in these prosperous cells at the expense of central cells. Opponents of the concept of programmed cell death in microorganisms could argue that cell death could bring no advantage for a unicellular organism, since the whole organism (one cell) dies and so this trait could

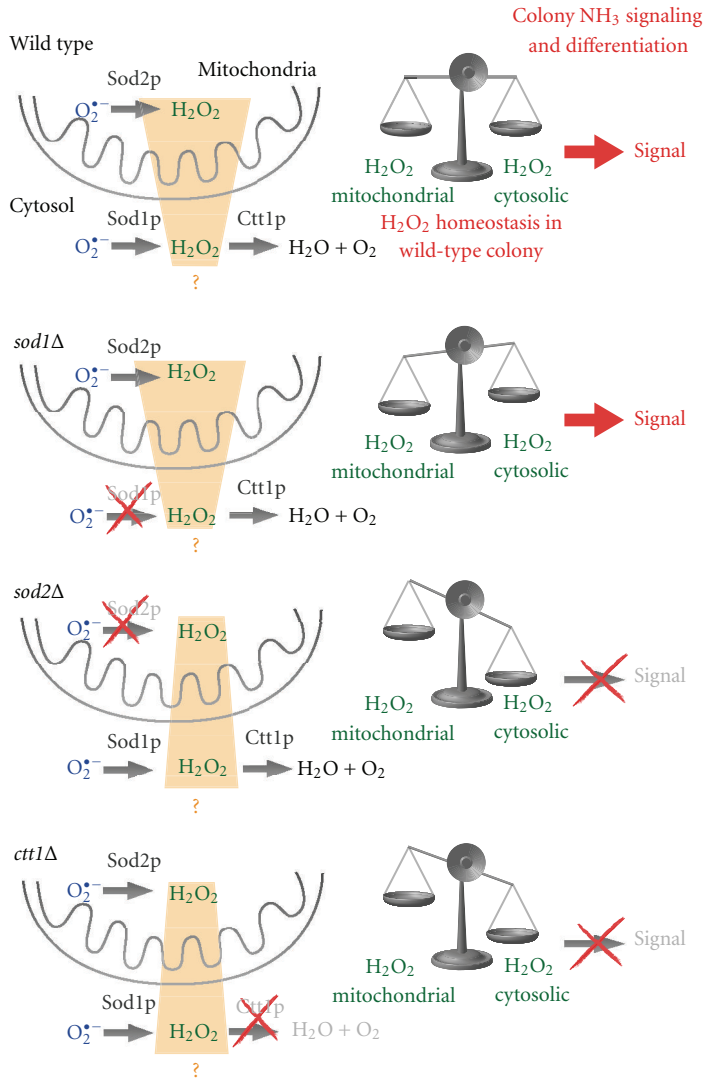

FIGURE 2: Hypothesis on the effect of H_2O_2 balance on induction of ammonia signaling. Left: predictions of changes in H_2O_2 concentration in cytosol and mitochondria of wild type and three oxidative-stress-defense-deficient strains based on reactions catalyzed by three main stress defense enzymes, cytosolic and mitochondrial superoxide dismutase Sod1p and Sod2p, respectively, and cytosolic catalase Ctt1p. Orange arrows indicate predicted H_2O_2 gradient between mitochondria and cytosol in wild-type strain and the three mutants. H_2O_2 gradient in wild-type cells is proposed on the basis of the prediction that mitochondria are the major site of ROS production in starving respiring cells from acidic-phase colonies. Right: the balances compare the mitochondrial-cytosolic H_2O_2-homeostasis of the particular mutant strain and the wild-type colony. The H_2O_2 imbalance towards the higher H_2O_2 concentration in the cytosol abolishes the induction of ammonia signaling and proper colony development and differentiation.

not be a subject to natural selection. However, when we consider unicellular organisms in terms of populations, it makes sense that the death of some cells could increase the prospects for survival of other cells in the same population. Moreover, microbial communities are often of clonal origin in nature; that is, they originate from one or a few cells and thus it is highly probable that the nutrients released by the sacrificed cells would benefit the kin of these cells, making this programmed cell death evolutionarily sustainable.

3. *Candida* Biofilms

Biofilms formed by *Candida* sp. can be serious problems in medical treatment, as they are usually highly resistant to extracellular toxins and drugs. ROS presumably play some role in *Candida* biofilms. Transcriptomic studies revealed that the biofilm population increases the expression of stress-defense genes, in particular those involved in combating oxidative stress, when compared to planktonic cultures [57].

Similar results were confirmed at the proteomic level [58]. The ROS level is dramatically decreased in biofilm when compared to a planktonic cultivation [58]. Whether this is a consequence of the activation of antioxidant mechanisms in biofilms or whether life in the biofilm *per se* results in a decreased ROS production (as with life in a colony) is unclear. It seems that a common mechanism for oxidative stress resistance and multicellular behavior exists in *C. albicans*, since cell adhesion, biofilm formation, and oxidative stress resistance are influenced by a common factor, the cell wall protein Hwp2p [59]. As the release of ROS during phagocyte respiratory burst is a crucial part of the immune response, adaptation to oxidative stress and oxidative stress defense enzymes help the yeast cells to survive respiratory burst and are thus important factors in pathogen virulence [60]. The increased oxidative stress defense of biofilms could also be responsible for their increased resistance to antifungal agents such as azoles, the toxicity of which involves the production of ROS [61].

3.1. Farnesol Signaling and ROS. Intercellular signaling by farnesol is involved in the induction of oxidative stress defense. Farnesol is a sesquiterpene alcohol produced by *Candida* sp. that acts as a quorum sensing (QS) molecule [62]. Quorum sensing is a synchronized transcriptional response of a microbial population to the presence of a small molecule called an autoinducer. Given that the autoinducer is produced continuously by all cells in the population, its concentration is proportional to the cell density.

Farnesol in *C. albicans* was shown to inhibit hyphae formation [63], to inhibit biofilm growth [64], to induce programmed cell death [65], to evoke ROS production [66], and to promote resistance to oxidative stress [67]. The latter involves farnesol-induced catalase expression via inhibition of the Ras-cAMP pathway [66] and, in parallel, farnesol-induced ROS production, which adapts cells to oxidative stress and induces protective mechanisms [68]. Farnesol thus acts as an intercellular adaptive signal that confers oxidative stress resistance to the cells within the same population. It is possible that the farnesol-induced high level of ROS and membrane-permeable hydrogen peroxide, in particular, participate in the farnesol signaling pathway, thus behaving as another intercellular signaling molecule. The possible signaling role of H_2O_2 in *C. albicans* is illustrated by the findings that low concentrations of hydrogen peroxide can induce the yeast-to-hyphal morphological transition [69], while higher concentrations induce programmed cell death [70, 71]. It was shown that H_2O_2 activates the AP-1-like stress-responsive transcription factor Cap1p, the stress-activated protein kinase Hog1p and also the checkpoint kinase Rad53p, which regulates hyperpolarized bud growth and filamentation [72–74]. Interestingly, these H_2O_2-regulated pathways are regulated and coordinated by the antioxidant enzyme thioredoxin, which appears to be a master regulator of redox signaling in *C. albicans* [72].

Farnesol also induces ROS production in other fungal and bacterial species [75–77]. Farnesol can thus act like an antibiotic, killing competing microbes and, in parallel, it induces mechanisms (e.g., cAMP-mediated oxidative stress adaptation) that protect the producing cells from farnesol's toxic effect. A similar strategy was described in killer toxins produced by different yeast species [78]. Different outcomes described for farnesol signaling (i.e., adaptation, differentiation or apoptotic cell death) could be the consequences of various concentrations of farnesol and combination of the farnesol signal with other factors, for example, other signaling molecules, cell physiology, nutrient status, and cell location in the biofilm.

4. Bacterial Biofilms

The stages of biofilm formation, that is, attachment, maturation, and dispersal of the bacterial biofilm, are all regulated by environmental cues as well as by intercellular signaling molecules [6, 79]. The role of various QS signals, indole, and polyamine signaling in the regulation of biofilm development has been described [80–82]. Beside these signaling molecules, ROS are another possible signal involved in biofilm formation (Figure 3). The role of ROS in cell death and the generation of genetic variants within a biofilm is well-described, while ROS' signaling function and cross-talk with other signaling pathways as well as their role in microbe-microbe, host-pathogen, or host-symbiont types of interactions are slowly emerging but mostly remain to be discovered.

4.1. ROS-Induced Diversity and Differentiation of Cells within a Biofilm. Many bacterial species develop genetic variability when growing within a biofilm, but not during cultivations of planktonic cells [83–86]. Variability was demonstrated as the frequency of the different colony morphotypes, resistance to antibiotics, swimming and sliding motility and exopolysaccharide production. Since different environmental conditions require different cell adaptations, genetic variability increases the chances of the community surviving under a broader spectrum of conditions. In the biofilms of *Pseudomonas aeruginosa*, the emergence of their genetic variability is dependent on oxidative-stress-induced DNA double-strand breaks and on their repair by the RecA system, which introduces genome rearrangements [84]. Interestingly, increasing resistance to oxidative stress or adding an antioxidant to the medium significantly reduced cell variance in the biofilm, while deletion of the catalase gene increased the variance [84]. ROS- and RecA-dependent biofilm cell variation was also described in *Listeria monocytogenes* [87]. Similar results were obtained from studies of *Staphylococcus pneumoniae* biofilm phenotypic variation. In this case, the "suicide" gene *spxB* encodes for pyruvate oxidase, which produces high amounts of hydrogen peroxide and which is responsible for the unusually high death rate in *S. pneumoniae* stationary cultures and possibly also in biofilms [88]. Likewise, SpxB-mediated production of H_2O_2 induces the cell death of about 10% of the population, leading to the release of DNA from the cells in two oral bacterial species, *Streptococcus sanguinis* and *Streptococcus gordonii* [89, 90]. This extracellular DNA (eDNA)

is an important part of the biofilm extracellular matrix, it enhances cell-cell adhesion, regulates biofilm dispersal, serves as a nutrient source, and is available to be taken up and incorporated into the chromosome by competent cells [91–95]. Given that streptococcal biofilms contain a high percentage of competent cells [96], eDNA release could be an important factor in creating genetic variability in biofilms. Moreover, the mutagenic activity of H_2O_2 [97] towards eDNA even increases this variability. Interestingly, *spxB* expression is controlled by the catabolic repression regulator CcpA, linking the roles of metabolism and H_2O_2 in biofilm development [98]. Hydrogen-peroxide-induced genetic variation and cell death were also reported in biofilms of *Pseudoalteromonas tunicata*, *Marinomonas mediterranea, Caulobacter crescentus,* and *Chromobacterium violaceum*, depending on the presence of the lysine oxidase encoded by the *alpP* gene and its homologues [99]. This hydrogen peroxide-producing enzyme is common among bacterial species, which makes variability and cell death regulated by ROS a common bacterial trait. Interestingly, AlpP-mediated cell death is also important for dispersal of the biofilm, that is, the release of planktonic cells from the biofilm. Cell death in the biofilm center presumably provides nutrients that increase the size, metabolic activity, and phenotypic variability of the dispersed cells [100]. Remarkably, besides H_2O_2, lysine oxidase also produces ammonia, but its possible signaling function in biofilms has not been explored. *P. aeruginosa* biofilm dispersal and cell differentiation are also regulated by the signaling molecule nitric oxide, a radical that could give rise to a spectrum of oxidants called reactive nitrogen species (RNS). Low concentrations of NO caused *P. aeruginosa* biofilm dispersal and enhanced swimming and swarming cell motilities, while higher NO concentrations induced cell death [101]. At least some of these effects are probably induced by NO-derived RNS, as RNS were detected in the biofilm. NO is produced by *P. aeruginosa* cells under anaerobic conditions through anaerobic respiration from nitrates and nitrites and is further reduced by NO reductase. Since the interior of a *P. aeruginosa* biofilm is a hypoxic environment [102, 103], the level of anaerobic NO and RNS production should be proportional to biofilm depth. In this way, cell position within the biofilm could be sensed and dispersal and cell death could be coregulated with biofilm growth [101].

Endogenous ROS production in the biofilm is the source of the high variability of biofilm cells, and ROS could act as a signal that mediates the cell death of a sensitive subpopulation in the deeper layers of the biofilm and metabolic differentiation in the upper part of the biofilm. The bacterial biofilm communities thus strikingly resemble metabolic differentiation and ammonia-regulated cell death in *S. cerevisiae* colonies described above. Whether ROS-mediated cell death is part of programmed colony/biofilm development or it is simply the inability of a sensitive subpopulation to withstand the accumulation of toxic byproducts of metabolism remains an unanswered question. The findings from yeast colonies and bacterial biofilms showing that cell death in one subpopulation leads to metabolic activity and variability in the other subpopulation, and thus increase fitness of the population as a whole, argue for the former option.

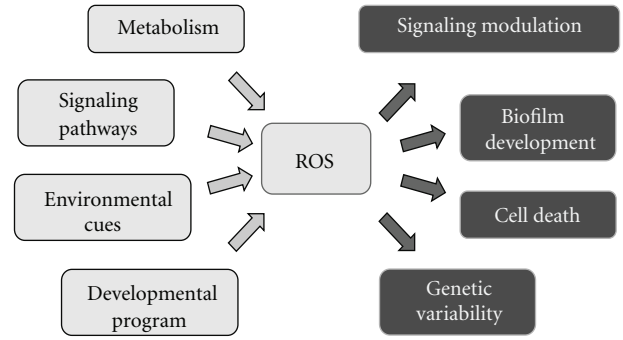

Figure 3: Inputs and outputs of ROS production in biofilms. Left: summary of processes inducing ROS production. Right: processes affected by ROS.

4.2. ROS-Dependent Signaling in Biofilm. Biofilm development is probably governed by both environmental cues as well as by intercellular signaling molecules. The best-studied intercellular signal is QS, which also plays a role in biofilm formation and development [6, 79]. A variety of autoinducer molecules have been identified in bacteria, of which the most studied are the species-specific acylated homoserine lactones (AHLs) found in many Gram-negative bacteria and the furanosyl borate ester AI-2 produced and recognized by both Gram-positive and Gram-negative species. It has been proposed that many autoinducers are able to induce ROS production, making ROS possible downstream signals or effectors of QS pathways [104]. QS plays an important role in the processes of biofilm formation and dispersal. For example, QS signaling through AI-2 in *Vibrio cholerae, Listeria monocytogenes, Bacillus cereus,* and *Staphylococcus epidermidiss* and the autoinducer protein AIP in *Staphylococcus aureus* inhibit biofilm formation. In contrast, a positive effect on biofilm formation was described for AI-2 signaling in *Bacillus subtilis, Lactobacillus rhamnosus* as well as in AHL-mediated QS in *P. aeruginosa* [79, 105]. Interestingly, ROS seem to be able to modulate quorum sensing in various ways. ROS can inhibit autoinducer peptide signaling in *S. aureus in vitro* [106]. In a study of the mouse skin infection model, ROS-producing enzymes of the immune system were indispensable for defense against infection by the wild-type *S. aureus* strain, but not necessary for defense against a QS-deficient strain, indicating that QS molecules could be a target for oxidation by the immune system *in vivo* [106]. Superoxide also decreases the expression of QS locus *comQXP* in *B. subtilis* [107]. On the other hand, ROS have the potential to increase the QS signal, as certain derivates of AHL oxidation by ROS exhibit increased biological activity [108]. In addition, QS regulates the expression of oxidative stress defense genes in various bacterial species [109–112].

Additional indications of ROS-dependent signaling pathways regulating biofilm growth have recently appeared. *Enterococcus faecalis* biofilm formation is dependent on the presence of the *xdh* gene, presumably encoding for a

selenoprotein xanthine dehydrogenase involved in purine metabolism and uric acid utilization and possibly evoking ROS production [113]. Cells in an *E. faecalis* biofilm produce high levels of ROS via a mechanism that is reliant on the presence of xanthine dehydrogenase, its cofactors selenium and molybdenum and its substrate uric acid. An intriguing model was proposed, in which uric acid in the environment is metabolized by *E. faecalis* cells with concomitant H_2O_2 production, which in turn induces biofilm formation. H_2O_2 thus would be a metabolic byproduct with a signaling function. Since uric acid is abundant in blood and urine, that is, preferred environments for *E. faecalis*, it makes sense that detecting this metabolite triggers the formation of the biofilm to successfully colonize the host [113].

In multispecies oral biofilms, streptococci produce H_2O_2 from pyruvate as a mean of biochemical warfare against other species, as well as a regulator of its own development as described above. However, the oral pathogen *Aggregatibacter actinomycetemcomitans* uses this *streptococci*-produced H_2O_2 as a signal that activates the expression of the compliment resistance protein ApiA, which helps *A. actinomycetemcomitans* to resist the host's nonspecific immune response [114].

Some results suggest that ROS play a role in modulating the indole signaling pathway. Indole acts as an intercellular signal in many bacterial species [80]. Similarly to other signaling molecules, indole even induces a response in some species that do not synthesize it, and thus acts as an interspecies signaling molecule. In *Escherichia coli*, indole, which is synthetized by the enzymes coded by the *tnaAL* operon, inhibits biofilm growth regulates pathogenicity and the expression of multidrug resistance genes [115, 116]. Among other signals, *tnaA* expression is induced by ROS and repressed by growth in a biofilm [117, 118]. Indole was proposed to act as an oxidant in membranes and to induce membrane rearrangements [119]. Furthermore, some antibiotics induce indole production, which consequently inhibits biofilm formation in *E. coli,* via a mechanism involving hydrogen peroxide [120], showing that indole signaling and H_2O_2 cooperate in a pathway inhibiting biofilm growth. The opposite results, that is, indole-induced biofilm formation in *E. coli* and other species, were reported by others [121].

4.3. Biofilms and Oxidative Stress Adaptation. As with *C. albicans* biofilms and *S. cerevisiae* colonies, there is obviously a connection between the life of bacteria within a multicellular structure (biofilm) and their adaptation to oxidative stress. A number of genes have been identified that are important for both biofilm growth and oxidative stress resistance. Examples are the transcriptional repressor Rex and trigger factor RpoS in *Streptococcus mutans* [122, 123], *in-silico*-identified genes *uspE* and *gadX* in *E. coli* [124], posttranslational regulator CsrA in *Campylobacter jejuni* [125] and two-component systems GacS-GacA in *Pseudomonas* sp. and ColR-ColS in *Xanthomonas citri* [126, 127]. The redox-sensitive DNA-binding protein OxyR is a well-studied transcription regulator that mediates oxidative stress response in many Gram-negative bacteria [128]. OxyR is activated by forming an intramolecular disulfide bond upon reaction with H_2O_2, which leads to the expression of OxyR-regulated genes. The role of OxyR in biofilm formation has been described in several bacterial species. In *E. coli*, OxyR induces biofilm formation by activating expression of the surface adhesin Ag43, which is responsible for cell-to-cell attachment and surface adherence [129]. Similarly, OxyR regulates cell attachment by increasing the expression of adherent fimbriae in *Serratia marcescens* and *Klebsiella pneumoniae* [130, 131] and OxyR's function in biofilm formation was also reported in *Neisseria gonorrhoeae* and *Tannerella forsythia* [132, 133].

5. Conclusions

Both endogenous and exogenous reactive oxygen species are important stress factors in the life of microorganisms. Endogenous ROS production is an inevitable consequence of microbial life in the presence of oxygen and can be even potentiated by some antibiotics that induce ROS production in sensitive microbes [134–136]. Exogenous ROS can be encountered during immune response to the presence of microbes inside the animal or plant body. In addition, many bacterial species release ROS as an oxidative weapon against competitors in multispecies populations. In these cases, ROS are produced by specialized enzymes. ROS are thus widely used as a means of biochemical warfare in nature. In order to defend against the deleterious effects of ROS, microorganisms have evolved efficient mechanisms of ROS removal. On the other hand, various pieces of data suggest that ROS could play an active and important role in processes like growth autoinhibition, cell death, and biofilm/colony development in both yeast and bacteria. In such cases, the enzymes producing ROS are tightly regulated as part of a biofilm developmental program and ROS are the effectors of some intrinsic regulation. Finally, ROS can act as signaling molecules either by targeting specific signaling pathways (e.g., kinases or transcription factors) or by, for example, modifying other signaling molecules such as quorum sensing factors. The large number of ROS-producing enzymes and the many different responses to ROS suggest that ROS-mediated processes are universal in the microbial world. Improving our understanding of the regulation and signaling driven by ROS could thus provide deeper insight into complex biological processes including the formation of biofilms, multicellular structures with important implications in medicine and other fields. The possibility of interfering with the signaling involved in biofilm formation or biofilm dispersal with ROS-producing or ROS-scavenging agents is especially attractive.

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Review Article

Cardiac Response to Chronic Intermittent Hypoxia with a Transition from Adaptation to Maladaptation: *The Role of Hydrogen Peroxide*

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Obstructive sleep apnea (OSA) is a highly prevalent respiratory disorder of sleep, and associated with chronic intermittent hypoxia (CIH). Experimental evidence indicates that CIH is a unique physiological state with potentially "adaptive" and "maladaptive" consequences for cardio-respiratory homeostasis. CIH is also a critical element accounting for most of cardiovascular complications of OSA. Cardiac response to CIH is time-dependent, showing a transition from cardiac compensative (such as hypertrophy) to decompensating changes (such as failure). CIH-provoked mild and transient oxidative stress can induce adaptation, but severe and persistent oxidative stress may provoke maladaptation. Hydrogen peroxide as one of major reactive oxygen species plays an important role in the transition of adaptive to maladaptive response to OSA-associated CIH. This may account for the fact that although oxidative stress has been recognized as a driver of cardiac disease progression, clinical interventions with antioxidants have had little or no impact on heart disease and progression. Here we focus on the role of hydrogen peroxide in CIH and OSA, trying to outline the potential of antioxidative therapy in preventing CIH-induced cardiac damage.

1. Introduction

Obstructive sleep apnea (OSA) is a highly prevalent respiratory disorder of sleep, characterized by recurrent episodes of complete or partial upper airway obstruction. It may develop at different ages from the premature infant to the elderly, with a prevalence related to age and sex, increasing significantly in the elderly (5–9%) [24]. The prevalence of OSA in children is generally 1–3% [25] and even higher (e.g., 5-6%) in certain conditions [26]. The prevalence of OSA in the general population of adult men and women is 3–7% and 2–5%, respectively [27, 28] and keeps increasing in the elders [24]. OSA was often found in the patients with diabetes and obesity since it is involved in the development of insulin resistance, glucose intolerance, type 2 diabetes, and metabolic syndrome independently of adiposity [29–32]. OSA was also considered as a causal factor in cardiovascular diseases [33, 34].

Collapsibility of the upper airway during sleep can be increased by underlying anatomical alteration and/or disturbances in upper airway neuromuscular control, both of which play key roles in the pathogenesis of OSA [35]. OSA-induced recurrent episodes of complete or partial collapse of the upper airway during sleep are associated with reductions in ventilation, which often leads to repetitive apneas and hypopneas. Each of these obstructive respiratory events results in recurrent episode of hypoxia. Reoxygenation occurs when the episode is terminated by an arousal that restores the airway patency. The recurrence of these hypoxia and reoxygenation episodes produces a characteristic pattern of nocturnal intermittent hypoxia that is unique to OSA. Generally, both apneas and hypopneas events can produce the same pattern of chronic intermittent hypoxia (CIH), sleep arousals also called sleep fragmentation, hemodynamic changes [36–38], and symptoms of disruptive snoring and daytime sleepiness. Clinical consequences of the disorder

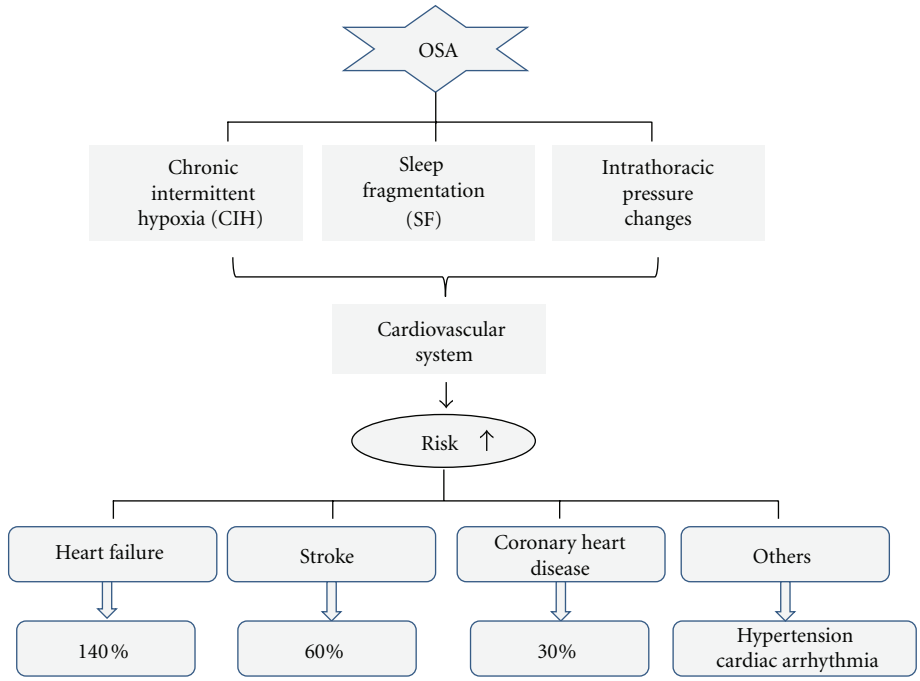

Figure 1: OSA-induced cardiovascular diseases. OSA increases the risk of cardiovascular diseases through three main pathological processes related with OSA: chronic intermittent hypoxia (CIH), sleep fragmentation (SF), and intrathoracic pressure changes. It can increase the risk of heart failure by 140%, stroke by 60%, and coronary heart disease by 30%. It can also increase the risk of other cardiovascular diseases such as hypertension and cardiac arrhythmia.

cover a wide spectrum, including daytime hypersomnolence, neurocognitive dysfunction, cardiovascular disease, metabolic dysfunction, and corpulmonale [37].

It has been recognized that OSA is a potential life-threatening condition and has become one of the most common public health problems [39, 40]. Since OSA is emerging as a cardiovascular risk factor, it became an important target for public health interventions aiming at reducing cardiovascular diseases [41].

Mammalian heart is an obligate aerobic organ and a constant supply of oxygen is indispensable for cardiac viability and function [42]. The heart as a contractile pump has the highest O_2 consumption among all body organs, which can be increased eight-fold or more under maximal workload conditions [43]. Consequently, cardiac tissue is susceptible to lack of O_2 and also highly dependent on oxidative metabolism to maintain its normal function [44]. Since CIH is one of the main events of OSA, OSA has been considered as an independent risk factor for cardiovascular morbidity [41, 45–47], such cardiovascular diseases including hypertension [45–53], stroke [54], and even glucose metabolic abnormality [55], and so forth. For instance, it has been reported that OSA can increase the risk of heart failure (HF) by 140%, stroke by 60%, and coronary heart disease by 30% [56].

As outlined in Figure 1, three major components of OSA associated with cardiovascular events are large swings in intrathoracic pressure, postapneic arousals, and CIH [57]. A body of experimental evidence has indicated that CIH is a unique physiological state with a profile of biological consequences distinct from other types of hypoxia [46, 47, 51]. In Figure 1, the negative cardiovascular consequences of OSA are illustrated, all of which were considered critically related to the unique pattern of CIH [38, 52, 53, 58]. In this paper, therefore, we would like to summarize the several features of CIH-induced cardiac changes at the early and later stages, based on both epidemiological and experimental animal information.

2. Cardiac Response to CIH: A Transition from Adaptation to Maladaptation

It has been reported that innate defense systems (adaptation) that are induced by exposure to repeated and relatively brief episodes of hypoxia act as a powerful temporal protective phenomenon [59]. Adaptation to chronic hypoxia was also reported to increase cardiac tolerance to all major deleterious consequences of acute oxygen deprivation such as myocardial infarction, contractile dysfunction, and ventricular arrhythmias [60]. However, long-term exposure to CIH may be detrimental to cardiac function (maladaptation). OSA exposes the cardiovascular system to CIH, oxidative stress, and systemic inflammation. With the extension of exposure duration to CIH, these factors can compel the heart to induce a transition from cardiac compensative (such as cardiac hypertrophy) to decompensative change (such as HF), that is, a transition from cardiac adaptation to cardiac maladaptation. OSA-related CIH has been suggested to play the main role in the development of left ventricular

Table 1: The effects of CIH on cardiac I/R-induced injuries.

Authors	Strains	Exposure time	Effects	Reference
Wang et al.	Male SD rats	4 wks	Protective effects	[1]
Ding et al	Male SD rats	42 days	Protective effects	[2–4]
Neckar et al.	Male Wistar rats	8 h/day, 5 days/wks;	Protective effects	[5]
Asemu et al.	Male Wistar rats	2 wks and 5 wks	Protective effects	[6]
Park and Suzuki	C57BL/6 mice	1, 2 wks and 4 wks	Protective effects (4 wks) Deleterious effects (1, 2 wks)	[7]
Guo et al.	male guinea pigs	28 days	Protective effects	[8]
Guo et al.	Male guinea pigs	28 days	Protective effects	[9]
Zong et al.	Dogs	20 days	Protective effects	[10]
Wang et al.	Male SD rats	14, 28 and 42 days	Protective effects	[11–13]
Joyeux-Faure et al.	Male Wistar rats	7 wks	Deleterious effects	[14]

* wks: weeks.

remodeling including cardiac hypertrophy, cardiac fibrosis, and cardiac dysfunction [61, 62]. This transition can impair myocardial contractility and cause development and progression of HF [63].

2.1. Cardiac Adaptation to CIH

2.1.1. CIH Effectively Protects the Heart against Ischemia/Reperfusion or Hypoxia/Reoxygenation-Induced Injury. It is reported that CIH can induce preconditioning-like effect to effectively protect the heart against ischemia/reperfusion (I/R) or hypoxia/reoxygenation-induced injury [1–6, 8–11, 64, 65], including the prevention of I/R-induced cardiac apoptosis and necrosis [3, 5] arrhythmias [6]. CIH also can improve postischemic recovery of cardiac function [1].

For instance, Park and Suzuki exposed C57BL/6 mice to CIH for 1, 2, and 4 weeks and then observed the effect on I/R injury. Results demonstrated that CIH exposure for 1-2 weeks resulted in increased susceptibility of the heart to I/R injury, while the heart appeared to adapt to CIH for 4 weeks by normalizing its susceptibility to I/R injury [7]. Their results imply that heart also has the ability to adapt to the condition of the CIH-mediated enhancement of the susceptibility to I/R. Guo et al. found that although cardiac hypertrophy may not occur in the right and left ventricles, the adaptation of guinea pigs to CIH significantly increased cardiac tolerance to I/R, shown by an improved recovery of contractile function, an increased coronary flow and a reduced level of reactive oxygen species (ROS) in cardiomyocytes [9]. These results are consistent with those observed in dogs and rats [4, 10, 11]. These data clearly show that CIH-induced cardiac protection against I/R-induced injury universally exists in different strains of animal models. CIH can promote recovery of cardiac contractile function from I/R, limiting cardiac infarction and arrhythmia caused by I/R [10, 12, 13]. Table 1 presents a summary of the protective effect of CIH on cardiac I/R-induced injuries. It seems that extensive cardiac protection by short-term CIH (less than 6 weeks) may be very similar to ischemic preconditioning, most likely sharing common signaling pathways [66].

2.1.2. CIH Induces Compensatory Increase of Cardiac Function. Naghshin et al. [15] exposed C57BL/6J mice to CIH for 4 weeks and then assessed cardiac function by echocardiography and pressure-volume loop analyses. They found that left ventricular (LV) ejection fraction (LVEF) and other measures of LV contractility were increased in CIH-exposed animals compared to controls. There was no change in contractile proteins, atrial natriuretic peptide levels, LV posterior wall thickness, or heart weight following the exposure to CIH. These results indicated that there was a compensatory increase in LV cardiac contractility that occurred independent of ventricular hypertrophy in the mouse model of CIH. It has been proposed that this adaptation, at least in part, results from activation of cardiac β-adrenergic pathways [15]. Lee et al. [16] showed that short-term IH (for 1 and 4 days) has protective effects on the heart. Campen et al. [17] found that CIH has produced a 26% increase in right ventricle weight and 10% increase in LV + septum weight, resulting in a significant increase by14% of the right ventricle/LV + septum ratio. Table 2 summarized previous studies available in the literature, which showed that cardiac hypertrophy may initially represent an adaptive response, but with the extent of exposure time, the hypertrophy ultimately is responsible for ventricular dilatation and HF. These results suggest that there is transition for cardiac response to CIH from adaptation to maladaptation.

2.2. Cardiac Maladaptation to CIH

2.2.1. CIH Enhances Cardiac Susceptibility to I/R-Induced Injury. While short-term CIH was found to elicit a preconditioning-like events [2, 3, 67], Joyeux-Faure et al. [14] reported that CIH increased the heart susceptibility to I/R-induced injury. They exposed Wistar male rats to CIH for 7 weeks and then exposed the heart to ischemia and reperfusion for 30 and 120 min. Cardiac infarct sizes were found to be significantly higher in CIH group in comparison to the control group. This was the first study to show that CIH makes the heart more sensitive to I/R injury and suggested that although short-period exposure to CIH can stimulate protective mechanism to reduce subsequent

Table 2: Effect of CIH on cardiac functions.

Authors	Strains	Exposure time	Effects	Reference
Naghshin et al.	C57BL/6J mice	4 wks	Protective effects	[15]
Lee et al.	SD rats	1, 4 days and 1, 2 wks	1, 4 days: protective effects 1, 2 wks: deleterious effects	[16]
Campen et al.	C57BL/6J mice		Deleterious effects	[17]
Chen et al.	Male SD rats	5 wks	Deleterious effects	[18, 19]
Williams et al.	Male SD rats	5 wks, 6 wks	Deleterious effects	[20]
Chen et al.	Male SD rats	10 days	Deleterious effects	[21]
Yin et al.	FVB mice	4 wks and 8 wks	Deleterious effects	[22]
Yang et al.	Patients	4 wks and 8 wks	Deleterious effects	[23]

* wks: weeks.

lethal I/R-induced injury as discussed above, prolonged CIH beyond protective periods might turn to a condition with increased susceptibility to oxidative stress such as I/R-induced cardiac injury (Table 1).

2.2.2. CIH-Induced Cardiac Dysfunction. Similar to hypoxia-provoked maladaptive consequences on cardiorespiratory homeostasis [68–70], long-term exposure to CIH may decrease cardiac functions [18, 19]. OSA-induced CIH and hypercapnia are related to continuous changes in pulmonary volume, intrathoracic pressure, and microarousals. These repetitions of respiratory events and rapid changes in alertness are the main reasons why OSA induces acute hemodynamic modifications, including the heart rate, blood pressure, and cardiac output. In addition, CIH also contributes, in the long run, to an increase in autonomous nervous system drive, generation of ROS, endothelial dysfunction, and metabolic abnormalities, which in turn increase both the blood pressure and the cardiovascular risks [71]. Short-term changes of hemodynamic parameters include hypoxia, hypercapnia, negative intrathoracic pressure, and microarousal, while long-term changes of hemodynamic parameters include sympathetic activity, metabolic and hormonal changes, oxidative stress, inflammation, endothelial dysfunction, hypercoagulability, and genetic effects [71].

Rodent models of CIH exposure lasting from days to weeks have exhibited multiple adverse outcomes, including hypertension [17, 72], insulin resistance [73, 74], hyperlipidemia [75], atherosclerosis [76], and increased size of experimentally induced cardiac infarction [7, 14]. In rats, CIH leads to several cardiovascular consequences that are also observed in human OSA, including blood pressure elevation, biventricular hypertrophy, and LV contractile dysfunction [18–20, 53]. These data suggest that CIH exposure is detrimental to many physiological processes. Accumulating data indicate that CIH can induce cardiac dysfunction as its maladaptation [18, 19, 21]. Several studies have focused on the point that CIH induces cardiac dysfunction, but conclusions were inconsistent. Lee et al. [16] showed that short-term CIH exposure of rats for 1 and 4 days appeared to exert protective effects on the hearts, whereas long-term CIH exposure for 1 and 2 weeks appeared to exert deleterious effects. Williams et al. also used rats to confirm that after exposure to CIH for 10 days, cardiac function was decreased [20]. Fagan reported that treatment of CB57BL/6J mice with 2 min cycles of 10% alternating with 21% oxygen for 8 h per day for 4 weeks increased right ventricular systolic pressure, right ventricular mass, and neovascularization of distal pulmonary vessels, but had no effect on LV mass [77]. Campen and coworkers [17] reported that CB57BL/6J mice exposed to a 5-week regimen of 60-second cycles of 21% alternating with 5% oxygen for 12 h per day did develop significant increases in both systemic and pulmonary vascular pressures showing more prominent right than LV hypertrophy. Chen's group exposed male Sprague-Dawley rats to CIH for 4 and 8 weeks and found that abnormal myocardial architecture and increased interstitial space were observed after 4 weeks and became more obvious at 8 weeks [21]. In addition, a decreased ratio of wall thickness to cavity diameter and cardiac dilatation were observed after 8 weeks. They have proposed that a longer duration of CIH could induce abnormal myocardial architecture and even lead to the transition from eccentric cardiac hypertrophy to dilated cardiac hypertrophy. In our own study, we used 8–10-week-old FVB mice for exposure to CIH for 4 and 8 weeks. Exposure to CIH for 4 weeks induced cardiac hypertrophy, cardiac fibrosis, cardiac inflammation, and even cardiac dysfunction. Beside the above changes, exposure to CIH for 8 weeks induced cardiac apoptosis, oxidative stress and damage along with cardiac dysfunction, showing progressive pathological changes [22].

The diversity of observations among different research groups implies that the difference of cardiac responses to CIH may be related to many factors: (1) different resistance to hypoxia of different animals [15, 16, 21, 77]; (2) age of the animals that were used for starting the experiments; (3) the different protocols of CIH (including oxygen level, cycle length, number of hypoxic episodes per day, and number of exposure days, etc.). For example, Zhang et al. [78] have exposed postnatal male Sprague-Dawley rats to intermittent hypobaric hypoxia (IHH) at 3000 m high-altitude (P_B = 525 mm Hg, P_{O_2} = 108.8 mm Hg) or at 5000 m high-altitude (P_B = 404 mm Hg, P_{O_2} = 84 mm Hg), and sham control, and then perfused the isolated hearts in the Langendorff system with 30 min. global ischemia and 60 min. reperfusion. They found that the recovery of cardiac function was enhanced,

coronary flow was increased and lactate dehydrogenase activity was decreased in 3000 m-IHH group at 60 min after I/R. In addition, cardiac function restored better in 3000 m-IHH group after 42 days of IHH than that after 28 days of IHH. In contrast to the above, the recovery of cardiac function was lower, coronary flow was decreased, and lactate dehydrogenase activity was increased in 5000 m-IHH group. The results suggest that the effect of CIH on I/R could be affected by the modes of IH exposure. In addition, the duration of CIH was the key determinant for the different effects on the I/R-induced cardiac damages and functional changes [15, 17, 21].

Although there are some differences of CIH-induced cardiac functional changes among animal models, Yang et al. [23] have examined the cardiac function of OSA patients with echocardiogram and found that the LVEF, fractional shortening (FS), and the ratio of early-to-late diastolic filling in patients with severe OSA was lower than in those with moderate OSA and in healthy controls. The inner diameters of the main pulmonary artery and right ventricle as well as the thickness of anterior wall of the right ventricle were increased in patients with severe OSA compared to those with moderate disease, which was worsened as a function of time with disease. The tissue Doppler imaging-derived Tei index and pulmonary artery systolic pressure were also increased along with the severity of OSA. LVEF and FS in patients who had suffered from OSA over 10 years were decreased compared to those suffering from OSA for a shorter time. LVEF and FS in patients with secondary hypertension were decreased significantly relative to nonhypertensive OSA patients and healthy controls. The ratio of early-to-late diastolic filling was decreased in OSA patients whether they had secondary hypertension or not. These results suggest that OSA affects the LV diastolic function in the early stage of the disease. Extended exposure to OSA resulted in LV systolic dysfunction with increased hypertension. Right ventricle dysfunction and abnormalities became more severe as the disease progressed. The results confirm the conclusion from clinical data that the duration of OSA is the key factor for OSA-induced cardiac damage.

HF is frequently observed in OSA patients, with a prevalence of 11–37% [79, 80]. Data obtained in the Sleep Heart Health Study have shown a 2.38 times increased likelihood of having HF in association with OSA, independent of other risk factors. OSA is not only a consequence of HF but indeed represents a risk factor for this condition [81], independent of hypertension [82]. Several grades of cardiac alterations have been reported in OSA patients, from silent or subclinical echocardiographic LV abnormalities to symptomatic systolic dysfunction (Table 2).

Based on the above discussion, we propose that the longer exposure to OSA-relevant CIH, the worse of the cardiac dysfunction. With the extension of exposure time to CIH, cardiac functions transit from compensative to decompensative phase as from cardiac hypertrophy to cardiac dilation even cardiac failure at the end. The development of contractile dysfunction is an important feature of cardiac decompensation (maladaptation) during CIH exposure to stress and also a hallmark of adverse cardiac remodeling [83].

3. Oxidative Stress Is the Predominant Mechanism of Cardiac Response to CIH: Role of Hydrogen Peroxide

Although OSA has been implicated in the pathogenesis of various cardiovascular diseases, mechanisms by which OSA affects the cardiovascular system are largely unknown. Oxidative stress, endothelial dysfunction, and inflammation are long-term consequences that mediate cardiovascular disease in patients with OSA [38]. Now, numerous studies have shown that oxidative stress is the main mechanism of cardiac I/R injury [84–86]. Because there is resemblance between the patterns of CIH associated with OSA and I/R injury, potential mechanisms of oxidative stress in OSA have been postulated to be related directly to CIH in a manner similar to I/R injury or indirectly via inflammatory response. The increased sympathetic tone and elevated catecholamine level might also be associated with increased ROS production [38]. Most recent studies in patients with OSA and animal models of CIH confirm that OSA is associated with oxidative stress, which generally correlates with the severity of sleep apnea, and improves with treatment [38, 87].

Oxidative stress represents an imbalance between the production of ROS and the antioxidant capacity of a biological system to buffer ROS. On the other hand, ROS are involved in signaling cascades, so that subphysiological ROS production may lead to reductive stress which has recently also been suggested to be detrimental in certain cardiac conditions [43, 88]. A fine balance between redox state and metabolism is more important than oxidative stress itself, and an imbalance in either the oxidative or the reductive direction could be detrimental [43]. In the past, ROS were considered solely injurious, but now it is generally accepted that they may exert both deleterious and beneficial actions [89]. In the last decade, they were consistently described as regulators of signal transduction and as second messengers in many signaling pathways in all cells that mediate cardioprotection [90–93]. The data of Kolar and Ostadel suggest that ROS not only contribute to I/R injury in normoxic rat hearts, but also are involved in the protective mechanisms induced by CIH [60]. During adaptation to CIH, repetitive cycles of hypoxia and reoxygenation (sublethal stresses) may lead to the production of ROS in the hypoxic heart, and the latter trigger a cascade of events that lead to increased antioxidant enzyme activity. These findings indicate that the ROS produced in cardiac tissue during sub-lethal stress may participate and activate signal transduction pathways [94] that form a positive feedback loop consisting of ROS and transcriptional factors [95]. This kind of strictly regulated generation of ROS at low levels can mediate physiological functions, such as increased level of antioxidative protection (enzymatic and nonenzymatic), growth, differentiation, and metabolism in cardiomyocytes [42, 96]. It may be involved in potentially adaptive processes such as adaptation to hypoxia and modulation of excitation—contraction coupling. On the other hand, the generation of higher levels of ROS and/or more potent oxidants such as hydroxyl radical may result in pathological changes as a result of macromolecular damage

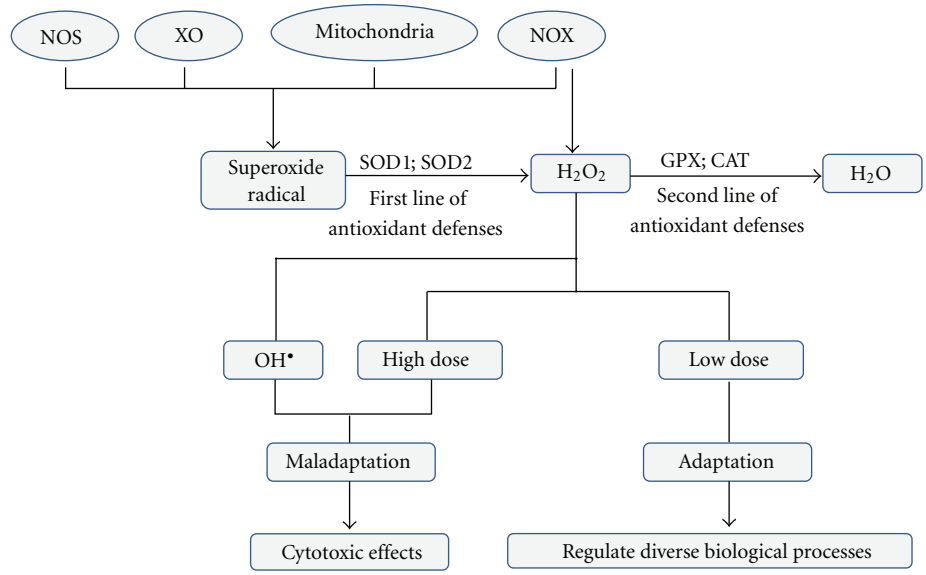

Figure 2: The schematic diagram of H_2O_2 source and effects. SOD can catalyze the dismutation of the superoxide radical to H_2O_2 and molecular oxygen. Then it can be converted to H_2O by CAT and GPX. H_2O_2 also can be converted either to water or OH•. Low dose of H_2O_2 can induce adaptation, regulating diverse biological processes. High dose of H_2O_2 together with its conversion to OH• can induce maladaptation and cause cytotoxic effects. NOX: NADPH oxidase; NOS: uncoupled NO synthases; XO: xanthine oxidase; H_2O_2: Hydrogen peroxide; OH•: hydroxyl radical; SOD: superoxide dismutase; CAT: catalase; GPX: glutathione peroxidase.

as well as inadequate signaling [43]. It is important to note that in addition to ROS, reactive nitrogen species, such as nitric oxide, play crucial roles in the regulation of cardiac functions. The metabolism of reactive nitrogen species is intertwined with ROS.

Sources of ROS and RNS in cardiomyocytes include mitochondria [97, 98], NADPH oxidase [99, 100], xanthine oxidase [101, 102] and uncoupled nitric oxide synthases [103, 104]. ROS include superoxide radical anion, hydroxyl radical, and hydrogen peroxide (H_2O_2). The superoxide is a relatively nonreactive, particularly targeting metalloproteins. It can be dismutated to H_2O_2 by superoxide dismutase. ROS exerts biological effects either by causing nonspecific oxidative damage to DNA, proteins, lipids, and macromolecules or through specific modulation of cellular signaling pathways (redox signaling). Cellular levels of ROS and their effects are regulated by a variety of specific and antioxidant systems (e.g., catalase, superoxide dismutase, glutathione peroxidase, peroxiredoxin, thioredoxin, and various vitamins) [105].

H_2O_2 is membrane permeable and diffusible, less reactive and longer-lived than hydroxyl radical or superoxide radical anion, and it is best suited for intra- and even inter-cellular signaling [106]. The physiological range of intracellular H_2O_2 concentrations appears to be remarkably conserved in different forms of life [107]. Among ROS, H_2O_2 is the only species that is generated and removed by several specific enzymes, which suggests that the intracellular concentration of H_2O_2 is tightly regulated and may serve specific cellular functions. Superoxide dismutase can catalyze the dismutation of the superoxide radical to H_2O_2 and molecular oxygen. Then it can be converted to H_2O by catalase and glutathione peroxidase (Figure 2). H_2O_2 may react with transition metals, such as iron or copper to produce the highly reactive hydroxyl radical. In living organisms, besides its well-known cytotoxic effects, H_2O_2 also plays an essential role as a signaling molecule in regulating diverse biological processes such as immune cell activation, vascular remodeling, and apoptosis [108–110].

Although H_2O_2 can contribute to I/R injury, it appears to play the part of activator in processes in which CIH upregulates the antioxidant enzymes. Using a cultured chick embryonic cardiomyocyte H/R model, Zhang et al. demonstrated that H_2O_2 is involved in ischemic preconditioning [111]. Preconditioning protects ischemic cardiomyocytes through H_2O_2 by opening mitochondrial K-ATP channels via activating PKC-ε pathway [111]. Park and Suzuki presented evidence that a redox regulator, thioredoxin, which can scavenge H_2O_2, is upregulated in adapted hearts in response to I/R and downregulated in the heart showing increased susceptibility to I/R following 2 weeks of CIH [7]. Their results suggested that thioredoxin plays a role in this adaptive mechanism as a scavenger of H_2O_2 and also represent a proof about H_2O_2's role in cardiac maladaptation. The Janus character of H_2O_2 as a mediator of growth and apoptosis suggests specificity of its biological activity. A number of studies have illustrated that, at concentrations in the high physiological range, H_2O_2 induces adaptive changes, increasing resistance of biological systems not only to oxidative stress but also to many other stimuli [43, 106]. The capability of H_2O_2 to induce a large number of protein syntheses and to provide cross-resistance implies that living systems may "intentionally" produce H_2O_2 as a component of adaptation in response to different fluctuations and perturbations shifting the system away from homeostasis. However, if the concentration of H_2O_2 exceeds

the physiological concentration or the stress persists for a longer period of time, it can induce cardiac maladaptation. Thus, H_2O_2 is an important ROS in the transition of cardiac response to CIH from the adaptive phase to maladaptive phase or from cardiac hypertrophy to cardiac failure.

As H_2O_2 is generated by many compartmentalized enzymes, local variations in the concentration of H_2O_2 could also be crucial for the activation of specific targets [110]. Treatment of different primary cells with increasing doses of exogenous H_2O_2 induces proliferation, senescence, or apoptosis. Studies using other experimental models also showed H_2O_2 as a ROS having a flag potential for adjustments related to hypertrophy and/or cell death depending on its intracellular concentration [112, 113].

4. Effects of Antioxidants on the CIH-Induced Cardiac Response

OSA is recognized as an oxidative stress-related disorder [114]. In addition, the concept that oxidative stress resulting from an imbalance between increased ROS generation and inadequate endogenous antioxidant pools contributing to HF is well established [43]. Therefore, agents able to abolish oxidative stress are attractive for prophylactic and therapeutic intervention of OSA. Antioxidant pharmacotherapy for OSA is considered as a viable and attractive clinical option, especially in the light of poor compliance of continuous positive airway pressure—the gold standard treatment for OSA.

Understanding of complex actions of CIH, which can exert both protective and detrimental effects, may reveal important information for developing therapeutic strategies for a better management for OSA patients [7]. Cardiac response to CIH should help developing therapeutic strategies to prevent and/or treat OSA-induced cardiovascular complications. A number of studies have been conducted using CIH-treated animal models to explore the effects of antioxidants, but the outcomes of these studies are controversial.

In the study of Skelly et al. [115], tempol (1 mM, superoxide dismutase mimetic) was applied in the treatment of rats exposed to CIH. It was found that antioxidant treatment may be beneficial as an adjunct OSA therapy. Doehner et al. found that allopurinol, a xanthine oxidase inhibitor, free radical scavenger, and lipid peroxidation inhibitor [116], was associated with the improvement of CIH-related oxidant stress, cardiac dysfunction, and apoptosis in rats [20]. However, Kolor and coworkers demonstrated that the pretreatment of antioxidant N-acetylcysteine completely prevented the development of cardioprotection in CIH rats although the infarct size was reduced [117]. This clearly implies the dual roles, adaptive and maladaptive, of ROS in CIH. The suppression of ROS production prior to hypoxia prevented the adaptive responses to be activated, but on the other hand, on the long run, it showed positive effects on cardiac function. Inamoto et al. [118] confirmed in their study using CIH-treated mice model that pitavastatin preserved, at least partially, the morphological structure of the LV myocardium in lean mice exposed to CIH, through its antioxidant effect. Unlike in the acute experiment, a chronic antioxidative treatment with N-acetylcysteine during the adaptation to hypoxia led to a significant attenuation of the improvement in tolerance to lethal myocardial injury. Generally, the dual effects might, at least partially, explain why clinical trials with antioxidants failed to confirm promising data obtained in a number of animal studies. It is obvious that beneficial consequences of antioxidant supplementation in normal healthy heart cannot be used to predict an outcome in adapted or diseased hearts [117].

In clinical studies, the results of antioxidants treatments were also inconsistent. From a clinical perspective, much attention has focused on the concept that oxidative stress may be a driver of cardiac disease progression (e.g., HF), but clinical interventions with antioxidants have had little or no impact on heart disease risk and progression [43]. Results of relatively few controlled clinical trials with antioxidants such as vitamin C, α-tocopherol (vitamin E), and coenzyme Q_{10} are also controversial [119, 120]. Oral N-acetylcysteine administration appears to have a therapeutic potential in the treatment of OSA. It is proposed that long-term treatment with N-acetylcysteine in patients with OSA may reduce their dependency on continuous positive airway pressure therapy [121].

5. Prospective

Excessive oxidative stress undoubtedly exerts toxic and detrimental effects through modification of biomolecules or triggering damaging signals and also plays important roles in the pathogenesis of certain oxidative stress-related diseases, such as OSA-elicited cardiac dysfunction. Thus, supplementation of exogenous antioxidants may be used to balance cellular redox status. However, low levels of ROS such as H_2O_2 also function as signal molecules and induce adaptive response to protect the cells. Under such condition, we may not supply exogenous antioxidants since they may attenuate or completely block the adaptive signals, as proposed in a recent review [122]. Antioxidants may be considered at higher H_2O_2 concentrations related to pronounced oxidative damage, but if applied too early they may interfere with adaptive processes. However, the threshold of H_2O_2 or other ROS associated with a switch from mild/transient to severe/persistent oxidative stress has not yet been identified in OSA patients or OSA-relevant CIH models with cardiac dysfunction, which could be a good molecular diagnosis and/or monitoring in the development and progression of this disorder. Nevertheless, the concept of cardiac response to chronic intermittent hypoxia with a transition from adaptation to maladaptation will help us to understand the pathogenic mechanisms of OSA-induced cardiac damage and shed light on potential prevention and therapy. Since OSA is considered as a life-threatening condition and as an independent risk factor of cardiovascular diseases, we anticipate an intervention of effective exogenous antioxidants and/or induction of endogenous antioxidants in heart at the right time and proper dose may have protective effect against OSA-induced cardiac damage.

Abbreviations

OSA: Obstructive sleep apnea
CIH: Chronic intermittent hypoxia
HF: Heart failure
I/R: Ischemia/reperfusion
LV: Left ventricular
LVEF: Left ventricular ejection fraction
IHH: Intermittent hypobaric hypoxia
FS: Fractional shortening
ROS: Reactive oxygen species
H_2O_2: Hydrogen peroxide.

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