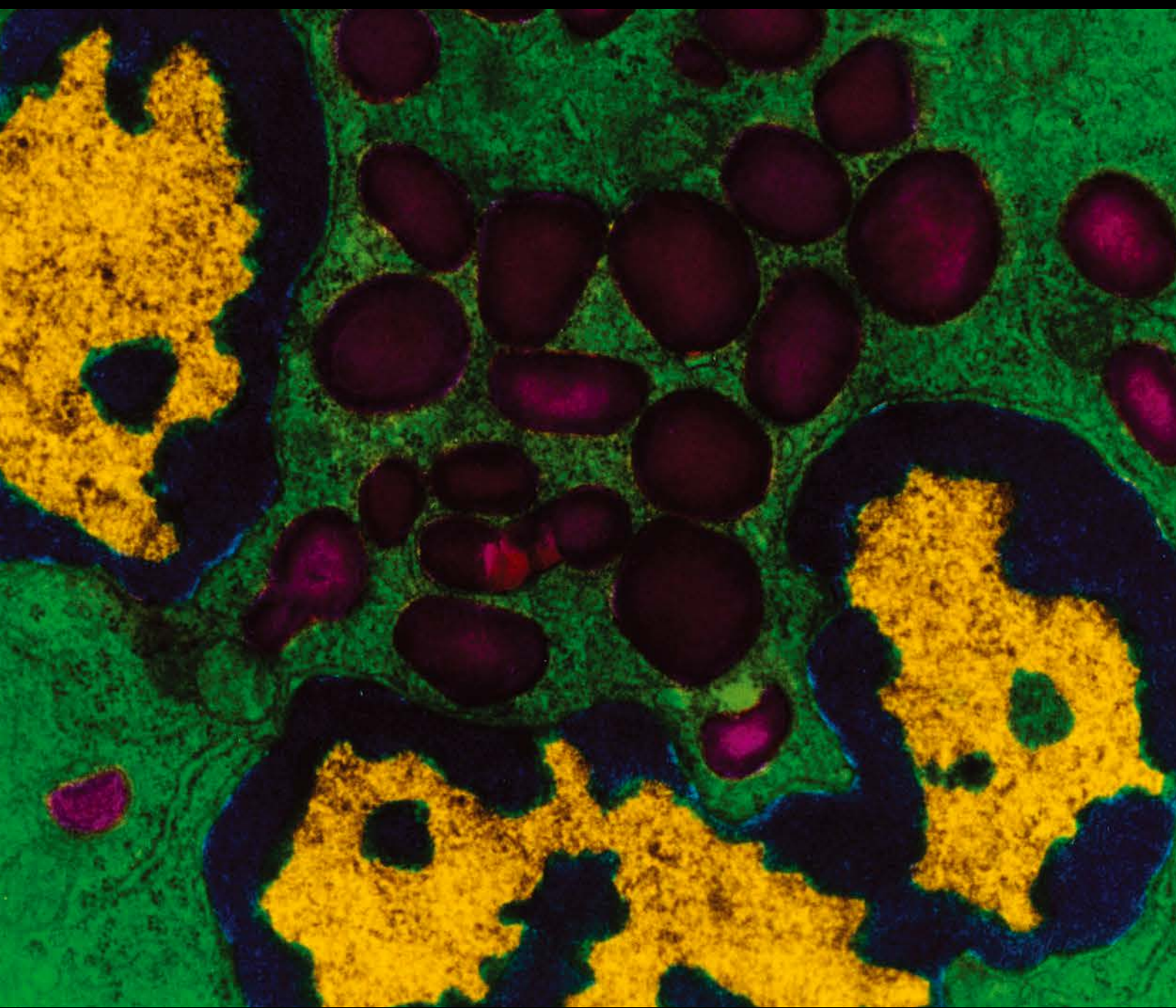
Mediators of Inflammation

The Role of Endocrine System in the Inflammatory Process

Guest Editors: Joilson O. Martins, Christian Bowman-Colin, and Luis A. Salazar



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Editorial Board

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Editorial

The Role of Endocrine System in the Inflammatory Process

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Inflammation is a general tissue response to a wide variety of stimuli. In situations in which inflammation is not properly regulated, inflammatory response may be exaggerated or ineffective, leading to immune dysfunction, recurrent infections, and tissue damage, both locally and systemically. Various hormones, cytokines, vitamins, metabolites, and neurotransmitters are known to be key mediators of the immune and inflammatory responses in endocrine as well as in paracrine fashions. Therefore, exploring the mechanisms underlying the production and response to these mediators might broaden the horizons for the development of novel therapeutic options that target disease states in which the immune/inflammatory responses are compromised or dysregulated.

This special issue covers the most current research aimed at elucidating the cellular and molecular mechanisms underpinning the endocrine/paracrine networks of regulatory immune mediators and their targets.

In this journal edition in disease states, Y.-S. Lee and H.-S. Jun reviewed the current status of glucagon-like peptide-1-(GLP-1-) based therapies and their impact on the treatment and management of type 2 diabetes mellitus. GLP-1 is an incretin hormone mainly secreted by intestinal L cells in response to nutrient ingestion, which has beneficial effects for glucose homeostasis by stimulating insulin secretion from pancreatic beta-cells, delaying gastric emptying, decreasing plasma glucagon, reducing food intake, and stimulating glucose catabolism. Beyond their metabolic effects, it is reviewed herein that GLP-1-based therapies have displayed anti-inflammatory properties through promoting downregulation of proinflammatory responses in a cell-autonomous as well as a systemic manner, especially in the context of inflammation-related diseases.

A. Mancini et al. report in this issue that thyroid hormones play particularly important roles in the antioxidant balance, since both hyper- and hypothyroidism have been shown to be associated with oxidative stress (OS) in humans and animals. In this context, the pathophysiological mechanisms of the nonthyroidal illness syndrome (NTIS) typically manifest as reduced conversion of thyroxine (T4) to triiodothyronine (T3) in several acute and chronic systemic conditions. This syndrome, along with the deiodinases that catalyze the conversion of T4 to T3, is reviewed herein.

Female development and reproductive function is well documented to be modulated by estrogens. In particular, 17β-estradiol (E2) is the main sex hormone regulating reproduction in females. However, E2 is also deeply involved in several other pathologies, such as cancer and autoimmune and infectious diseases, in which the innate immune response is a key player. I. Medina-Estrada et al. reported in this issue that E2 induces anti-inflammatory responses of bovine mammary epithelial cells during *S. aureus* internalization and that effect is dependent, at least in part, on the estrogen receptor α (ESRα).

Like estradiol, progesterone levels fluctuate dramatically during pregnancy. In this issue, M. Wu et al. report that the known increase in serum progesterone levels during pregnancy exacerbates gingival inflammation. This effect of progesterone is shown to be independent of crevicular fluid levels of both interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α).

Diabetic retinopathy (DR) is the most common microvascular complication of diabetes and is a leading cause of blindness across the globe. Genetic predisposition has been found to contribute to DR pathology, since specific haplotypes cosegregate with disease onset within affected families. M. M. Yang et al. reported herein that a polymorphism in the C5 gene (rs17611) represents a novel putative susceptibility locus for DR, particularly predisposing to the clinically relevant proliferative DR subtype. On the other hand, it was shown that polymorphism of SERPING1, which encodes for one well-known component of the Complement system, has only marginal to no contribution to the development of DR.

Endoplasmic reticulum (ER) stress facilitates fibrotic remodeling through the promotion of inflammatory responses. Aldosterone (Aldo), a known ER stressor, is thought to be involved in fibrotic renal injury by upregulating the production of inflammatory mediators such as IL-1β and IL-6. H. Guo et al. reported an important role for Aldo responses in ER stress and renal inflammation in the pathogenesis of renal fibrosis. In addition, the ER stress can be inhibited by Tauroursodeoxycholic Acid (TUDCA) and this effect is associated with downregulation of collagen I, collagen IV, fibronectin, transforming growth factor-β (TGF-β) expression, and Nlrp3 inflammasome markers such as the apoptotic speck protein (ASC), IL-1β, and IL-18. Altogether, these findings suggest that these inflammatory pathways are involved in Aldo-induced chronic kidney disease.

In summary, the original research articles and literature reviews featured in this special issue will hopefully enhance our knowledge about the roles of the endocrine system in the inflammatory process, shedding light on potential avenues for the development of novel therapies.

Acknowledgments

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Research Article

Endoplasmic Reticulum Chaperon Tauroursodeoxycholic Acid Attenuates Aldosterone-Infused Renal Injury

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Aldosterone (Aldo) is critically involved in the development of renal injury via the production of reactive oxygen species and inflammation. Endoplasmic reticulum (ER) stress is also evoked in Aldo-induced renal injury. In the present study, we investigated the role of ER stress in inflammation-mediated renal injury in Aldo-infused mice. C57BL/6J mice were randomized to receive treatment for 4 weeks as follows: vehicle infusion, Aldo infusion, vehicle infusion plus tauroursodeoxycholic acid (TUDCA), and Aldo infusion plus TUDCA. The effect of TUDCA on the Aldo-infused inflammatory response and renal injury was investigated using periodic acid-Schiff staining, real-time PCR, Western blot, and ELISA. We demonstrate that Aldo leads to impaired renal function and inhibition of ER stress via TUDCA attenuates renal fibrosis. This was indicated by decreased collagen I, collagen IV, fibronectin, and TGF-β expression, as well as the downregulation of the expression of Nlrp3 inflammasome markers, Nlrp3, ASC, IL-1β, and IL-18. This paper presents an important role for ER stress on the renal inflammatory response to Aldo. Additionally, the inhibition of ER stress by TUDCA negatively regulates the levels of these inflammatory molecules in the context of Aldo.

1. Introduction

The chronic administration of aldosterone (Aldo) leads to the production of reactive oxygen species (ROS) and causes endothelial dysfunction, disruption of the glomerular filtration barrier, proteinuria, and tubular damage and regeneration, leading to the progression of chronic kidney disease [1–5]. Aldo exhibits these classic actions by combining with the mineralocorticoid receptor (MR), a member of the nuclear receptor family of proteins. Additionally, an MR antagonist can attenuate renal injury via reducing the level of ROS generation [6]. Recently, ROS was identified as an initiator and major contributor to ER stress [7]. Reports have shown that endoplasmic reticulum (ER) stress facilitates fibrotic remodeling through the promotion of inflammatory responses [8]. Excessive ER stress may result in fibrosis through activating CHOP-mediated apoptosis and a subsequent response of inflammatory and profibrotic cytokines [9]. ER stress can elicit an inflammatory response through the induction of the ASC and Nlrp3 inflammasome [10, 11]. This in turn promotes the maturation and secretion of proinflammatory cytokines, including IL-1β and IL-18, to initiate innate immune defenses and subsequently results in cellular injury [12]. The Nlrp3 inflammasome is a crucial event in the progression of kidney disease [13–16], including unilateral ureteral obstruction (UUO) [13], ischemia/reperfusion injury [17, 18], and proteinuric animal model [19]. In those models, Nlrp3$^{-/-}$ mice were remarkably resistant to renal injury, possibly via the inhibition of the inflammatory response. Aldo-driven renal injury also consists of inflammatory components involving IL-1β and IL-6 upregulation. These effects are attenuated by eplerenone, supporting the protective effect of an Aldo blockade in renal disease via inhibiting inflammatory cytokines [20].

Our previous studies have demonstrated that TUDCA could ameliorate renal injury and ER stress-mediated uremic cardiomyopathy [10]. Chiang and coworkers also demonstrated the inhibition of ER stress by TUDCA, an ER stress

inhibitor, protected against UUO-induced renal fibrosis [21]. Collectively, these studies indicate that inhibition of ER stress may be one of the possible therapeutic targets against renal fibrosis and injury. However, the molecular mechanisms leading to the renal inflammatory changes that occur in response to ER stress have not been reported in detail. The present study explores the potential role of ER stress in Aldo-infused renal inflammation and fibrosis.

2. Materials and Methods

2.1. Animal Models. All experiments were performed in accordance with the Fudan Medical University Guide for Laboratory Animals. Eight-week-old C57BL/6J mice weighting between 25 and 30 g were purchased from the Institute of Animal Care at Fudan University and underwent a right uninephrectomy under anesthesia with sodium pentobarbital (50 mg/kg, IP). After two weeks of recovery, all mice were given drinking water containing 1% NaCl and randomly treated with one of the following for four weeks: group 1, Sham+V (0.5% ethanol subcutaneously, saline vehicle i.p., $n = 6$); group 2, Sham+TUDCA (0.5% ethanol subcutaneously, 250 mg/kg/d of TUDCA i.p., Sigma-Aldrich, USA, $n = 6$); group 3, Aldo+V (Aldo 0.75 μg/h subcutaneously, Sigma-Aldrich, USA, saline vehicle i.p., $n = 6$); and group 4, Aldo+TUDCA (Aldo 0.75 μg/h subcutaneously, 250 mg/kg/d of TUDCA i.p., $n = 6$) [10]. At the end of the experiment, the mice were anesthetized and the body and kidney weight were measured. Twenty-four-hour urine samples were collected after a 24 h acclimatization period in the metabolic cages. Urinary protein excretion was determined using enzyme-linked immunosorbent assay (ELISA) kits (Exocell). Additionally, the plasma was centrifuged for testing creatinine, urea nitrogen, IL-18, and IL-1β. Kidney samples were immediately frozen in liquid nitrogen and stored at −80°C.

2.2. Kidney Histopathological Analysis. Kidney tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sliced at a thickness of 3 μm per section. They were stained with periodic acid-Schiff (PAS) according to a standard protocol [22]. The severity of glomerular injury from each mice kidney section was assessed using light microscopy. Then, the sections were rated for glomerular proliferative lesions on a scale from 0 to 4 as follows: 0 equated to no proliferation, whereas 1+, 2+, 3+, and 4+ corresponded to 1%–25%, 26%–50%, 51%–75%, and 76%–100% of segmental lesion per glomeruli, respectively.

2.3. Detection of ROS. We detected renal thiobarbituric acid reactive substances (TBARS) using commercial kits (Cayman Chemical Company). Moreover, we measured serum malondialdehyde (MDA) and 8-OHdG using commercial kits (Jiancheng Bioengineering Research Institute) according to the manufacturer's protocol.

2.4. Quantitative Real-Time PCR. The total RNA was extracted from the kidneys using a kit (Fermentas, Glen Burnie, MD, USA) according to the manufacturer's protocol. Real-time PCR amplification was performed using the SYBR Green master mix (ABI, USA) and the Prism 7300 real-time PCR detection system (Applied Biosystems). Oligonucleotide sequences were provided by Invitrogen and the primer pairs were presented in Table 1 [10]. The expression of mRNA levels was normalized by subtracting the corresponding GAPDH as a control and calculated by using the comparative cycle threshold method.

2.5. Western Blotting and ELISA. The kidney tissues were homogenized and the supernatant was collected after centrifugation at 12,000 ×g at 4°C for 20 min [23]. We separated the lysates on 10% polyacrylamide gels before immunoblotting using anti-Nlrp3 (AdipoGen company, San Diego, CA), anti-ASC (AdipoGen company, San Diego, CA), anti-CHOP (Cell Signaling Technology, USA), anti-caspase-12 (Cell Signaling Technology, USA), anti-IL-1β (Affinity Biosciences, USA), and anti-IL-18 (Affinity Biosciences, USA) antibodies at a dilution of 1 : 500. The expression levels of CHOP, caspase-12, ASC, Nlrp3, IL-1β, and IL-18 were analyzed using an ECL advance system (Amersham, Little Chalfont, UK). The relative protein expression levels were determined by normalization to β-actin. Serum IL-1β and IL-18 were measured with ELISA kits (RayBiotech, Norcross, GA) according to the manufacturer's instruction.

2.6. Statistical Analysis. Results were expressed as means ± the standard error of the mean (SEM). A one-way ANOVA was used to compare mean values, and a value of $p < 0.05$ was determined to be statistically significant.

3. Results

3.1. Effects of TUDCA on Renal Function in Aldo-Infused Mice. Renal damage was assessed by PAS staining, serum creatinine, albumin/creatinine, and BUN. As shown in Figure 1, Aldo-infused mice showed markedly expanded mesangial regions and glomerulosclerosis (3.02 ± 0.16) compared to the Sham+V group (Glomerular Injury Score: 0.13 ± 0.05). However, treatment with TUDCA significantly mitigated renal injury and reduced the Glomerular Injury Score (0.52 ± 0.09). Consistent with this finding for PAS staining, levels of BUN and albumin/creatinine were also significantly increased in Aldo+V mice (65.4 ± 3.96 mg/dL and 102.5 ± 12.77, resp.) compared to Sham+V mice (24.5±0.83 mg/dL and 25.5±3.04, resp.). Levels of BUN and albumin/creatinine were markedly decreased in Aldo+TUDCA mice (37.4 ± 1.97 mg/dL and 44.8 ± 5.26, resp.) relative to Aldo+V mice. The creatinine concentration was higher in Aldo-infused mice (0.30 ± 0.02) compared to Sham+V mice (0.21 ± 0.01), and treatment with TUDCA decreased the level of creatinine in the Aldo-treated group (0.26 ± 0.01) (Table 2).

3.2. Effects of TUDCA on Renal Fibrosis. Compared with the Sham+V mice, the Aldo+V group demonstrated significantly increased mRNA levels. mRNA levels of fibronectin, transforming growth factor-β (TGF-β), collagen I, and collagen IV

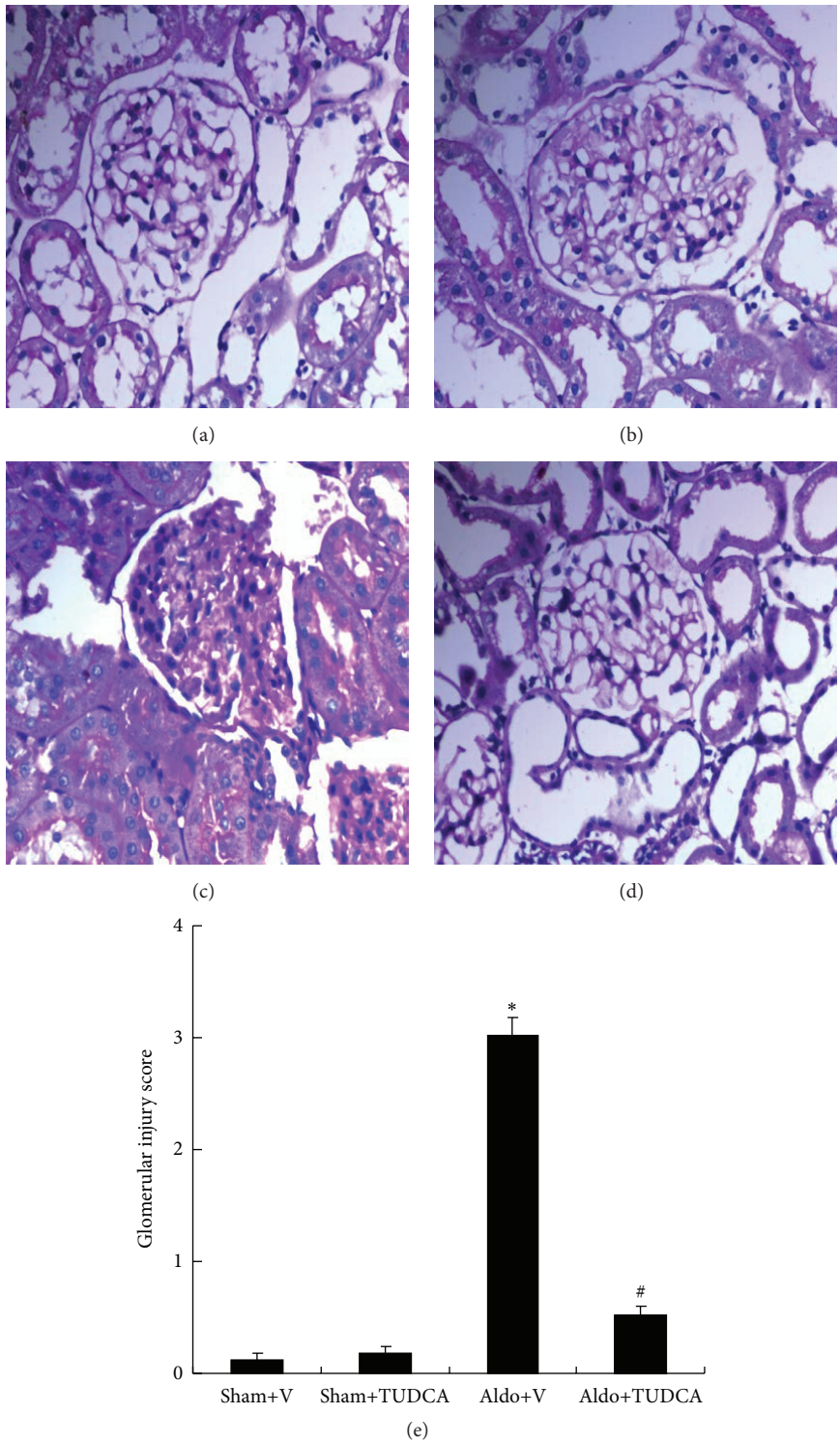

Figure 1: Physiologic parameters of the mice at the end of week 4. Representative photomicrographs (magnification: 400x) of PAS-stained renal injury. (a) Sham+V group; (b) Sham+TUDCA group; (c) Aldo+V group; (d) Aldo+TUDCA group; (e) Glomerular Injury Score. $^{*}p < 0.05$, Sham+V group versus Aldo+V group, $^{\#}p < 0.05$, Aldo+V group versus Aldo+TUDCA group.

Table 1: Oligonucleotides synthesized by Invitrogen (Carlsbad, CA, USA).

	Forward	Reverse
Transforming growth factor-beta	5′-AGCTTTGCAGGGTGGGTATC-3′	5′-CCTTCGGGTGAGACCACAAA-3′
Fibronectin	5′-GCGACGGTATTCTGTAAAGTGG-3′	5′-GGACAGGGCTTTGGCAGTT-3′
Collagen I	5′-AGGGTCATCGTGGCTTCTCT-3′	5′-CAGGCTCTTGAGGGTAGTGT-3′
Collagen IV	5′-ATCGGATACTCCTTCCTCATGC-3′	5′-CCAGGGGAGACTAGGGACTG-3′
GRP78	5′-CTGCTGAGGCGTATTTGGGAAA-3′	5′-TCAATGGTGAGAAGAGACACATCG-3′
GRP94	5′-GTCGTGGAACAACAATTACTCTTG-3′	5′-GCTTCATCATCAGATTCTTCTTTCTC-3′
IL-1β	5′-AGCCTTTGTCCTCTGCCAAGT-3′	5′-CCAGAATGTGCCACGGTTTT-3′
IL-18	5′-GGGATGGGAGGAACGCTACTA-3′	5′-ACAGGTTGTACTGGAAAAGCC-3′
Glyceraldehyde 3-phosphate dehydrogenase	5′-TCAGCCGCATCTTCTTTTG-3′	5′-AAATCCGTTGACTCCGACC-3′

Table 2: Biological parameters in Aldo-infused mice at week 4.

	Sham+V	Sham+TUDCA	Aldo+V	Aldo+TUDCA
Body weight (g)	27.06 ± 1.25	27.18 ± 1.32	26.54 ± 1.85	26.88 ± 1.98
Kidney weight/body weight ratio (mg/g)	10.04 ± 0.21	10.11 ± 0.28	14.73 ± 0.22*	11.15 ± 0.25#
Albumin/creatinine (ug/mg)	25.5 ± 3.04	26.4 ± 2.72	102.5 ± 12.77*	44.8 ± 5.26#
Serum creatinine (mg/dL)	0.21 ± 0.01	0.25 ± 0.01	0.30 ± 0.02	0.26 ± 0.01
BUN (mg/dL)	24.5 ± 0.83	25.1 ± 1.07	65.4 ± 3.96*	37.4 ± 1.97#

Data are presented as mean ± SEM; ($n = 6$); * $p < 0.05$, Sham+V versus Aldo+V; # $p < 0.05$, Aldo+V versus Aldo+TUDCA.

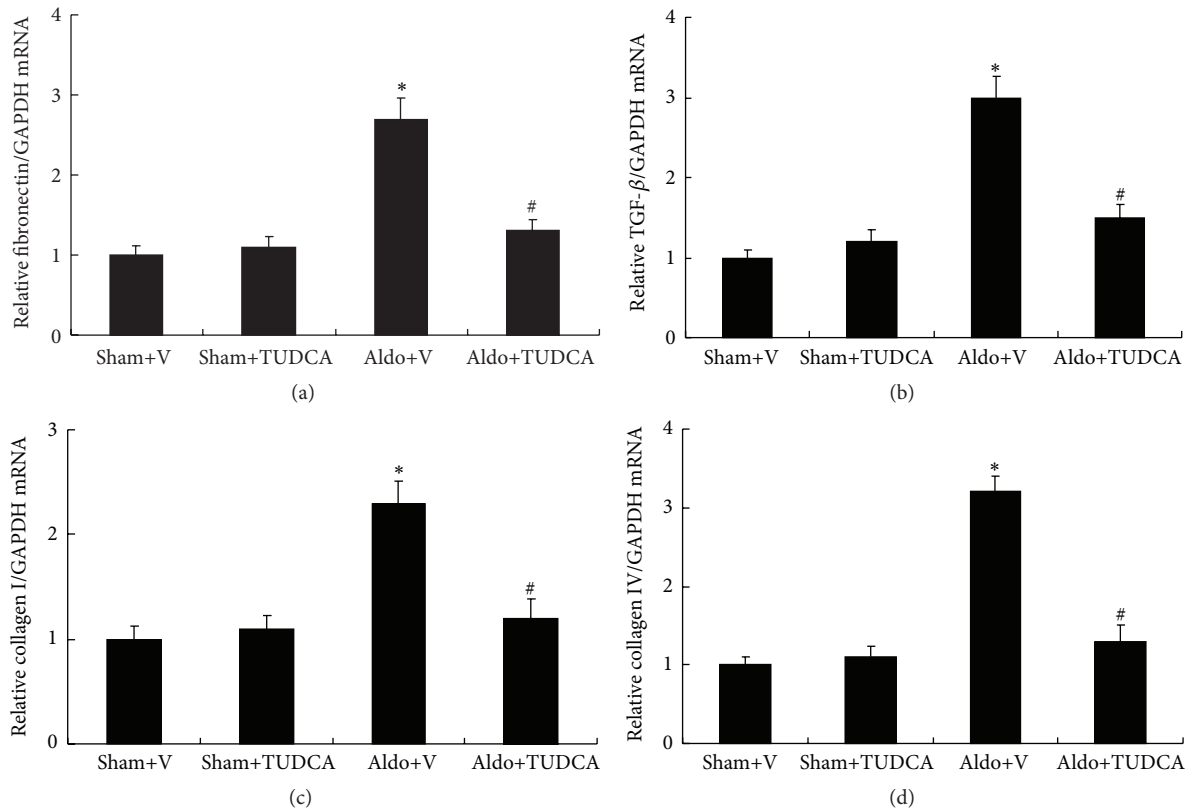

Figure 2: Expression of fibrotic cytokines in the kidney. (a) mRNA expression of fibronectin, (b) TGF-β, (c) collagen I, and (d) collagen IV was detected by real-time PCR and normalized to expression of glyceraldehyde 3-phosphate dehydrogenase. Values are mean ± SEM ($n = 6$). * $p < 0.05$, Sham+V group versus Aldo+V group, # $p < 0.05$, Aldo+V group versus Aldo+TUDCA group.

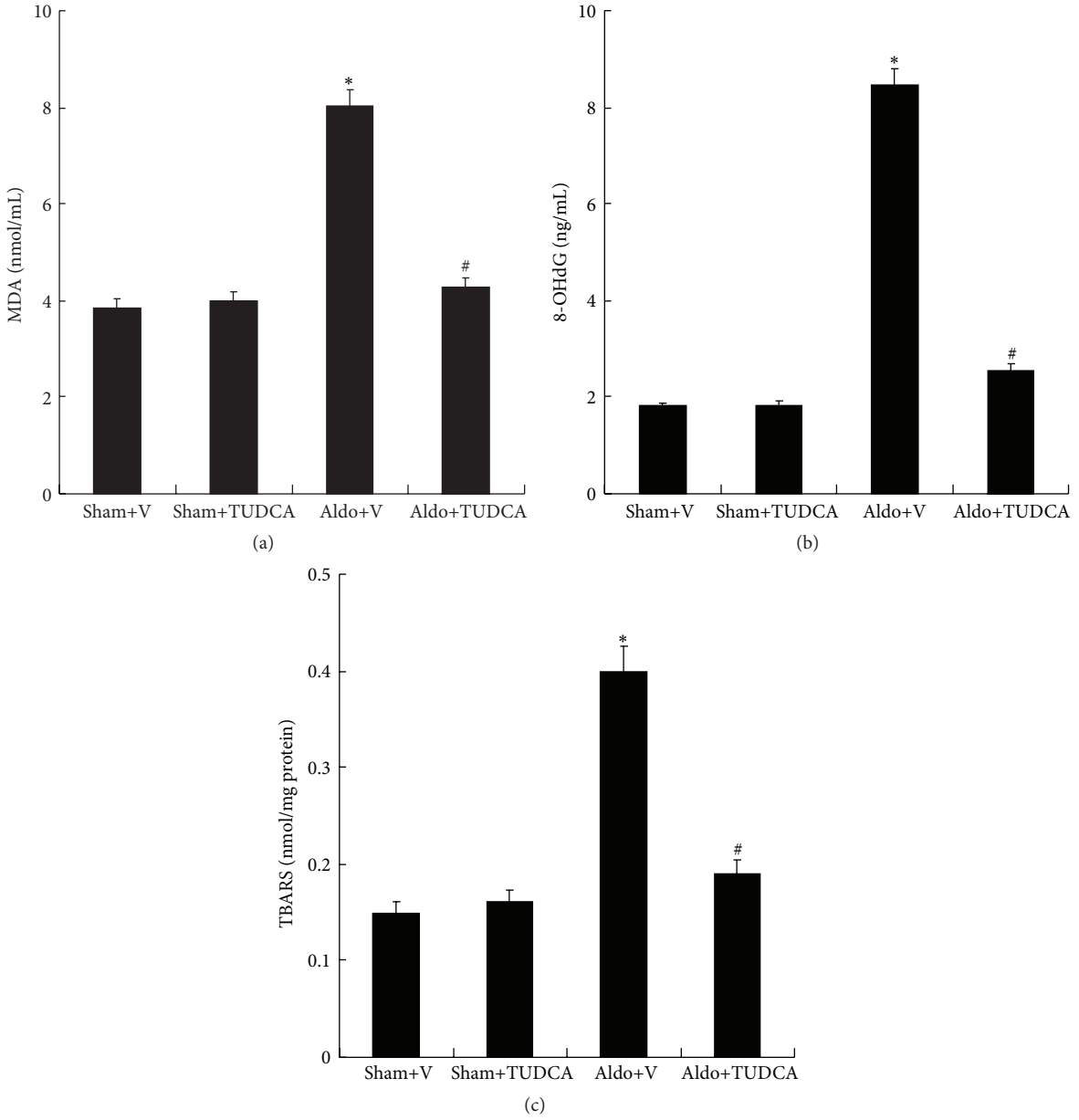

Figure 3: ROS levels in the mouse kidney. (a) MDA, (b) 8-OHdG, and (c) TBARS were detected according to the manufacturer's protocol. Values are mean ± SEM ($n = 6$). $^{*}p < 0.05$, Sham+V group versus Aldo+V group, $^{\#}p < 0.05$, Aldo+V group versus Aldo+TUDCA group.

were increased remarkably in Aldo+V group (2.7-fold, 3.0-fold, 2.3-fold, and 3.2-fold, resp.) compared to the Sham+V mice. In addition, TUDCA treatment significantly decreased the mRNA levels of fibronectin, TGF-β, collagen I, and collagen IV compared with those of Aldo+V mice (Figure 2).

3.3. Effects of TUDCA on Kidney ROS. Indices of ROS production, including MDA (8.03 ± 0.33), 8-OHdG (8.46 ± 0.32), and TBARS (0.4 ± 0.03), were significantly increased in Aldo+V group compared with the Sham+V group (3.86 ± 0.18, 1.81 ± 0.08, and 0.15 ± 0.01). In contrast, treatment with TUDCA significantly attenuated the Aldo-induced elevated levels of MDA, 8-OHdG, and TBARS (Figure 3).

3.4. Effects of TUDCA on the ER Stress-Induced Apoptotic Pathway in Mouse Kidney. GRP 78 and GRP 94 as ER stress markers are critical regulators of ER function. Expression of GRP78 (4.2-fold) and GRP94 (3.9-fold) are increased markedly in mouse kidneys of Aldo+V mice relative to Sham+V mice. However, TUDCA treatment reduced the levels of GRP 78 and GRP 94 significantly (Figure 4). Activation of ER stress can initiate apoptosis via CHOP and caspase-12 pathways. Levels of CHOP (3.4-fold) and caspase-12 (2.8-fold) were increased significantly in the Aldo+V group compared with the Sham+V mice. Moreover, TUDCA treatment reduced the levels of CHOP and caspase-12 remarkably (Figure 4). This demonstrates the important role of CHOP and caspase-12 signaling in Aldo-driven renal injury.

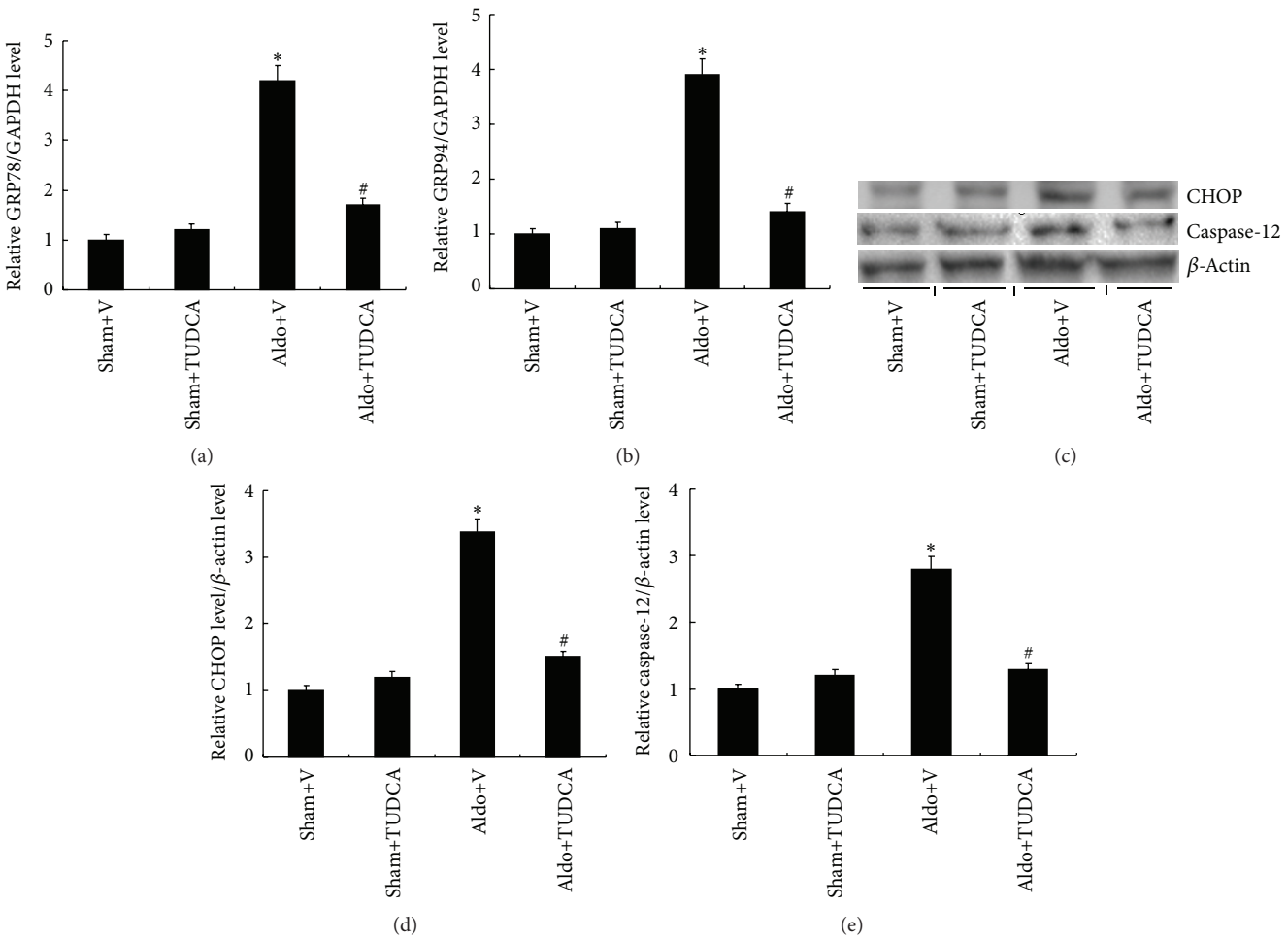

Figure 4: TUDCA ameliorated Aldo-induced ER stress. (a) Relative mRNA expression of GRP78 compared to expression of GAPDH. (b) mRNA expression of GRP94 relative to the expression of GAPDH. (c) Representative Western blots of CHOP and caspase-12. (d) Relative expression of CHOP to expression of β-actin. (e) Relative expression of caspase-12 to expression of β-actin. Values are mean ± SEM ($n = 6$). $^{*}P < 0.05$, Sham+V group versus Aldo+V group, $^{\#}P < 0.05$, Aldo+V group versus Aldo+TUDCA group.

3.5. Effects of TUDCA on Nlrp3 Inflammasome Activation in the Kidney. The real-time PCR analysis demonstrated that mRNA expression of Nlrp3 inflammasome-related genes, including IL-1β (4.6-fold) and IL-18 (4.1-fold), was significantly increased in the kidneys of Aldo+V mice relative to Sham+V mice. Treatment with TUDCA markedly decreased the levels of IL-1β and IL-18 (Figure 5). Similarly, the levels of serum IL-1β (62.2 ± 3.5) and IL-18 (150.5 ± 7.6) were significantly increased in Aldo+V mice compared with Sham+V mice (16.8 ± 1.5 and 66.3 ± 3.2, resp.), whereas the levels of IL-1β and IL-18 were markedly decreased in Aldo+TUDCA mice. Since activated Nlrp3 and ASC proteins cause the subsequent maturation of proinflammatory cytokines, the protein levels of mature IL-1β and IL-18 were measured in the mouse kidneys by Western blot. The protein levels of IL-1β (4.5-fold) and IL-18 (5.6-fold) were higher in Aldo+V mice compared to the Sham+V group, while TUDCA decreased both cytokines in Aldo-infused mice. Similarly, Nlrp3 (3.4-fold) and ASC (5.2-fold) protein expression were also increased in the Aldo+V group compared with the Sham+V group. Treatment with TUDCA was able to significantly decrease these protein levels in Aldo+V mice (Figure 6).

4. Discussion

This study demonstrated that ROS, ER stress, and renal Nlrp3 inflammasome were increased in Aldo-infused mice. In addition, treatment with TUDCA, an ER stress inhibitor, was shown to prevent Nlrp3 inflammasome activation and its related cytokines. This indicates that TUDCA may ameliorate Aldo-infused renal injury via inhibiting activation of the NLRP3 inflammasome.

ROS overproduction has been correlated with a variety of renal injury models, including diabetic kidney disease, focal segmental glomerulosclerosis, and membranous nephropathy [24]. Several reports have demonstrated that excessive Aldo in animal models was associated with mesangial cell and podocyte injury, due to ROS activation [24, 25]. Previous studies have also shown that ROS overproduction leads to

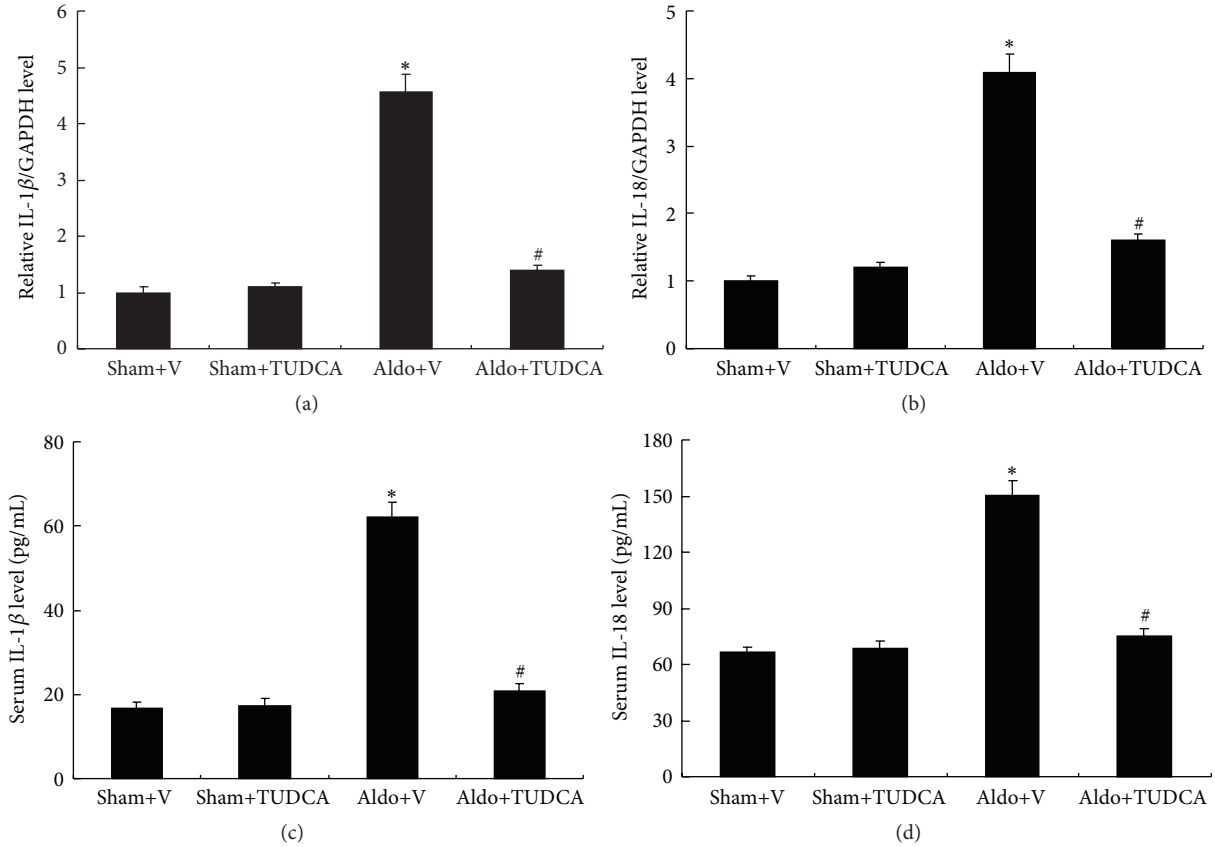

Figure 5: TUDCA decreased IL-1β and IL-18 levels in Aldo-infused mice. (a) Relative mRNA expression of IL-1β to expression of GAPDH. (b) Relative mRNA expression of IL-18 to the expression of GAPDH. (c) Serum IL-1β levels and (d) serum IL-18 levels. Values are mean ± SEM ($n = 6$). $^{*}p < 0.05$, Sham+V group versus Aldo+V group, $^{\#}p < 0.05$, Aldo+V group versus Aldo+TUDCA group.

oxidative stress and triggers redox-sensitive cell signaling cascades that elicit an inflammatory response, mitochondrial dysfunction, and fibrogenesis. Moreover, antioxidants and free radical scavengers partially improved proapoptotic outcomes of Aldo [26]. ROS was also identified as a potential trigger of the Nlrp3 inflammasome in podocytes [13]. Therefore, these results indicate that it is likely that ROS is involved in the activation of the inflammasome in Aldo-driven renal injury [13, 27–30]. The present study demonstrated that ROS markers were activated following Aldo-infused renal injury. Oxidative stress is a known inducer of ER stress. Oxidant stress disrupts the ER homeostasis and activates ER stress in the kidneys and is associated with Aldo-driven renal injury. Although the detailed mechanisms between ROS and ER stress in Aldo-induced inflammation remain unknown, these findings provide important insight into the renal injury in response to Aldo.

Increasing evidence has demonstrated that ER stress and Nlrp3 inflammasome activation are important pathogenic factors in multiple kidney diseases [2, 16–18]. Previous studies have also reported that the levels of ER stress protein in human and experimental animal models were significantly upregulated following Aldo treatment [31, 32]. CHOP, an ER stress activation marker, is commonly expressed at low levels and is robustly activated in a wide variety of organs as part of the natural stress response. The present study showed that Aldo exposure substantially increased CHOP expression. However, how ER stress contributes to Aldo-evoked renal injury remains unknown. Previous reports demonstrated that ER-induced renal injury can induce autophagy [7, 31]. However, the precise mechanism by which this occurs was not determined. In addition, activation of autophagy in animal experiments exhibited only a partial resistance to ER stress-induced renal injury, indicating that autophagy was not a unique pathway of fibrosis.

Inflammation appears to be an important destructive process that mediates injury in the kidneys. Moderate tubulointerstitial inflammation and fibrosis have been shown to be mediated through Nlrp3 inflammasome activation as indicated by the elevated mRNA and protein levels of Nlrp3 and ASC [33]. Aldo is able to facilitate the initiation and maintenance of inflammatory cells into the vascular wall to promote smooth muscle cell proliferation and decrease endothelial function. This in turn accelerates the development of atherosclerosis and stimulates the progression of tissue injury [34–36]. Consistent with previous studies, this study also showed that the Nlrp3 inflammasome was present in the Aldo-infused model of renal injury. Similar to previous findings, increased protein levels of Nlrp3 and ASC and the activation of matured IL-18 and IL-1β were observed. Taken

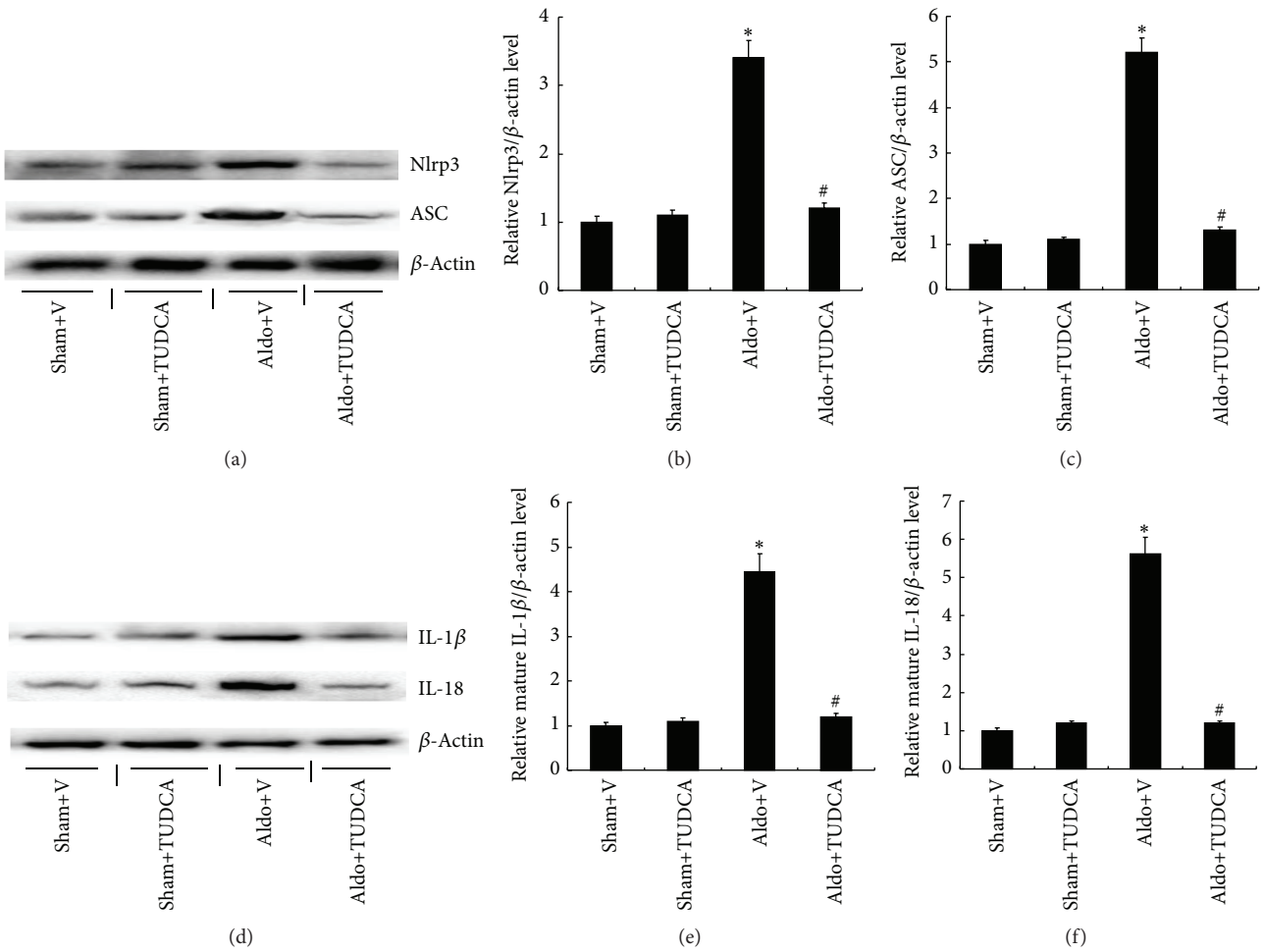

Figure 6: TUDCA attenuated Nlrp3 inflammasome activation in Aldo-infused mice. (a) Representative Western blots of Nlrp3 and ASC. (b) Relative expression of Nlrp3 to the expression of β-actin. (c) Relative expression of ASC to the expression of β-actin. (d) Representative Western blots of IL-1β and IL-18. (e) Relative expression of IL-1β to expression of β-actin. (f) Relative expression of IL-18 to the expression of β-actin. Values are mean ± SEM ($n = 6$). $^{*}p < 0.05$, Sham+V group versus Aldo+V group, $^{\#}p < 0.05$, Aldo+V group versus Aldo+TUDCA group.

together, these results suggest that the Nlrp3 inflammasome may contribute to Aldo-induced renal injury. Treatment with TUDCA markedly attenuated kidney disease and decreased Nlrp3 inflammasome activation, indicating that the Nlrp3 inflammasome may be downstream of the ER stress pathway in Aldo-induced kidney disease.

Prolonged ER stress contributed to fibrosis via increased inflammatory responses and the generation of profibrotic cytokines [36]. In addition, activation of ER stress in immune cells induced the production of proinflammatory cytokines. ER stress was also identified to play an important role in inducing inflammation and the release of TGF-β in the CHOP-mediated activation of apoptosis [9].

We previously demonstrated that TUDCA significantly ameliorated renal function and uremic cardiomyopathy via inhibiting ER stress pathway [10]. Fang et al. [37] showed that albuminuria induced inflammasome activation via ER stress signaling in renal proximal tubular cells. The present study demonstrated that TUDCA alleviated ER stress response driven by Aldo in renal injury. Treatment with TUDCA decreased ER stress proteins including GRP78, GRP94, CHOP, and caspase-12. In addition, TUDCA alleviated inflammation induced injury via downregulating ASC and NLRP3. Blocking the ER stress pathway can inhibit Aldo-driven production of IL-18 and IL-1β. The results above suggest that Aldo may activate the Nlrp3 inflammasome via the ER stress response, indicating the important crosstalk between ER stress pathway and Nlrp3 inflammasome activation in Aldo-induced renal injury. This data provides indirect evidence supporting the pathological role of the Nlrp3 inflammasome induced by Aldo in renal injury. Further *in vitro* studies are necessary to identify the possible involvement of ER stress in Aldo-driven Nlrp3 inflammasome.

In conclusion, the present study presents an important role of ER stress on renal inflammation, responses to Aldo, and ER stress inhibitors in the context of inflammation ameliorated renal fibrosis. The inhibition of ER stress, as well as Nlrp3 and ASC, suggests that redundant inflammatory pathways are involved in Aldo-induced chronic kidney disease. Furthermore, treatment with TUDCA significantly

attenuates the Nlrp3 inflammasome, suggesting that the ER stress pathway may mediate Nlrp3 inflammasome activation in Aldo-infused renal injury.

Competing Interests

The authors declare that there is no conflict of interests.

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Review Article

Anti-Inflammatory Effects of GLP-1-Based Therapies beyond Glucose Control

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Glucagon-like peptide-1 (GLP-1) is an incretin hormone mainly secreted from intestinal L cells in response to nutrient ingestion. GLP-1 has beneficial effects for glucose homeostasis by stimulating insulin secretion from pancreatic beta-cells, delaying gastric emptying, decreasing plasma glucagon, reducing food intake, and stimulating glucose disposal. Therefore, GLP-1-based therapies such as GLP-1 receptor agonists and inhibitors of dipeptidyl peptidase-4, which is a GLP-1 inactivating enzyme, have been developed for treatment of type 2 diabetes. In addition to glucose-lowering effects, emerging data suggests that GLP-1-based therapies also show anti-inflammatory effects in chronic inflammatory diseases including type 1 and 2 diabetes, atherosclerosis, neurodegenerative disorders, nonalcoholic steatohepatitis, diabetic nephropathy, asthma, and psoriasis. This review outlines the anti-inflammatory actions of GLP-1-based therapies on diseases associated with chronic inflammation *in vivo* and *in vitro*, and their molecular mechanisms of anti-inflammatory action.

1. Introduction

Glucagon-like peptide-1 (GLP-1) is produced by posttranslational proteolytic cleavage of the *proglucagon* gene product and mainly secreted from the enteroendocrine L cells in the distal intestine in response to nutrient ingestion. GLP-1 is an incretin hormone, which increases glucose-stimulated insulin secretion [1, 2]. GLP-1 is quickly degraded by dipeptidyl peptidase-4 (DPP-4), and inhibition of this proteolytic enzyme enhances its biological half-life [3]. GLP-1 has many beneficial effects on the control of blood glucose levels including stimulation of insulin secretion and inhibition of glucagon secretion, expansion of the beta-cell mass by stimulating beta-cell proliferation and differentiation and inhibiting beta-cell apoptosis, delay of gastric emptying, and reduction of food intake [4–6]. Therefore, GLP-1 has been extensively studied as a possible treatment of type 2 diabetes, and GLP-1 analogues and DPP-4 inhibitors are now widely in clinical use in these patients [7–11].

Expression of the GLP-1 receptor is widely detected in various cells and organs including the kidney, lung, heart, hypothalamus, endothelial cells, neurons, astrocytes, and microglia as well as pancreatic beta-cells [12–17], suggesting that GLP-1 might have additional roles other than glucose-lowering effects. It was reported that GLP-1 shows anti-inflammatory effects on pancreatic islets and adipose tissue, contributing to lowering glucose levels in diabetes [18–20]. In addition to these tissues, emerging data suggest that GLP-1-based therapies also showed anti-inflammatory effects on the liver, vascular system including aorta and vein endothelial cells, brain, kidney, lung, testis, and skin by reducing the production of inflammatory cytokines and infiltration of immune cells in the tissues [17, 21–25]. Thus, GLP-1 therapy may be beneficial for the treatment of chronic inflammatory diseases including nonalcoholic steatohepatitis, atherosclerosis, neurodegenerative disorders, diabetic nephropathy, asthma, and psoriasis [14, 26–32]. Drugs which are GLP-1 receptor agonists or DPP-4 inhibitors are shown in Table 1. In

Table 1: GLP-1-based drugs.

GLP-1-based drugs	Generic name	Disease	References
GLP-1 receptor agonists	Exenatide (synthetic form of exendin-4)	Diabetes	[43, 46, 47]
		Vascular disease	[61, 65, 70]
		Nonalcoholic steatohepatitis	[61, 93, 96]
		Nephropathy	[99, 101, 105, 107]
		Neurodegenerative brain disorder	[87, 92]
	Liraglutide	Diabetes	[48]
		Vascular disease	[8, 23, 58–60]
		Neurodegenerative brain disorder	[24, 84, 89]
		Nonalcoholic steatohepatitis	[9, 28, 95]
		Nephropathy	[14]
		Asthma	[30, 108]
		Psoriasis	[31, 111, 112]
	Lixisenatide	Neurodegenerative brain disorder	[90]
	Albiglutide		
	Taspoglutide		
	Dulaglutide		
DPP-4 inhibitors	Sitagliptin	Diabetes	[7, 19, 45]
		Vascular disease	[26, 64, 65, 72]
		Neurodegenerative brain disorder	[91]
		Nephropathy	[25, 107]
	Des-fluoro-sitagliptin	Vascular disease	[66]
	Alogliptin	Vascular disease	[67]
		Nephropathy	[105, 106]
	Linagliptin	Nephropathy	[104]
		Vascular disease	[63]
	Vildagliptin (PKF-275-055)	Diabetes	[49]
		Nephropathy	[103]
	NVP-DPP728	Diabetes	[44]
	Anagliptin	Nephropathy	[105]
	Saxagliptin	Nephropathy	[102]

this review, we will introduce some of the chronic inflammatory diseases and then discuss evidence for beneficial effects of GLP-1-based therapies focusing on its anti-inflammatory actions.

2. Diabetes

Type 1 diabetes is caused by autoimmune-mediated destruction of pancreatic beta-cells [33], and type 2 diabetes is caused by both insulin resistance and relative deficiency of insulin [34–36]. Inflammation can be a mediator of insulin resistance and beta-cell damage by high glucose, fatty acids, or adipokines released from adipose tissues [37–39]. Thus, inflammation is an important factor for the pathogenesis of both type 1 and type 2 diabetes, and inhibition of inflammation can be a therapeutic strategy for treatment of diabetes.

The proinflammatory cytokines, such as interleukin-1 beta (IL-1β), interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α), inhibit glucose-stimulated insulin secretion and proliferation of beta-cells [40–42]. Treatment

of isolated mouse islets with palmitate induced the expression of proinflammatory cytokines TNF-α, IL-1β, and IL-6. Liraglutide (100 nM), a long-acting GLP-1 analogue, inhibited the palmitate-induced expression of these inflammatory factors and p65 expression [43]. Treatment of cultured human islets with exendin-4 (50 nM), a GLP-1 receptor agonist, suppressed the expression of inflammatory genes such as NFκB1(p105), NFκB2(p100), RelA (also termed p65), TNF receptor superfamily member 1A, and receptor-interacting serine/threonine kinase 2. As well, exendin-4 (50 nM) and cyclic adenosine monophosphate (cAMP) response element-binding protein overexpression additively protected transplanted human islets in streptozotocin- (STZ-) induced diabetic nude mice [44]. Treatment of nonobese diabetic mice with the DPP-4 inhibitor, NVP-DPP728 (30 mg/kg), significantly increased the levels of plasma transforming growth factor beta-1 (TGF-β1), an anti-inflammatory cytokine, and increased CD4+CD25+FoxP3+ regulatory T cells, contributing to the remission of diabetes [45]. Treatment of diet-induced obese mice with sitagliptin (4 g/kg), a DPP-4 inhibitor, significantly reduced the expression of inflammatory genes including monocyte chemotactic protein- (MCP-) 1, IL-6, IL-12(p40), IL-12(p35), and IFN-γ-induced protein 10 (IP-10) in pancreatic islets and improved glucose-stimulated insulin secretion in isolated islets [19]. Treatment of STZ-induced diabetic rats with another DPP-4 inhibitor, vildagliptin (10 mg/kg), significantly reduced plasma TNF-α concentration and decreased nitric oxide concentration in serum and pancreatic homogenates compared with untreated diabetic rats [46]. Treatment with sitagliptin (20 mg/kg) increased serum GLP-1 levels in STZ-induced diabetic monkeys and showed significantly protective effects on STZ-induced islet injury *in vivo* and *in vitro* via activation of the insulin-like growth factor receptor (IGFR)/AKT/mammalian target of rapamycin (mTOR) signaling pathways [47]. These results suggest that GLP-1-based therapies suppress inflammatory cytokines and increase anti-inflammatory mediators in the pancreas.

C-X-C motif chemokine 10 (CXCL10/IP10), which is induced by IFN-γ, has an important role in recruiting activated T cells into the islets in type 1 diabetes. Exendin-4 (100 nM) decreased IFN-γ-induced signal transducer and activator of transcription-1 (STAT1), which is important for CXCL10 expression in the pancreatic beta-cell line, MIN6 cells, and human islets. Therefore, suppression of CXCL10 production by exendin-4 could reduce islet inflammation by decreasing cytotoxic T lymphocyte recruitment into the islets in autoimmune type 1 diabetes [48].

Serine proteinase inhibitor-9 plays an important role in the survival of cells against attack by natural killer cells and cytotoxic T lymphocytes, which play a direct role in the destruction of pancreatic beta-cells in type 1 diabetes. The GLP-1 receptor agonist, exenatide (a synthetic form of exendin-4) (10 nM), induces the expression of serine protease inhibitor-9 in human islets [49]. These results suggest that GLP-1-based therapies not only directly regulate the expression of inflammatory mediators, but also regulate the recruitment of immunocytes and protect from immunocyte attack, contributing to the preservation of pancreatic islets.

The abundance of proinflammatory cytokines and chemokines in adipose tissue is a key contributor to insulin resistance in type 2 diabetes, and blocking of inflammatory signaling pathways or immune cell infiltration in adipose tissue improves insulin sensitivity [50–52]. Administration of a recombinant adenovirus producing GLP-1 (4×10^9 PFU/mouse) to *ob/ob* mice reduced the macrophage population and production of TNF-α, MCP-1, and IL-6 in adipose tissue via inhibition of nuclear factor-kappa B (NF-κB) activation and phosphorylation of ERK1/2 and c-Jun N-terminal kinases [18]. Sitagliptin (4 g/kg) also showed similar effects and reduced the expression of mRNA for inflammatory cytokine genes and macrophage infiltration in adipose tissue of high fat diet- (HFD-) induced obese mice [19]. In patients with type 2 diabetes, sitagliptin (100 mg/day) therapy significantly reduced the plasma levels of C-reactive protein (CRP), IL-6, IL-18, secreted phospholipase-A2, soluble intracellular adhesion molecule- (ICAM-) 1, and E-selectin compared with placebo. The inflammatory score and the homeostatic model assessment index for insulin resistance were significantly reduced in sitagliptin-treated type 2 diabetes patients [7]. Therefore, suppression of inflammatory mediators in adipose tissue by GLP-1-based therapies might contribute to the improvement of insulin sensitivity.

GLP-1-based therapies for diabetes contribute to reduce inflammation and have additional beneficial effects such as islet preservation and improvement of insulin sensitivity in addition to glucose-lowering effects. However, some rare cases of acute pancreatitis and neoplasms have been reported [53–55]; thus the establishment of safety of GLP-1-based therapy should be validated by sufficient further studies.

3. Vascular Disease

Inflammation is known to be a risk factor for vascular diseases such as atherosclerosis. Atherosclerotic cardiovascular disease is caused by proinflammatory stimuli in the vascular endothelial cells and is associated with increased plasma levels of TNF-α, IL-6, CRP, and circulating endotoxin (i.e., lipopolysaccharide (LPS)) [56, 57]. Atherosclerosis is a chronic inflammatory condition resulting from the invasion and accumulation of white blood cells (foam cells) in the walls of arteries and therefore is a syndrome affecting arterial blood vessels [58].

GLP-1 (5.0 μM) perfusion attenuates LPS-induced microvascular permeability via the cAMP protein kinase A (PKA) pathway [59]. Liraglutide (100 μM) reduced the mRNA expression of adhesion molecules such as vascular cell adhesion molecule- (VCAM-) 1, ICAM-1, and E-selectin in TNF-α- or LPS-stimulated human aortic endothelial cells and human umbilical vein endothelial cells [60–62]. Liraglutide (100 nM) induced phosphorylation of calcium/calmodulin-dependent protein kinase I and 5′ adenosine monophosphate-activated protein kinase (AMPK), and inhibition of calcium/calmodulin-dependent protein kinase kinase β (CAMKKβ) abolished the inhibitory effect of liraglutide on the expression of VCAM-1 and E-selectin. In addition, knockdown of AMPK with short hairpin AMPK RNA

abolished the liraglutide activation of AMPK and anti-inflammatory effects. These results demonstrate that the anti-inflammatory effects of liraglutide in human aortic endothelial cells is dependent on activation of CAMKKβ and AMPK, which are cAMP/Ca^{2+} signaling pathways [60]. In addition, it was reported that liraglutide (100 nM) inhibited TNF-α- or hyperglyceamia-mediated induction of plasminogen activator inhibitor type-1 in human vascular endothelial cells [23]. Exendin-4 (50 μg/kg/day) treatment resulted in a reduction of atherosclerosis development and the number of monocytes adhering to the endothelium wall in the aortic root in western-type diet-fed APOE*3-Leiden.CETP(E3L.CETP) mice [63].

Sitagliptin (25 μM), NVP-DPP728 (270 μM), or liraglutide (1000 ng/mL) treatment significantly reduced oxidized-low-density lipoprotein-induced or PKC activator-induced protein expression of nucleotide-binding domain-like receptor with a pyrin domain 3 (NLRP3), toll-like receptor 4 (TLR4), and IL-1β in a human monocytic cell line, THP-1, by decreasing phosphorylated-protein kinase C (PKC) [64]. Administration of linagliptin (10 mg/kg/day), a DPP-4 inhibitor, to $ApoE^{-/-}$ mice, an animal model of atherosclerosis, decreased inflammatory molecule expression and macrophage infiltration in the atherosclerotic aorta [65]. Another report showed that sitagliptin (576 mg/kg) reduced plaque macrophage infiltration and matrix metallopeptidase-9 (MMP-9) levels in $ApoE^{-/-}$ mice [26] and increased activation of AMPK and AKT signaling pathway but inhibited MAPK and ERK1/2 signaling in aorta of $ApoE^{-/-}$ mice [66]. This suggests that sitagliptin has protective actions against atherosclerosis through AMPK and MAPK-dependent mechanisms. In addition, sitagliptin (30 mg/kg/day) and exenatide (3 μg/kg/12 h) significantly inhibited advanced glycation end products-induced oxidative stress in aortic endothelials in high fat diet (HFD)/STZ diabetic rats by reducing endothelin-1 (ET-1) and inflammatory cytokine via RhoA/Rho-associated protein kinase (ROCK)/NF-κB signaling pathways and AMPK activation [67]. Des-fluorositagliptin (200 mg/kg/day) treatment reduced atherosclerotic lesion formation, infiltration of macrophage and T lymphocytes, and the expression of proinflammatory cytokines within plaques in $ApoE^{-/-}$ mice [68]. As well, treatment with alogliptin (20 mg/kg/day), a selective DPP-4 inhibitor, showed similar anti-inflammatory effects in the injured arteries of low-density lipoprotein receptor-deficient mice [69]. Interestingly, metabolite (9-37) of GLP-1 as well as the c-terminal GLP-1 split product (28-37) also reduced plaque inflammation and stabilized atherosclerotic lesions in $ApoE^{-/-}$ mice [70]. These suggest that GLP-1-based therapies have protective effects in atherosclerosis by decreasing macrophage infiltration in atherosclerotic lesions via inhibition of the expression of adhesion molecules.

The loss of sirtuin 6 (SIRT6), which regulates proinflammatory mediators, in human umbilical vein endothelial cells is associated with upregulation of the expression of proinflammatory genes [71]. Liraglutide (100 nM) treatment increased SIRT6 expression and reduced NF-κB expression compared with only high glucose-treated endothelial cells. In diabetic patients treated with GLP-1-based therapy, the protein level of SIRT6 in asymptomatic plaques was significantly increased and TNF-α and MMP-9 levels in lesions were significantly reduced compared with diabetic patients without treatment [8]. This result suggests that GLP-1-based therapy has anti-inflammatory effects by induction of SIRT6 expression in endothelial cells.

Cardiovascular disease is increased in type 2 diabetes, and hyperglyceamia is a critical promoter during the development of cardiovascular diseases. Inflammation is an important pathophysiologic factor in diabetic cardiomyopathy. Exendin-4 protects against cardiac contractile dysfunction in an experimental myocardial infarction model. Exendin-4 (5 μg/kg or 1 and 10 nM) inhibited high mobility group box I protein expression, a proinflammatory mediator, in myocardial ischemia and reperfusion in rats [72] and in high glucose-induced myocardial cell injury [73]. Sitagliptin (30 and 50 mg/kg/day) reduced the expression of TNF-α and IL-6 in the diabetic heart and had a myocardial protective effect in STZ/HFD-induced diabetic rats [74]. Therefore, GLP-1-based therapy have anti-inflammatory effects on vascular disease and may explain the vasoprotective properties.

4. Neurodegenerative Brain Disorder

Neurodegenerative central nervous system disorders are associated with chronic neuroinflammation [75–77]. Epidemiological and clinical studies have suggested a link between type 2 diabetes and Alzheimer's disease [78]. In patients with Alzheimer's disease, insulin receptors and insulin signaling in the brain are desensitized and impaired as found in type 2 diabetes patients. Therefore, drugs used for treatment of diabetes are expected to have a preventive effect against Alzheimer's disease. GLP-1 is known to be produced in the brain [79] and has many functions including neuroprotection [80–82]. In addition, GLP-1 and GLP-1 analogues enter the brain through blood brain barrier [83–86].

The glia may play a critical role in the central nervous system inflammatory responses including Alzheimer's disease, and GLP-1 receptor was observed in astrocytes and microglia [17, 87]. In astrocytes, GLP-1 (1 μM) prevented the LPS-induced IL-1β expression by increase of cAMP [17].

Models of Alzheimer's disease include intracerebroventricular injection of STZ [88], intracerebral injection of LPS [88], and the APPSWE/PS1ΔE9 mouse [84]. Exenatide (20 μg/kg/day) treatment inhibited brain TNF-α levels, which were induced by intracerebroventricular injection of STZ [89]. GLP-1 (7-36) amide (50 nM) protected the synaptic impairments induced by intracerebral injection of LPS in the rat hippocampus [90]. Liraglutide (25 nmol/kg/day) treatment significantly reduced the inflammatory response in the cortex as measured by the number of activated microglia and prevented degenerative processes in a 7-month-old $APP_{SWE}/PS1_{\Delta E9}$ mouse model of Alzheimer's disease [86]. In addition, in the 14-month-old $APP_{SWE}/PS1_{\Delta E9}$ mouse, inflammation was also markedly reduced and restorative effects were improved by liraglutide treatment [91]. The GLP-1 receptor agonist, lixisenatide, exerted neuroprotective

effects via reduction of oxidative stress and the chronic inflammation response in the brain of $APP_{SWE}/PS1_{\Delta E9}$ mouse [92]. In addition, sitagliptin (10 and 20 mg/kg) also showed similar anti-inflammatory effects in $APP_{SWE}/PS1_{\Delta E9}$ mouse [93]. This suggests that GLP-1-based therapies could have a preventive and restorative effect on the pathophysiology of Alzheimer's disease progression.

Irradiation of the brain causes a chronic inflammatory response. X-ray irradiation of the brain significantly increased IL-6, IL-1β, and IL-12p70 cytokine protein expression. Liraglutide (25 nmol/kg/day) treatment reduced the mRNA expression of proinflammatory cytokine genes, which was induced by X-ray irradiation [24].

Parkinson's disease is a chronic and neurodegenerative brain disorder, and inflammatory activity is one of important features of Parkinson's disease. Microglial activation plays a critical role in the pathogenesis of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyidine- (MPTP-) induced Parkinson's disease model and human Parkinson's disease [27]. Exendin-4 (10 μg/kg) treatment significantly decreased MPTP-induced microglial activation and suppressed MPTP-induced expression of TNF-α and IL-1β [94]. The inhibitory effect of exendin-4 on microglial activation may have therapeutic potential for the treatment of Parkinson's disease. These anti-inflammatory effects of GLP-1-based therapies on the brain may protect against neurodegenerative central nervous system disorders.

5. Nonalcoholic Steatohepatitis

Nonalcoholic steatohepatitis is associated with an inflammation of the liver by an aberrant accumulation of fat in the liver. GLP-1 receptor agonists reduced alanine aminotransferase and aspartate aminotransferase levels in patients with nonalcoholic fatty liver disease (or type 2 diabetes) and improved lipid metabolism and reduced fat mass [21]. Liraglutide (50, 100, and 200 μg/kg/12 h) treatment protected against nonalcoholic fatty liver disease by inhibition of ER stress-associated apoptosis in HFD-fed rats [28]. Liraglutide or exendin-4 (1 nmol/kg/day) treatment dose-dependently reduced steatosis and lobular inflammation in HFD-fed rats or mice compared with the saline-injected group [28, 95], probably due to an increase of SIRT1 [96]. As a matter of fact, exendin-4 (50 μg/kg/day) treatment increased the expression of SIRT1 and its downstream factor, AMPK, in exendin-4 treated mouse livers and hepatocytes. Exendin-4 treatment reduced hepatic expression of the inflammatory markers TNF-α, IL-1β, and IL-6 and macrophage markers, cluster of differentiation 68 (CD68), and F4/80 in the liver of mice fed a western-type diet [63].

In nonalcoholic steatohepatitis patients with glucose intolerance, liraglutide (0.9 mg/person/day) therapy for 96 weeks resulted in improvement of histological indicators of inflammation in seven subjects out of ten subjects [97]. CRP is produced by the liver and is a marker of inflammation. In a retrospective analysis of 110 obese patients with type 2 diabetes treated with liraglutide, the mean concentration of CRP declined after treatment with liraglutide for a mean duration of 7.5 months [9]. In addition, exenatide plus metformin resulted in a significant reduction in CRP and TNF-α compared with baseline [98]. These reports suggest that GLP-1-based therapies improve fatty liver disease by ameliorating inflammation in rodents and humans.

6. Nephropathy

Diabetic nephropathy is associated with a state of low-grade inflammation in the microvasculature of the kidney's glomeruli [99, 100]. The GLP-1 receptor is expressed in glomerular capillaries and vascular walls of the mouse kidney [14, 101] and in the glomerulus and proximal convoluted tubules of the rat and pig [29, 102]. GLP-1 receptor deficiency in the diabetic nephropathy-resistant C57BL/6-Akita mouse contributes to the development of diabetic nephropathy, and liraglutide treatment suppressed the progression of nephropathy of the KK/Ta-Akita mouse, which shows high susceptibility to diabetic nephropathy [14], suggesting that GLP-1 action might play an important role in prevention of diabetic nephropathy.

Various studies have shown that GLP-1-based therapies can reduce macrophage infiltration and inflammatory molecules in models of diabetic nephropathy. Exendin-4 (3 and 10 μg/kg/day) treatment significantly downregulated the gene expression of CD14, ICAM-1, and TGFβ1 in the renal cortex, prevented glomerular macrophage infiltration in glomeruli, and reduced oxidative stress and inflammation in tubular cells in STZ-induced diabetic animals [101, 103]. Treatment with the DPP-4 inhibitor, saxagliptin (10 mg/kg/day), reduced renal tubulointerstitial inflammation by NF-κBp65-mediated macrophage infiltration in STZ-induced diabetic $enos^{-/-}$ mice [104]. Administration of the DPP-4 inhibitor, PKF275-055 (3 mg/kg/day), or linagliptin in STZ-induced diabetic rats inhibited macrophage infiltration, inflammatory molecules, and NF-κB activity in the glomeruli [105] and significantly reduced glomerular leukocyte infiltration [106]. Sitagliptin (10 mg/kg/day) treatment decreased the expression of proinflammatory cytokine genes IL-1β and TNF-α in kidney of diabetic ZDF rat [25].

GLP-1-based therapies are also effective in nondiabetic models of kidney injury. In a nondiabetic glomerular injury model, alogliptin (20 mg/kg/day), anagliptin (300 mg/kg/day), or exendin-4 (10 mg/kg) significantly reduced infiltration of CD68-positive inflammatory macrophages in the kidney [107]. In the mouse cisplatin-induced renal injury model, treatment with alogliptin (10 mg/kg/day) significantly decreased cisplatin-induced renal injury via antiapoptotic effects [108]. In addition, after ischemia-reperfusion injury, the expression of proinflammatory cytokines, NF-κB and ICAM-1, as well as macrophage infiltration in the kidney was significantly decreased by exendin-4 (10 μg/kg) or sitagliptin (600 mg/kg) treatment [109]. Therefore, GLP-1-based therapies might be beneficial for nephropathy by reducing glomerular leukocyte infiltration and proinflammatory mediators.

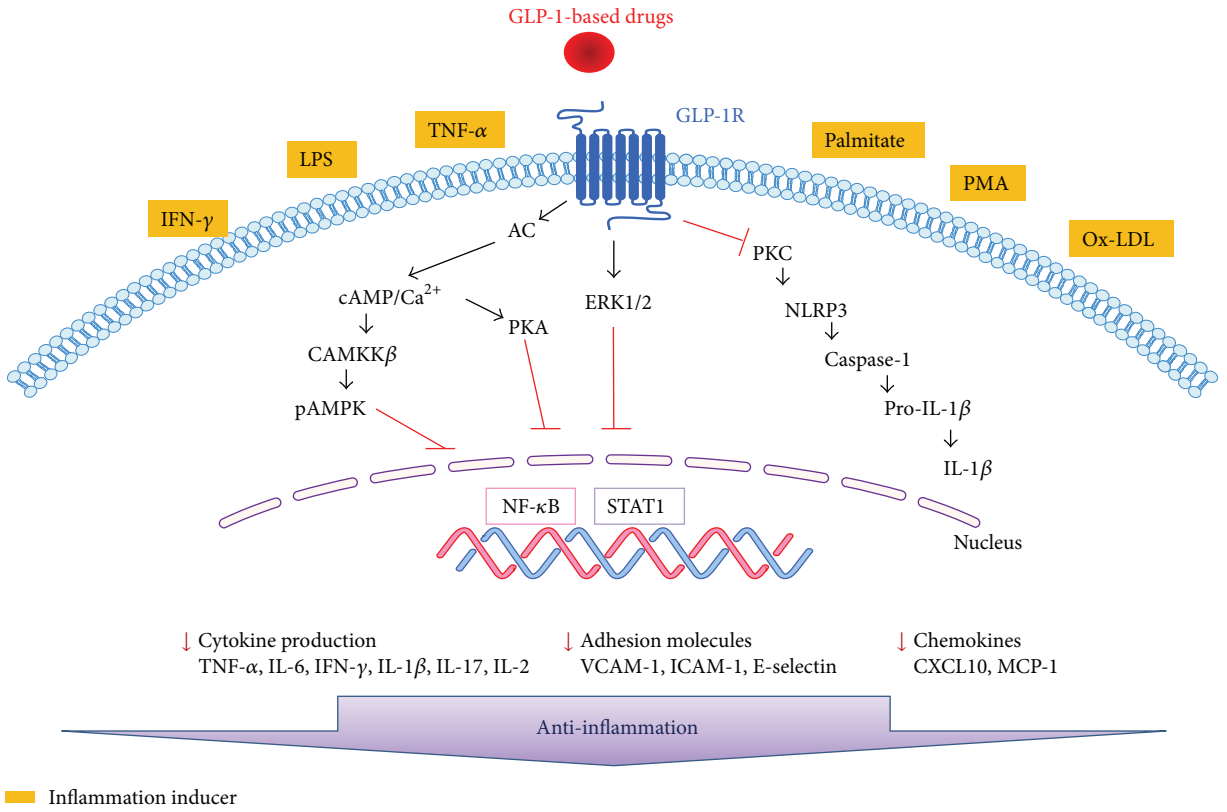

Figure 1: Molecular signals underlying the anti-inflammatory effects of GLP-1-based drugs. DPP-4 inhibitors increase GLP-1 levels in plasma. GLP-1 and GLP-1 receptor (GLP-1R) agonists bind to the GLP-1 receptor, which blocks PKC or NF-κB activation and subsequent expression of NLRP3, IL-1β, TNF-α, IL-6, VCAM-1, IFN-γ, and MCP-1. In addition, GLP-1R signaling activates cAMP/Ca^{2+}, CAMKKβ, and pAMPK, which induces anti-inflammatory effects on monocyte adhesion.

7. Other Diseases

Asthma is a chronic pulmonary inflammatory disease. Liraglutide (2 mg/kg) reduced immune cell infiltration and protein expression of E-selectin, TNF-α, IL-4, IL-5, and IL-13 in the lung tissue or bronchoalveolar lavage fluid in an ovalbumin-induced chronic asthma model. Liraglutide treatment decreased NF-κB activation, which was reversed by PKA inhibitor, H-89, suggesting that the cAMP-PKA pathway is involved in inhibition of NF-κB activation, and subsequently the inhibition of inflammation [110]. In addition, in mice with bleomycin-induced pulmonary fibrosis, liraglutide treatment inhibited infiltration of immune cells and decreased the content of TGF-β1. Liraglutide treatment markedly attenuated bleomycin-induced VCAM-1 and NF-κB activation [30]. These results suggest that GLP-1-based therapies might have beneficial effects on asthma but need to be validated by clinical studies.

Obesity can reduce the quality and count of men's sperm [111, 112]. The expression of TNF-α, MCP-1, and F4/80 mRNA levels is increased in the testis and significantly decreased the sperm motility and activity in diet-induced obesity mice, and exenatide (24 nmol/kg/day) treatment suppressed the expression of TNF-α, MCP-1, and F4/80 mRNA levels in testis and improved sperm quality in diet-induced obesity mice [111].

In type 2 diabetes patients, GLP-1 and liraglutide also improve clinical symptoms of psoriasis, a skin inflammatory disease, by downregulation of invariant natural killer T cells [31, 113, 114].

GLP-1 (100 nM) or exendin-4 (10 nM) treatment inhibited TNF-α-induced expression of receptor for advanced glycation end products (RAGE), ICAM-1, and VCAM-1 in human retinal pigment epithelial cells [32], suggesting that GLP-1-based therapies might have beneficial effects on diabetic retinopathy.

Treatment with the DPP-4 inhibitors, linagliptin (5 mg/kg/day) and sitagliptin (50 mg/kg/day), and the GLP-1 analogue, liraglutide (200 μg/kg/day), significantly reduced inflammatory markers such as inducible NO synthase, cyclooxygenase, and VCAM-1 via the AMPK pathway in LPS-induced endotoxemic shock in rats as a model of human sepsis [115].

These reports suggest that GLP-1-based therapies have anti-inflammatory effects in the lung, testis, skin, and eye.

8. Conclusion

Inflammation is a protective process including immune system, vascular system, and molecular mediators. However out-of-control inflammation and chronic inflammation can

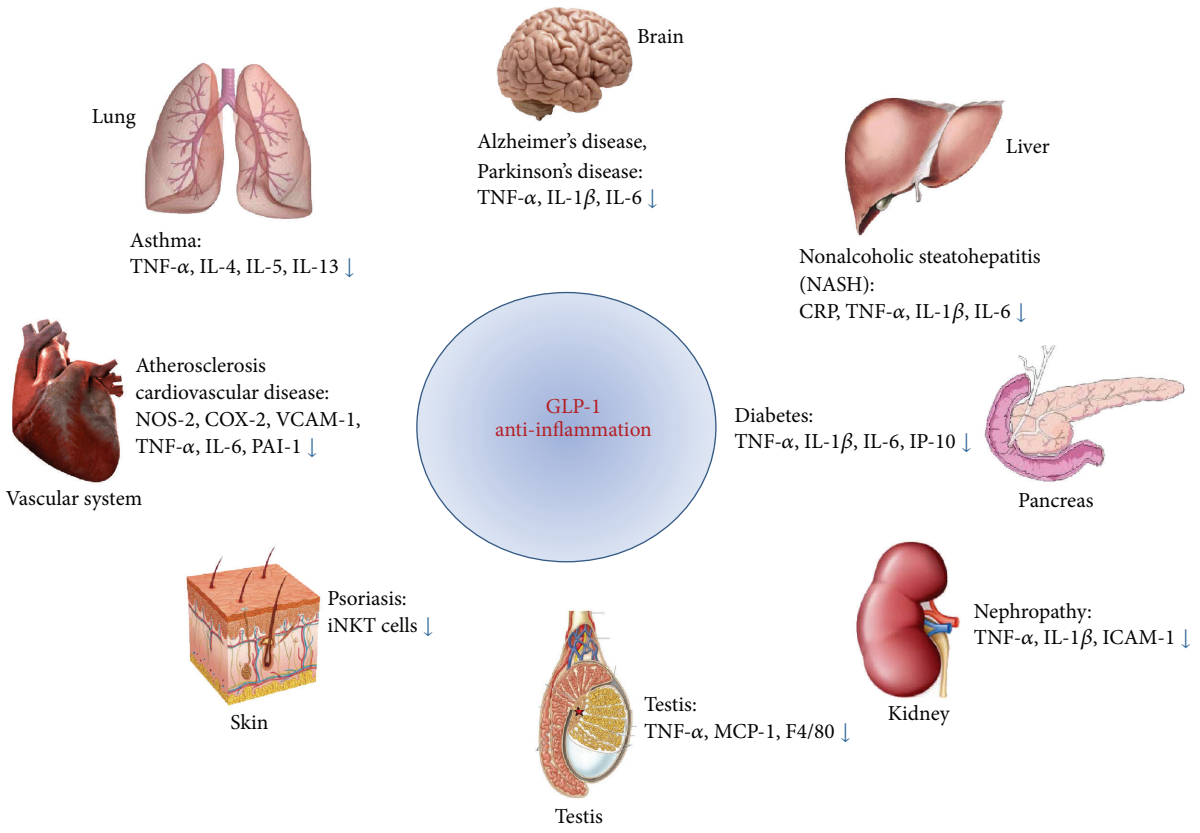

Figure 2: GLP-1-based therapies, including GLP-1, GLP-1R agonists and DPP-4 inhibitors, have anti-inflammatory functions in several organs.

cause pathological disease. Inflammation is a risk factor for diabetes, atherosclerosis, cardiovascular disease, neurodegenerative central nervous system disorders, nonalcoholic steatohepatitis, and nephropathy.

GLP-1-based therapies have many attractive and beneficial effects including their antidiabetic actions on pancreatic beta-cells. However, beyond their metabolic effects, GLP-1-based therapies have been shown to have anti-inflammatory effects via several molecular pathways (Figure 1) in several organs, tissues, and cells (Figure 2). GLP-1-based therapies downregulate proinflammatory responses in inflammatory related diseases. This review concludes that GLP-1-based therapy has beneficial effects on inflammatory disease. Thus GLP-1, GLP-1R agonists, and DPP-4 inhibitors might have important roles as mediators of inflammation.

Abbreviations

AMPK: 5′ adenosine monophosphate-activated protein kinase
CAMKKβ: Calcium/calmodulin-dependent protein kinase kinase β
CD: Cluster of differentiation
CRP: C-reactive protein
CXCL10: C-X-C motif chemokine 10
cAMP: Cyclic adenosine monophosphate
DPP-4: Dipeptidyl peptidase-4
Erk: Extracellular signal-regulated kinase
GLP-1: Glucagon-like peptide-1
HFD: High fat diet
ICAM: Intercellular adhesion molecule
IFN: Interferon
IL: Interleukin
LPS: Lipopolysaccharide
MCP-1: Monocyte chemotactic protein
MPTP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyidine
NF-κB: Nuclear factor-kappa B
NLRP3: Nucleotide-binding domain-like receptor with a pyrin domain 3
PKA: Protein kinase A
PKC: Protein kinase C
SIRT: Sirtuin
STZ: Streptozotocin
TLR4: Toll-like receptor 4
TGF-β: Transforming growth factor beta
TNF-α: Tumor necrosis factor alpha
VCAM: Vascular cell adhesion molecule.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Review Article

Thyroid Hormones, Oxidative Stress, and Inflammation

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Inflammation and oxidative stress (OS) are closely related processes, as well exemplified in obesity and cardiovascular diseases. OS is also related to hormonal derangement in a reciprocal way. Among the various hormonal influences that operate on the antioxidant balance, thyroid hormones play particularly important roles, since both hyperthyroidism and hypothyroidism have been shown to be associated with OS in animals and humans. In this context, the nonthyroidal illness syndrome (NTIS) that typically manifests as reduced conversion of thyroxine (T_4) to triiodothyronine (T_3) in different acute and chronic systemic conditions is still a debated topic. The pathophysiological mechanisms of this syndrome are reviewed, together with the roles of deiodinases, the enzymes responsible for the conversion of T_4 to T_3, in both physiological and pathological situations. The presence of OS indexes in NTIS supports the hypothesis that it represents a condition of hypothyroidism at the tissue level and not only an adaptive mechanism to diseases.

1. Introduction

Oxidative stress (OS) is defined as an unbalance between the production of prooxidant substances and antioxidant defenses. The most important prooxidants are the reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1]. The ROS family includes superoxide anion, hydroxyl radical, hydrogen peroxide, and hypochlorous acid. The first three substances are produced *in vivo* mainly by the mitochondrial respiratory chain during the oxidative metabolism of energetic substrates [2, 3]. They are regulators of redox-sensitive pathways involved in cellular homeostasis [4] and influence some transcription factors, in addition to the endogenous antioxidant pool [4–7]. RNS are peroxynitrite, produced by the reaction of nitric oxide (NO) with superoxide, and nitrosoperoxycarbonate, formed by the reaction of peroxynitrite with carbon dioxide. ROS and RNS are considered important pathogenetic factors in different diseases [8]. Among them, a particular pathogenetic role is played by the free radicals, that is, superoxide anion and hydroxyl radical, that are molecules characterized by high chemical reactivity due to a single unpaired electron in the external orbital.

In some cell types, such as leukocytes, endothelial and mesangial cells, fibroblasts, thyrocytes, oocytes, Leydig cells, and adipocytes, ROS generation could play functional roles [9]. Dual oxidases (DUOX), enzymes crucial for hydrogen peroxide generation, are essential for thyroid peroxidase- (TPO-) catalyzed hormone synthesis [10]. Two oxidases of such family are present in thyroid (DUOX1 and DUOX2). They work in conjunction with DUOXA1 and DUOXA2, which are maturation factors that allow DUOX enzymes to translocate to the follicular cell membrane and exert their enzymatic activity [10]. In addition, NADPH oxidase 4 (NOX4) [11] is a new intracellular ROS generating system recently described in the human thyroid gland.

An increased ROS production by the respiratory chain resulting from the rise of the energetic demand or substrate availability [12], as occurs in obesity, or mitochondrial dysfunction or impairment, can produce cell damage and contribute to the pathophysiology of different diseases, such as

inflammatory (e.g., rheumatoid arthritis) and cardiovascular (e.g., myocardial infarction) diseases [2]. A pathophysiological role of ROS has been also suggested in diabetes mellitus, in which oxidation accompanies glycation *in vivo* and the antioxidant capacity is decreased, resulting in increased susceptibility to oxidative stress [13].

Different defensive mechanisms that protect against the free radical damage have been characterized in various cellular localizations, including the endoplasmic reticulum, mitochondria, plasma membrane, peroxisomes, and cytosol [2]. Enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and transition-metal binding proteins, such as transferrin, ferritin, and ceruloplasmin, prevent the production of or rapidly inactivate free radicals. SOD accelerates the dismutation process of superoxide anion in hydrogen peroxide and molecular oxygen that normally occurs with a rate constant 10^4-fold lower. CAT detoxifies hydrogen peroxide by transforming it in water and molecular oxygen. GPx also participates in hydrogen peroxide detoxification when hydrogen peroxide levels are high. In addition, GPx detoxifies lipid peroxides by transforming them in the corresponding alcohols. "Scavengers" molecules, including both water-soluble, such as albumin, bilirubin, ascorbic acid, urates and thiols, and liposoluble, such as Vitamin E and coenzyme Q_{10} (CoQ_{10}), substances interrupt the lipid-peroxidation chain by reacting with and neutralizing the intermediate radicals. The high diffusion rate of scavengers, particularly the liposoluble ones in biological membranes, allows them to intercept radicals and transform them into more stable molecules, thus stopping the radical chain. Sometimes scavengers can be regenerated. A third defensive mechanism uses processes which remove the molecules damaged by the oxidative attack, allowing the reconstitution of normal structures (e.g., specific phospholipases remove the peroxidized fatty acids, making the enzymatic reacylation of damaged molecules possible) [2].

The production of ROS and RNS can occur at the cellular level in response to metabolic overload caused by the overabundance of macronutrients. In addition, mitochondrial dysfunction and endothelial reticulum stress contribute to adipose tissue metabolic derangement in obese patients [14, 15]. ROS generation is further maintained by an inflammatory response, feeding a vicious circle. This picture is worse in pre- and postpubertal children, because puberty alters some inflammatory markers associated with endothelial dysfunction (adipocytokine levels, OS, and insulin sensitivity).

Recent findings suggest that mitochondrial reactive species are signalling molecules that mediate the production of proinflammatory cytokines, thus connecting OS and inflammation. This topic has been extensively studied in cardiovascular diseases [16].

However, besides inflammation, OS can be related to hormonal derangement in a reciprocal way. Some hormones influence antioxidant levels; on the other hand, OS can modify synthesis, activity, and metabolism of hormones. Therefore, OS is related to both systemic inflammation and hormonal derangement. In particular, thyroid hormones play important roles in antioxidant modulation, as demonstrated in different *in vitro* and *in vivo* studies. Reduced glutathione (GSH) is an important cofactor of both antioxidant enzymes and deiodinases, the enzymes responsible for the conversion of thyroxine (T_4) to triiodothyronine (T_3). Moreover, plasma levels of small antioxidant molecules, such as Vitamin E and CoQ_{10}, and thyroid hormones are closely related to each other [2, 17]. Both hyperthyroidism and hypothyroidism have been shown to be associated with OS and special cases are the autoimmune thyroiditis or the functional picture of low-T_3 syndrome, observed in acute and chronic nonthyroidal illness syndrome (NTIS) [17–19]. It is still debated whether NTIS represents an adaptive response or a real hypothyroidism at the tissue level. Therefore, studies on OS in NTIS are important to gain knowledge about the pathophysiology of the syndrome itself.

In this review, we firstly examine the relationships between OS and inflammation. Then, we present available data on thyroid hormones and antioxidant regulation. Finally, we report the results of investigations on the relationships between inflammatory mediators and OS in NTIS, in the attempt of hypothesizing a reciprocal influence between tissue hypothyroidism (as primary cause or secondary to inflammation) and OS. Thus, the aim of our review is to discuss and clarify the relationships between thyroid hormones and parameters of OS in the context of the inflammatory diseases.

2. Oxidative Stress and Inflammation

Different mediators produced by the adipose tissue may potentially cause an increase of systemic and local ROS and RNS. Thus, the dysregulation of signalling pathway originating in adipocytes, as observed in obese patients, can induce and perpetuate inflammation and OS. Recent studies clearly indicate that the adipose tissue can be considered as an endocrine organ producing different proteins (adipokines) with wide biologic activities. In addition, after maturation from the stage of preadipocytes, the adipocytes gain functions similar to those of macrophages, including the ability to be activated by components of the bacterial wall and to synthesize and secrete cytokines [20]. Moreover, during the periods in which weight gain or loss occurs, the cellular composition of the adipose tissue dynamically changes, showing variations in the levels of various cell types represented in the tissue, in particular vascular and immune cells. The levels of the latter, in particular the macrophages, importantly increase in obese patients. The macrophages seem to play important roles in the pathogenesis of insulin resistance associated with obesity, through the production of Monocyte Chemoattractant Protein-1 (MCP-1) and the modulation of the spreading and the growth of the adipose tissue itself [21]. Monocytes mobilized and attracted by MCP-1, together with neutrophils and lymphocytes T present in the adipose tissue, originate an inflammatory response that is reinforced by the stimulation of the synthesis and secretion of tumor necrosis factor (TNF) by macrophages, in turn induced by the increased production of free fatty acids (FFAs) by adipocytes. In addition, a two-way interaction between

adipocytes and macrophages seems to develop, by which the macrophages stimulate the expression and release of MCP-1 from the adipocytes through ROS production. By this way a vicious circle is established, which may promote a chronic inflammatory status gradually more and more intense, typical of obesity and its complications. Finally, the macrophages regulate the remodelling of the adipose tissue when a chronic positive energetic balance ensues. Different pathways are activated in adipocytes depending on whether subtype M1 or M2 macrophages are stimulated, that regulate adipocyte proliferation, growth, and survival. The induced changes are responsible for the appearance of a hypertrophic or hyperplastic obesity. In case of the prevalence the M1 proinflammatory macrophagic subtype, the reduced survival and proliferation of the preadipocytes will cause an inadequate adipocyte reserve; consequently, the energetic backlog, through an excessive hypertrophy, will produce a dysfunctional adipose tissue, which will perpetuate the inflammatory process and, in the long term, produce insulin resistance. Conversely, if the M2 macrophagic subtype is prevalent, the functional pool of preadipocytes will be favoured. They will differentiate into adipocytes, contributing to the formation of an adequate hyperplastic adipose tissue with preserved cell functions and insulin sensitivity [22].

Therefore, obesity is associated with increased secretion of proinflammatory hormones and cytokines (leptin, resistin, TNF-α, and interleukin- (IL-) 6) and decreased release of adipokines that downregulate inflammation (adiponectin, IL-10). Recent studies [23] show that not only the amount but also the kind of adipose tissue, as well as the kinds of fats in the diet, influence in different ways this chronic inflammatory state.

Many other mechanisms reviewed by Siti et al. [16] reinforce the link between OS and inflammation. Among these, there is the overexpression of endothelin that induces ROS production in endothelial cells by increasing NADPH oxidase activity [24]; on the other hand, OS causes an increase in angiotensin converting enzyme [25], creating a loop with the previously cited mechanism. Another important mechanism is the OS-induced Ca^{2+} influx, responsible for inflammatory processes [26].

In diabetes, the chronic inflammation, the increase in FFA levels, and the overactivation of the renin-angiotensin system contribute to insulin resistance via OS [27]. TNF-α, an important mediator of inflammation, interferes with insulin signals through the activation of the PI3-kinase pathway in endothelial cells [28]. A systemic lipid infusion, that induces acute elevation of plasma FFA levels, causes the activation of the NF-kB pathway, OS, and impairment of endothelium-dependent vasodilatation. In addition, insulin effects on vasodilatation, NO production, and muscle capillary recruitment are blunted by the lipid infusion [29–32]. Regarding this subject, we have shown that a naturally enriched antioxidant diet is capable of improving insulin sensitivity and metformin effects in adult obese patients [33].

Other studies confirmed the link between OS, vascular inflammation, and hypertension-associated vascular changes [34]. Moreover, it is well known that oxidized LDL have a key role in the initiation and progression of the atheromatous plaque [16, 35]; a main role has been recently attributed to the lectin-like oxidized LDL receptor-1 (LOX-1), which is upregulated by the exposure to inflammatory stimuli [36]. The role of the renin-angiotensin system in OS-related injury of endothelial cells has been recently reviewed [37]. Elegant studies conducted in experimental animal models, such as the ApoE knock-out mouse, confirmed an oxidant/antioxidant unbalance in the atherosclerotic process [38–40]. A large number of studies have been published on this topic, which, however, is not among the subjects of the present review. Nevertheless, they overall confirm the association between inflammation and OS.

3. The Role of Thyroid Hormones in Antioxidant Regulation

The role of thyroid in the regulation of the antioxidant systems has been recently reviewed in the context of the reproductive endocrinology [41]. It is well known that thyroid function influences the ovarian activity. ROS play physiological roles in the ovary and hypothyroidism, or a low-T_3 syndrome, can induce ovarian dysfunction by interfering with the antioxidant systems.

OS has been shown to be associated with both hyperthyroidism and hypothyroidism [42]. However, the mechanisms by which OS is generated in these two clinical conditions are different: increased ROS production in hyperthyroidism and low availability of antioxidants in hypothyroidism.

Some complications of hyperthyroidism in target tissues are caused by OS [43]. Thyroid hormones *per se* can act as oxidants and produce DNA damage (contrasted by CAT), probably through the phenolic group, which is similar to that of steroidal estrogens [44]. Many other mechanisms, as previously reviewed [45], can be involved, in particular the enhanced Nitric Oxide Synthase (NOS) gene expression with NO overproduction and the activation of hepatic NF-kB with the consequent increase in cytokines levels which induces ROS production. On the other hand, other mechanisms regulated by thyroid hormones carry out a fine regulation of the oxidative status via autoloop feedback. Among them, we underline the role of Uncoupling Protein- (UCP-) 2 and Uncoupling Protein-3. Data obtained in plants and animals indicate that these molecules have antioxidant activity [46–48]. However, only T_3 seems to regulate UCP, whereas no effect is exerted by T_4 [49, 50]. An opposite effect is induced by estrogens, which increase ROS production by repressing UCP [51].

The increased turnover of mitochondrial proteins and mitoptosis also participate in the regulation of the oxidative status, by removing the mitochondria damaged by OS [52]. These processes are regulated by peroxisome proliferator-activated receptor gamma coactivator-1, which in turn is upregulated by T_3 administration [53].

Thyroid hormones influence lipid composition of rat tissues and consequently the susceptibility to OS. However, the response is tissue-specific, and discrepant effects of T_3 and T_4 have been reported. In rat liver, T_3-induced hyperthyroidism

was found to be associated with altered lipid-peroxidation indexes, including elevated levels of thiobarbituric reactive substances (TBARS) and lipid hydroperoxides that are byproducts of lipid peroxidation [45, 53–55]. On the contrary, no changes in TBARS production were found in homogenized livers from rats made hyperthyroid by administration of T_4 over a 4-week period [56]. No significant changes of TBARS or lipid hydroperoxides were observed in testes of hyperthyroid adult rats as well; however, hyperthyroidism promoted protein oxidation in testes, as indicated by the enhanced content of protein-bound carbonyls [57]. In addition, it should be emphasized that the effects of hyperthyroidism on the activity of antioxidant enzymes, including Mn- or Cu,Zn-SOD, CAT, and GPx, depend on the tissue investigated, with T_3 and T_4 having differentiated effects [58].

At the systemic level, hyperthyroidism has been associated with reduced circulating levels of alpha-tocopherol [59, 60] and CoQ_{10} [60, 61] in humans. CoQ_{10} showed a trend toward higher levels in hypothyroidism [61]. Thus, it seems to be a sensitive index of tissue effect induced by thyroid hormones in situations in which drug interference, such as treatment with amiodarone [62], or systemic illness inducing low-T_3 conditions [63] complicate the interpretation of thyroid hormone levels.

On the other side, data on hypothyroidism and OS in humans are conflicting. In a group of patients with primary hypothyroidism, Baskol et al. [64] found high plasma levels of malondialdehyde (MDA), an OS marker that is formed by lipid peroxidation, and NO, low activity of paraoxonase-(PON-) 1, an enzyme synthetized in the liver with antioxidant properties, and SOD levels not significantly different from those of controls. Interestingly, the treatment with thyroid hormones decreased MDA levels and increased PON-1 activity, even though values similar to those observed in controls were not reached [64]. They hypothesized that in patients with hypothyroidism the prooxidant environment could play a role in the development of atherosclerosis. Elevated MDA levels were also shown in subclinical hypothyroidism [65]. In this setting, the increased OS was attributed primarily not only to the decrease in antioxidants levels, but also to altered lipid metabolism, since a significant correlation among MDA and LDL-cholesterol, total cholesterol, and triglyceride levels was found. Total antioxidant status (TAS) was similar in overt hypothyroidism, subclinical hypothyroidism, and controls.

Excess TSH is known to directly produce OS [66]. Other studies confirmed the lipid peroxidation both in overt hypothyroidism and in subclinical hypothyroidism [67] as indicated by MDA elevation; protein oxidation has been reported as well, with elevation of protein carbonyls [67]. In this study, the correlation analysis suggested that both the TSH increase and the MDA elevation contribute to protein damage. Finally, different studies reported NO elevation [68, 69].

Data on other parameters are more conflicting. As far as PON-1 is concerned, a decreased activity of this enzyme was observed both in hypothyroidism and in hyperthyroidism [70], whereas no significant differences with respect to controls were shown in other studies [68]. Increased levels of TBARS, but also antioxidants, such as SOD, CAT, and Vitamin E, have been also reported [71]. All these parameters correlated with T_3 and the correlation between T_3 and CAT remained significant also when corrected for total cholesterol. TBARS elevation was shown in both overt hypothyroidism and subclinical hypothyroidism [69, 72], but these findings were not confirmed in other studies [68, 73].

Another matter of discussion is whether OS is related to hypothyroidism *per se* or to lipid profile alterations caused by thyroid disfunction, as reported above. Indeed, Santi et al. [74] reported OS in subclinical hypothyroidism, as shown by reduced arylesterase and increased TBARS and CAT, but they attributed this pattern to hypercholesterolemia.

We showed low total antioxidant capacity (TAC) levels in hypothyroid patients [75] and increased CoQ_{10} plasma levels in secondary hypothyroidism. This latter finding is mainly to be put in correlation with the metabolic role of CoQ_{10} in the mitochondrial respiratory chain and its consequent reduced cell use in hypothyroid patients. In secondary hypothyroidism, the picture is complicated by concomitant alterations of other pituitary-dependent axes, which can have opposite effect on CoQ_{10} plasma levels. Acromegaly and hypoadrenalism are characterized by low CoQ_{10} plasma concentrations; however, when they are associated with hypothyroidism, this latter has a predominant effect [75, 76].

New perspectives concern DUOX, DUOXA, and NOX4. Cases of hypothyroidism due to mutation of DUOX or DUOXA genes have been reported in the literature [10, 11]. In addition, alterations of NOX4 could be associated with thyroid cancer (via activation by H-Ras oncogene) and Hashimoto's thyroiditis, in which the increased extracellular expression of this enzyme raises Intercellular Adhesion Molecule-1 (ICAM-1) expression and cytokine release [77, 78].

Finally, another study conducted on patients affected by subclinical hypothyroidism secondary to Hashimoto's thyroiditis did not show any difference in endogenous MDA levels between hypothyroid patients and controls; however, MDA induction by the prooxidant 2,2′-azobis-(2-amidinopropane) hydrochloride was markedly augmented in hypothyroid patients. This response in serum was not accompanied by a similar pattern in the LDL fraction: in fact, copper-induced MDA production did not differ in patients affected by subclinical hypothyroidism with respect to controls, whereas it was significantly different from controls in patients with overt hypothyroidism [79]. Studies on patients with thyroiditis should be, however, interpreted with caution, in that both tissue inflammation and systemic inflammation are present in this autoimmune disorder.

The experimental procedures by which hypothyroidism is induced affect the OS findings. Hypothyroidism obtained by surgical thyroid resection in rats was associated with decreased OS in heart [80] and kidney [81]. On the contrary, drug-induced hypothyroidism was associated with increased lipid peroxidation in amygdala [82] and hippocampus in rats [82, 83]. Other cerebral areas, including the cerebellum, remained unaffected [84]. The latter findings, however, were not confirmed in other studies [82, 83]. Similarly, cell damage in various organs, including heart, spleen, liver, lung, and

kidney, has been found in animals following methimazole treatment, but not after thyroidectomy [84]. Some studies, however, indicate that the organ damage is not consequent to the hypothyroidism *per se*, but to the drug itself [85, 86].

In the latest years, the attention has been concentrated on the damage induced by OS in certain organs, including liver, bone, skeletal muscle, and particularly the heart [53]. The metabolism of cardiomyocytes depends on serum T_3, in that these cells lack a significant deiodinase activity [87]. Increased, decreased, or unmodified levels of total SOD, Mn-SOD, Cu,Zn-SOD, GPx, GSH, or Vitamin E have been reported in cardiomyocytes in response to hypothyroidism [88]. Unchanged or decreased levels of various other antioxidant molecules or parameters, such CoQ_9, CoQ_{10}, and TAC, have been also reported. These findings indicate that the evaluation of a single OS parameter is not a reliable index of the cellular oxidative status and the evaluation of TAC depends on the measurement method used.

OS has been also involved in the pathophysiology of schizophrenia. In fact, higher plasma levels of MDA and total plasma peroxides have been found in schizophrenic patients with respect to control subjects, which showed a significant correlation with T_3 levels [89].

The thyroid itself can be damaged by OS, which occurs in case of iodine excess. This topic has been studied both *in vitro* and in animals fed with a diet rich in iodide [90, 91]. Iodide has a stimulatory action of on hydrogen peroxide generation in thyroid slices and induces thyroid cell apoptosis at high concentrations [92].

Vitamin E has been shown to be protective against the tissue damage induced by peroxyl radicals, mainly not only by preserving the polyunsaturated fatty acids in biological membranes, but also by reducing the activity of NADPH oxidase [53].

4. The Model of Low-T_3 Syndrome

Low-T_3 syndrome is a condition characterized by a reduced peripheral conversion of T_4 to T_3 in the presence of normal thyroid hormone secretion. It occurs in a variety of nonthyroidal illness (NTI) and is defined as nonthyroidal illness syndrome (NTIS). The most important acute conditions in which the low-T_3 syndrome occurs include starvation and eating disorders and critical illness. During starvation (especially carbohydrate deprivation) and nonthyroid illness, deiodination of T_4 to T_3 is rapidly inhibited, causing the low-T_3 syndrome. As the illness progresses to more and more severe stages, a more complex syndrome with low-T_3 and T_4 ensues. In critical illness, many other changes of the pituitary-thyroid axis have been shown, including attenuated response to TRH, low tissue uptake of thyroid hormones, and altered thyroid hormone metabolism. A low-T_3 syndrome caused by the reduced peripheral conversion from the prohormone T_4 is also observed in different chronic diseases, including chronic kidney disease, liver failure, and chronic inflammatory diseases.

A component of NTIS can be related to cachexia, which is common in chronic systemic inflammation, renal failure, and heart failure. This field has been widely investigated in cancer patients. Cachexia represents a hypermetabolic wasting syndrome with progressive depletion of adipose tissue and skeletal muscle mass, often accompanied by anorexia [93]. Among the mediators of cachexia in cancer patients there are several cytokines and hormones also involved in the pathophysiology of NTIS. They are produced by tumour cells or macrophages surrounding them, as expression of the interaction between the neoplasia and the host environment. The most important are TNF-α, IL-1, IL-6, interferon- (IFN-) γ, proteolysis-inducing factor (PIF), angiotensin II, and myostatin, a member of the transforming growth factor-β superfamily. Interestingly, the signal transduction pathways of many of these substances involve NF-kB, the activity of which is in turn related to ROS levels. In fact, it has been shown that hydrogen peroxide, PIF, and angiotensin II activate NF-kB in myotubes [94] and the treatment of myotubes exposed to TNF-α, PIF, or angiotensin II with antioxidants reduces the NF-kB binding to DNA [94, 95]. In addition, it has been reported that the treatment of MAC16 colon-tumour bearing mice with Vitamin E reduces protein degradation in skeletal muscle [95]. Finally, some cytokines, including TNF-α, IL-1, IL-6, and IFN-γ, mimic leptin signalling, inducing central suppression of appetite [96].

The condition of NTIS is considered as an adaptive response rather than true hypothyroidism. Thyroid replacement therapy is not usually required, but this topic is still debated, since indirect signs of true hypothyroidism at tissue level have been shown. Some molecular mechanisms of NTIS are known, but more studies are necessary to further elucidate its pathogenesis. Indeed, it is probable that a full understanding of the pathophysiological mechanisms at the tissue level will allow the identification of patients who would benefit from replacement therapy. Our discussion will focus on the roles of cytokines and OS in the pathophysiology of NTIS.

The roles of cytokines as key molecules involved in coordinating the hormone, immune, and inflammatory responses to a variety of stressful stimuli are well known [18]. In a series of septic patients studied shortly after admission to the ICU, total T_4 (tT_4), free T_4 (fT_4), total T_3 (tT_3), and TSH plasma concentrations were depressed, and plasma levels of IL-1β, sIL-2 receptor, and TNF-α were elevated [97], indicating the establishment of central TSH suppression. The hypothalamic-pituitary-adrenal axis was activated as expected. Continuous infusion of IL-1 in rats causes reduction of TSH, free T_3 (fT_3), and fT_4 plasma levels. Higher doses of IL-1 induced a febrile reaction and suppression of food intake, with a cascade of events altering thyroid hormone economy [98]. However, IL-1 did not decrease the hepatic 5′-deiodinase activity that, on the contrary, is typically reduced in NTIS.

TNF is another proinflammatory cytokine that is thought to be involved in many of the alterations associated with NTIS. Infusion of rTNF in man decreases serum T_3 and TSH and increases reverse-T_3 (rT_3) [99]. These findings suggest that TNF could be involved in the IL-6-mediated suppression of the hypothalamic-pituitary axis. However, the involvement of TNF in NTIS pathophysiology was not confirmed in other studies, in which the effects of endotoxin

on thyroid hormones in humans were not counteracted by TNF-α blockade through specific IgG fusion proteins [100]. TNF-α was found in *in vitro* studies to activate NF-kB [101], which in turn inhibits T_3-induced expression of deiodinase 1 (D1).

An important pathophysiological role in NTIS has been attributed to IL-6, which is often elevated in serum of NTIS patients [102] in an inversely proportional manner with respect to T_3 levels [103]. Short term infusion of rIL-6 to healthy volunteers [104] suppressed TSH secretion, whereas daily injections over a 6-week period only slightly decreased T_3 levels and transiently increased rT_3 and fT_4 concentrations.

Deiodinases are dimeric selenoproteins that catalyze the stereospecific removal of iodine atoms from the prohormone T_4, generating the active and inactive isomers of both T_3 and diiodothyronine (T_2). Different isoforms are expressed with tissue specificity: D1 and D2, via the deiodination of the outer ring, convert T_4 to active T_3; D3, via the inner ring deiodination, converts T_4 to inactive metabolites: rT_3 and 3,3′-T_2 [105, 106]. Phylogenetic analysis suggests that D1 is the oldest vertebrate deiodinase, while D2 is the most recent one; this is in agreement with the key role of D2 as the most specialized and finely regulated member of this enzyme family [106].

Deiodinases play pivotal roles in the regulation of the intracellular levels of active thyroid hormones [107]. D2 is located in the endoplasmic reticulum and plays the primary role in the conversion of T_4 to T_3. D1 has lower affinities for the substrates with respect to D2 and seems to be mainly a scavenger enzyme, involved in iodine recycling. Furthermore, the balance between D2 and D3 activities seems to be an important factor in determining the amount of T_3 available to bind the nuclear receptors. Different mechanisms regulate the expression of deiodinase genes (DIO1, DIO2, and DIO3), first of all the levels of thyroid hormones: hyperthyroidism suppresses D2 activity and DIO2 expression, whereas hypothyroidism exerts the opposite effects [108]. The ubiquitination of the enzymes, which can be reversible to assure the appropriate protein homeostasis, is a mechanism of finer regulation of deiodinase activity [109].

D2 plays important roles in the regulation of the energetic balance as well. It has been shown that animal exposure to low temperatures activates D2 in brown adipose tissue through catecholamine-induced cAMP production. The resulting increase in T_3 levels induces thermogenic genes, including UCP-1 [110]. In addition, DIO2 expression is upregulated by bile acids in the brown adipose tissue of mice through the increase in cAMP levels. When fed with a high fat diet supplemented with bile acids, the animals do not gain weight, showing a resistance to diet-induced obesity, and this effect is absent in D2 knock-out animals [111, 112].

Recent studies on the effects of IL-6 on both endogenous cofactor-mediated and dithiothreitol-stimulated deiodinase activity in human cell lines [112] have shown that T_3 generation by D1 and D2 is suppressed by IL-6, despite an increase in expression of deiodinases. The inhibitory action of IL-6 is prevented by the addition of N-acetyl-cysteine (NAC), an antioxidant that restores intracellular GSH concentrations, suggesting the involvement of prooxidant substances in IL-6-induced effects.

Finally, the interaction between the complex network of cytokines and the hypothalamic-pituitary-thyroid axis probably plays pathogenetic roles in NTIS, even though it is not possible to build a simplistic model [18]. Also the role of cytokines in eating disorders and related thyroid hormone alterations has been recently reviewed [113].

Different conditions in which NTIS develops are associated with OS, due to augmented production ROS or RNS [114]. Since thyroid hormones, as above discussed, increase ROS generation, low-T_3 could be viewed as a compensatory mechanism. In fact, low-T_3 concentrations would be associated with decreased metabolic rate that would reduce further radical generation. Cytosolic thiols, particularly GSH, and Thioredoxin (Trx), which are also deiodinase cofactors, contribute to the maintaining of a reducing intracellular environment. Thus, their depletion, consequent to their buffering effect on radical propagation, could interfere with the conversion of T_4 to T_3 [115]. The nuclear sequestration of SECIS binding protein 2 (SBP2), which reduces the incorporation of selenocysteine residues in the selenoproteins [116], might be another mechanism. It is well known that IL-6 induces OS, so that a unifying mechanism might be that cytokine-induced OS alters secondarily the expression and activity of deiodinases [115]. The contribution of the reduction in the levels of thiol cofactor of deiodinases, consequent to the increase in intracellular ROS concentrations, has been suggested by other authors [117].

On the basis of the pathophysiological studies available in the literature, we can conclude that the alterations of the pituitary-thyroid axis depend not only on the severity of the disease, but also on the inflammatory response and the patients' nutritional status. They also indicate that low-T_3 is simply not an adaptive mechanism, but it is associated with tissue hypothyroidism and OS.

A special, reevaluated role could be played by selenium. This essential trace element exerts complex effects on the endocrine system, due to its antioxidant capacity; it is a cofactor of GPx and Trx reductase (TrxR), enzymes that protect the cells from the oxidative damage [118]. On the other hand, selenium is involved in the mechanisms of deiodination: a proposed model involves the formation of selenenyl iodide intermediate [119], even though the catalytic mechanisms and the regulation of deiodinases by selenium are not fully understood [120]. Thus, because of its double function, molecules that compete with this element could, in a reciprocal way, connect hypothyroidism due to low-T_3 and OS. This hypothesis is supported by the evidence that NAC, an antioxidant that restores intracellular GSH levels, prevents the IL-6-induced effects on the intracellular redox state [121, 122]. In addition, the administration of sodium selenite in cells expressing deiodinases decreases the IL-6-induced ROS production and carbonyl protein content and enhances GPx and TrxR activities [123].

Also deiodinases may be involved in NTIS pathophysiology, with possible tissue specificity [124]. DIO1 is a T_3-responsive gene; thus, D1 activity and intracellular T_3

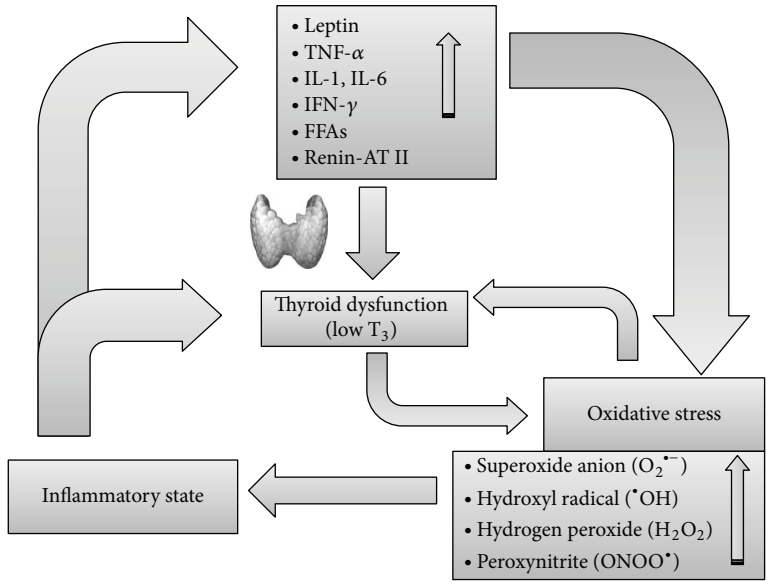

Figure 1: Proposed model of the interrelationships between inflammation, oxidative stress, and thyroid derangement. Inflammation, via hormone and cytokine changes, leads to oxidative stress and also affects thyroid function, causing nonthyroidal illness syndrome or pituitary-thyroid axis depression. At the tissue level, hypothyroidism reinforces the oxidative stress, which in turn worsens hypothyroidism by inhibiting deiodinases, thus establishing a vicious circle (see text for further explanations). AT: angiotensin; FFAs: free fatty acids; IFN: interferon; IL: interleukin; TNF: tumor necrosis factor.

concentrations can affect each other in a reciprocal way. D1 activity has been shown to be suppressed in hepatocytes. The activity of D2 has been reported to be reduced [125], unchanged [126], or increased [127] in skeletal muscle. An increase in DIO2 expression in skeletal muscle has been reported in mice during chronic inflammation that has been linked to enhanced CREB signaling [128]. On the contrary, skeletal muscle DIO2 expression was found to be decreased in sepsis and this decrease was related to the reduction in food intake [129]. DIO2 expression increases in lung and in endothelial cells following LPS-induced injury [130] and in hepatic resident macrophages during acute and chronic inflammation [128]. As far as D3 is concerned, a decrease in DIO3 mRNA levels has been reported in liver during inflammation and sepsis [131, 132]. On the contrary, hepatic expression and activity of D3 were found to be increased in rabbits with prolonged critical illness [133]. Similarly, D3 activity was found to be increased in the skeletal muscle of critically ill patients [134] and in patients after myocardial infarction [135, 136].

In summary, even if the picture appears to be quite complex, some of these changes are mediated by inflammatory pathways, such as NF-kB and AP-1, whereas the CREB pathway seems to be predominant in skeletal muscle [124]. On the other hand, overexpression of D2 in tanycytes, that has been observed in rats after LPS infusion [117, 137, 138], could be responsible for central suppression of the hypothalamic-pituitary-thyroid axis, thereby contributing to the complex picture of the regulation of thyroid function in this clinical condition.

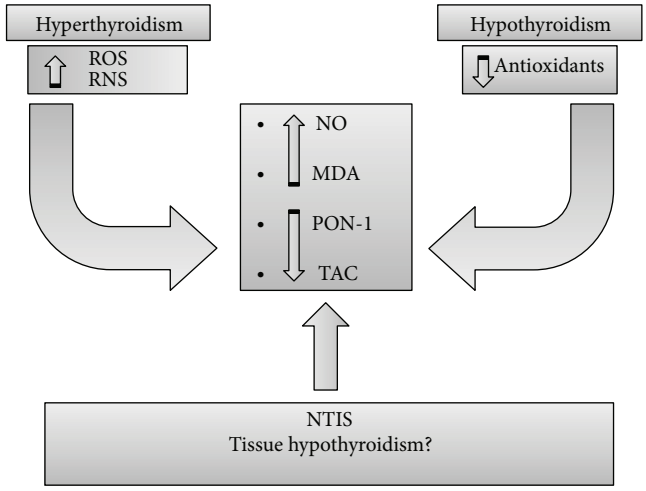

Figure 2: Both hyperthyroidism and hypothyroidism can cause oxidative stress but with different mechanisms. We speculate that nonthyroidal illness syndrome (NTIS) may represent a tissue hypothyroidism condition linked to intracellular and systemic oxidative stress. MDA: malondialdehyde; NO: nitric oxide; PON-1: paraoxonase-1; RNS: reactive nitrogen species; ROS: reactive oxygen species; TAC: total antioxidant capacity.

5. Conclusion

In conclusion, OS seems to be an important mechanism underlying the progress of inflammation. A vicious circle

creates a link between these two conditions. Thyroid hormones can have a protective role, modulating antioxidant levels; on the other side, a tissue hypothyroidism can worsen OS (Figure 1). An interesting model is represented by NTIS, in which IL production due to inflammation can reduce the expression of deiodinases, inducing low-T_3 levels and consequently a condition of tissue hypothyroidism. In turn, this latter could cause further OS (Figure 2). These pathophysiological observations suggest the possible therapeutic efficacy of antioxidants in the NTIS.

Conflict of Interests

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

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Research Article

Anti-Inflammatory and Antimicrobial Effects of Estradiol in Bovine Mammary Epithelial Cells during *Staphylococcus aureus* Internalization

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17β-Estradiol (E2), the predominant sexual hormone in females, is associated with the modulation of the innate immune response (IIR), and changes in its levels at parturition are related to intramammary infections, such as mastitis. In bovine mammary epithelial cells (bMECs), E2 regulates differentiation and proliferation, but its immunomodulatory functions have not been explored. *Staphylococcus aureus* is the predominant pathogen causing mastitis, which can persist intracellularly in bMECs. The aim of this work was to analyze whether E2 modulates the IIR of bMECs during *S. aureus* internalization. bMECs treated with E2 (50 pg/mL, 24 h) reduced bacteria internalization (~50%). The host receptors $\alpha5\beta1$ and TLR2 do not participate in this reduction. However, E2 activates ERα and modulates the IIR reducing the *S. aureus* induced-mRNA expression of TNF-α (~50%) and IL-1β (90%). E2 also decreased the secretion of these cytokines as well as IL-6 production; however, in infected bMECs, E2 induced the secretion of IL-1β. Furthermore, E2 upregulates the expression of the antimicrobial peptides DEFB1, BNBD5, and psoriasin S100A7 (~5-, 3-, and 6-fold, resp.). In addition, E2 induced the production of antimicrobial compounds in bMEC culture medium, which, together with the modulation of the IIR, could be related to the reduction of *S. aureus* internalization.

1. Introduction

Estrogens play crucial roles in the development and maintenance of normal sexual and reproductive function. 17β-Estradiol (E2) is the predominant and most potent sexual hormone during the reproductive stage in females [1]. Its main functions are associated with reproduction, although it is also involved with different pathologies, such as cancer, autoimmune diseases, and infectious processes, in which the innate immune response plays a key function [2]. In bovines, during the period around parturition, cows experience an increased susceptibility to inflammatory disorders in the mammary gland and uterus, for example, mastitis [3]. This increased susceptibility has been correlated with the decreased functionality of the components of the innate immune response and with abrupt changes in the levels of sex steroids. Parturition and the onset of lactation comprise extensive alterations in the sex steroid hormones. E2 levels rise abruptly in the last week before parturition, peak in the last 3 days before delivery, and fall rapidly after calving and regain basal values [3, 4]. These changes may be related to the incidence of intramammary infections such as mastitis. Accordingly, Lavon et al. [5] reported that cows with subclinical mastitis (without apparent signs) exhibit low circulating E2 levels. In addition, the immunomodulatory effects of E2 in cows with mastitis are associated with a reduction of neutrophil migration [6].

Most of the genomic actions of estrogens, including E2, are mediated by two estrogen receptors (ERs), ERα and ERβ, and bovine mammary epithelial cells express both [7]. However, there is little information concerning the immunomodulatory effects of E2 on epithelial cells from the bovine mammary gland. It is well known that E2 is required for mammary epithelial cell proliferation and ductal

development in the growing animal [7]. Bovine mammary epithelial cells (bMECs) play a relevant role during intramammary infections because they are in intimate contact with the pathogens responsible for mastitis and are a target for intracellular bacteria causing chronic and subclinical infections, such as *Staphylococcus aureus* [8, 9]. This pathogen can persist for long periods of time in cows and is internalized by a zipper-type mechanism depending on the presence of fibronectin-binding proteins (FnBPs) on the bacterial surface, as well as the interaction of fibronectin and the host-cell $\alpha5\beta1$ integrin, in which integrin is taken up together with its ligands [9]. This mechanism is employed during the internalization process of *S. aureus* by bMECs [10]. In addition, bMECs participate in the innate immune response of the bovine mammary gland producing pro- and anti-inflammatory mediators, as well as antimicrobial peptides, nitric oxide, and so forth [11, 12], but the role of E2 on this response has not been explored. Considering the immunomodulatory effects of E2 in different tissues and that one of its targets is the mammary epithelium, the aim of this work was to analyze whether this hormone modulates the innate immune response of bMECs and its implications during *S. aureus* internalization.

2. Materials and Methods

2.1. Reagents and Antibodies. 17β-Estradiol was acquired from Sigma, and working solutions were dissolved in 1% ethanol (1–500 pg/mL). For all of the experiments, 1% ethanol (vehicle) was used as a control. The LIVE/DEAD BacLight bacterial viability kit (Thermo Scientific) was obtained from Molecular Probes. The monoclonal blocking antibodies anti-$\alpha5\beta1$ integrin (MAB2514) and anti-TLR2 (TLR2.1) were obtained from Millipore and Abcam, respectively. The anti-phospho-ERα (2511S, Ser118) was obtained from Cell Signaling and the anti-ERβ (517700) was acquired from Life Technologies. The FITC-conjugated secondary antibodies against mouse and rat IgGs were purchased from Invitrogen and Thermo Scientific, respectively.

2.2. Staphylococcus aureus Strain. The *S. aureus* subsp. *aureus* (ATCC 27543) strain was used. This strain was isolated from a case of bovine clinical mastitis and has the capacity to invade bMECs [13]. *S. aureus* were grown at 37°C overnight in Luria-Bertani broth (LB Bioxon), and the CFUs were adjusted by measuring the optical density at 600 nm (OD 0.2 = 9.2×10^7 CFU/mL).

2.3. Primary Culture of Bovine Mammary Epithelial Cells (bMECs). bMECs were isolated from the alveolar tissue of the udders of healthy lactating cows as previously described [13]. Cells from passages 2–8 were used in all of the experiments. The bMECs were cultured in growth medium (GM) that was composed of a DMEM medium/nutrient mixture F12 Ham (DMEM/F12K, Sigma) supplemented with 10% fetal calf serum (Equitech Bio), 10 μg/mL insulin (Sigma), 5 μg/mL hydrocortisone (Sigma), 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 μg/mL amphotericin B (Invitrogen). The cells were grown in a 5% CO_2 atmosphere at 37°C. To perform the E2 and/or *S. aureus* challenge, polarized monolayers of bMECs (dishes covered with 6–10 $\mu g/cm^2$ rat-tail type I collagen, Sigma) were cultured in serum-free DMEM/F12K without phenol red (Sigma) and antibiotics (incomplete medium) for 24 h, and then they were treated with the hormone and/or infected with the bacteria.

2.4. Invasion Assays. For the invasion assays, bMEC polarized monolayers were used (~10,000 cells were cultured onto 96-well flat-bottom dishes (Corning)), which were incubated with different concentrations of E2 (1, 5, 10, 50, 150, 300, and 500 pg/mL) in DMEM/F12K (Sigma) without antibiotics, serum, and phenol red (incomplete medium) for 24 h and then were infected with *S. aureus* (MOI 30 : 1 bacteria per cell). For this, the bMECs were inoculated with bacterial suspensions from a broth of 9.2×10^7 CFU/mL and incubated for 2 h in 5% CO_2 at 37°C. Then, the cells were washed three times with PBS (pH 7.4) and incubated with incomplete medium that was supplemented with 80 μg/mL gentamicin for 1 h at 37°C to eliminate extracellular bacteria. Finally, the bMEC monolayers were detached with trypsin- (0.05%) EDTA (0.02%) (Sigma) and lysed with 250 μL of sterile distilled water. The bMEC lysates were diluted 100-fold, plated on LB agar in triplicate, and incubated overnight at 37°C. The number of CFUs was determined by the standard colony counting technique. The number of bMECs cultured in each well plate was calculated for each invasion assay using an automated cellular counter (BIO-RAD, TC20). The data are presented as the ratio of the CFU recovered per bMEC.

For the invasion assays in the presence of the blocking antibodies, one hour previous to the addition of *S. aureus* to the bMECs, the blocking antibodies anti-$\alpha5\beta1$ integrin or anti-TLR2 were added separately (10 and 5 μg/mL, resp.) to triplicate wells. Rat IgGs (purified from normal rat serum with protein A-sepharose beads (Sigma)) or mouse IgGs (purified from normal mouse serum and acquired from Pierce) were used as the negative controls. The invasion assays were performed using gentamicin protection assays as previously described [13].

2.5. bMEC Viability and S. aureus Growth Assays. To determine the effect of E2 on bMEC viability, 10,000 cells were incubated with different concentrations of the hormone in incomplete medium for 24 h at 37°C in 96-well plates. Then, 10 μL of a 5 mg/mL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) solution in PBS was added to each well and incubated for 4 h at 37°C. Finally, 100 μL of acid isopropanol (95% isopropanol and 5% 1 N HCl) were added to dissolve the formazan crystals. The optical density was measured with a microplate reader (BIO-RAD) at 595 nm.

The bMEC viability was also tested using the trypan blue exclusion assay and the cells were counted in an automated cell counter (BIO-RAD, TC20).

To analyze the effect of E2 on *S. aureus* growth, 9.2×10^7 CFU/mL were cultured at 37°C in LB broth. The bacterial suspensions were treated with different concentrations of the hormone (1, 5, 10, 50, 150, 300, and 500 pg/mL) and growth

was monitored turbidimetrically (600 nm) over 24 h (measuring the absorbance at 2, 4, 6, 8, 12, and 24 h). To evaluate the *S. aureus* viability in the presence of E2, bacteria were grown (as previously described) in LB broth and were treated with E2 for 24 h at 37°C overnight. Later, the bacteria were plated on LB agar in triplicate and were incubated overnight at 37°C. The number of CFUs was determined by standard colony counting.

Bacterial viability was also analyzed in the supernatants of the infection assays. To do this, the bMECs were treated with E2 and infected with *S. aureus* as described above. After 2 h of infection, the media were recovered and the bacteria pellet was obtained by centrifugation at 10,000 rpm for 10 min at 4°C and was washed three times with PBS. The *S. aureus* viability was measured using a LIVE/DEAD BacLight Bacterial viability kit (Thermo Scientific). The pellet was incubated with equal volumes (1.5 mL) of component A (SYTO 9 dye, live indicator) and component B (propidium iodide, dead indicator) at room temperature in the dark for 15 min. After staining the bacteria, the pellet was washed three times with PBS. The fluorescent signals of 10,000 events were measured and evaluated using a BD Accuri™ C6 cytometer. In addition, *S. aureus* growth was also analyzed using the E2-treated bMEC conditioned media. To do this, the bacteria were grown at 37°C overnight, and a *S. aureus* suspension containing 9.2×10^7 CFU/mL (OD 0.2 at 600 nm) was incubated at 37°C for 2 h with the conditioned media. Then, the bacteria were plated on LB agar in triplicate and were incubated overnight at 37°C. The number of CFUs was determined as described previously.

2.6. Analysis of Receptors by Flow Cytometry. The bMEC polarized monolayers were cultured in 24-well dishes (Corning) and were treated with E2 (50 pg/mL) for 24 h and/or *S. aureus* as described above. This concentration was selected considering the highest inhibitory effect on bacterial internalization into bMECs. After the treatment, the cells were washed three times with PBS, detached with trypsin- (0.05%) EDTA (0.02%) (Sigma), centrifuged at 2,500 rpm for 10 min at 4°C, and washed with PBS. The bMEC pellet was blocked with normal goat serum (5% in PBS, Pierce) for 30 min at 4°C with shaking, and then the cells were centrifuged and the pellet was incubated with the primary antibodies anti-TLR2 or anti-integrin separately. To analyze the presence of the ERs (ERα or ERβ), nuclei from the bMECs were obtained using the ProteoJet kit (Fermentas), and the procedure continued as described above. To measure the TLR2 membrane abundance (MA) or the nuclear presence of the ERs, the cells were incubated with the antibody at a dilution of 1 : 50 (PBS containing BSA 0.1%) for 1 h at 4°C with shaking. For the determination of integrin MA, invasion assays were performed using different infection times: 30, 60, and 120 min in control bMECs or E2-treated (50 pg/mL, during 24 h) bMECs. The invasion assay was completed as described previously. The cells were incubated with the anti-$\alpha5\beta1$ integrin at a concentration of 10 μg/mL for 2 h at 4°C with shaking. In all cases, after the primary antibody incubation, the bMECs were washed three times with PBS and incubated with the respective secondary antibody (diluted 1 : 50) (FITC-conjugated anti-mouse IgGs for TLR2 and ERα and FITC-conjugated anti-rat IgGs for ERβ and $\alpha5\beta1$ integrin) for 1 h at 4°C with shaking in the dark. The pellet was recovered by centrifugation, washed, and fixed with paraformaldehyde (4%) for 10 min at 4°C and finally washed three times with PBS. The cells were then suspended in 100 μL of PBS. The fluorescent signals of 10,000 events were measured and evaluated using the BD Accuri C6 cytometer.

2.7. Role of MAP Kinases in S. aureus Internalization into bMECs. The polarized bMEC monolayers were cultivated in 96-well flat-bottom plates that were coated (Corning-Costar) with 6–10 $\mu g/cm^2$ rat-tail type I collagen (Sigma). The bMECs were incubated with E2 (50 pg/mL for 24 h). Prior to the invasion assay (30 min, 1 or 2 h), pharmacological inhibitors of p38 (5 μM, SB203580), JNK (20 μM, SP600125), or ERK1/2 (2.5 μM, U0126) were added separately to the bMECs. The infection assays were performed with the gentamicin protection assays as described above. The cells lysates were plated on LB agar in triplicate and incubated overnight at 37°C. The CFU numbers were determined with the standard colony counting technique or *S. aureus* viability was measured using the LIVE/DEAD BacLight Bacterial viability kit as described above. 0.1% DMSO (vehicle) was used as a control.

To evaluate the MAP kinase activation levels by flow cytometry, the bMECs were treated with E2 (50 pg/mL), *S. aureus,* or both, and the samples (30 μg of protein) were prepared according to the manufacturer's protocol for adherent cells (Becton Dickinson, Germany). pp38 (T180/Y182), pJNK1/2 (T183/185), and pERK1/2 (T202/Y204) were quantitatively determined using antibodies from a Flex Set Cytometric Bead Array (Becton Dickinson) according to the manufacturer's protocol. The flow cytometric analyses were performed using the BD Accuri C6 and the CBA analysis FCAP software (Becton Dickinson). A total of 3,000 events were acquired following the supplied protocol. The minimum detection levels for each phosphoprotein were 0.38 U/mL for pJNK and 0.64 U/mL for pp38 and pERK.

2.8. RNA Isolation and RT-qPCR Analysis. Monolayers of bMECs were cultured in 6-well dishes ($\sim 5 \times 10^5$) and then were incubated with 50 pg/mL of E2 (24 h) and/or *S. aureus* (MOI 30 : 1) for 2 h, as described above. The RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions. DNA contaminations were removed from RNA samples with DNase I treatment (Invitrogen). The RNA was used to synthesize cDNA as described previously [10]. The RT-qPCR assay was performed using the comparative Ct method ($\Delta\Delta$Ct) with a StepOne Plus Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. The reactions were carried out with a SYBR Green PCR Master Mix (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The specific primer pairs were obtained from Invitrogen; their sequences and PCR conditions used to amplify the different bovine mRNAs are detailed in Table 1.

2.9. Enzyme-Linked Immunosorbent Assays (ELISA). For the measurement of the TNF-α, IL-1β, IL-6, and bovine β-defensin 1 (DEFB1) concentrations in the medium, the

Table 1: Oligonucleotides used in this study.

Gene (bovine)*	Primer	Sequence (5′ → 3′)	Fragment size (bp)	Tm (°C)	References
TAP	F	GCGCTCCTCTTCCTGGTCCTG	216	57	[14]
	R	GCACGTTCTGACTGGGCATTGA			
LAP	F	GCCAGCATGAGGCTCCATC	194	54	[14]
	R	CTCCTGCAGCATTTTACTTGGGCT			
DEFB1	F	CCATCACCTGCTCCTCACA	185	54	[14]
	R	ACCTCCACCTGCAGCATT			
BNDB4	F	GCCAGCATGAGGCTCCATC	278	54	This study
	R	CGTTTAAATTTAGACGGTGT			
BNBD5	F	GCCAGCTGAGGCTCCATC	143	55	[14]
	R	TTGCCAGGGCACGAGATCG			
BNBD10	F	GCTCCATCACCTGCTCCTC	152	54	[14]
	R	AGGTGCCAATCTGTCTCATGA			
GAPDH	F	TCAACGGGAAGCTCACTGG	237	57	[14]
	R	CCCCAGCATCGAAGGTAGA			
α5 integrin	F	TGCAGTGTGAGGCCGTGTATGAAG	230	58	[10]
	R	CGGGAGGGAGCGTTTGAAGAAT			
β1 integrin	F	TGCGAGTGTGGTTGCCTGTAAGT	123	60	[10]
	R	ATTGAAGGCTCGGCACTGAACA			
TLR2	F	CGACTGGCCCGATGACTACC	146	58.5	[15]
	R	TGAGCAGGAGCAACAGGAAGAG			
TNF-α	F	CCCCTGGAGATAACCTCCCA	101	56	[15]
	R	CAGACGGGAGACAGGAGAGC			
IL-1β	F	GCAGAAGGGAAGGGAAGAATGTAG	198	52	[15]
	R	CAGGCTGGCTTTGAGTGAGTAGAA			
IL-6	F	AACCACTCCAGCCACAAACACT	179	57	[15]
	R	GAATGCCCAGGAACTACCACAA			
IL-8	F	TTCCACACCTTTCCACCCCAA	149	53.5	[15]
	R	GCACAACCTTCTGCACCCACTT			
IL-10	F	GATGCGAGCACCCTGTCTGA	129	59	[15]
	R	GCTGTGCAGTTGGTCCTTCATT			
S100A7 (psoriasin)	F	GCAGCTCTCAGCTTGAGCAG	221	54	[14]
	R	CCAGCAAGGACAGGAACTCAG			
ERα	F	TTCGGAAGTGCTATGAGGTTG	139	55	This study
	R	GATATTCTTTGTGTTGGAGTTTTA			
ERβ	F	GACCCTACCAGACCTTTCAGTG	153	60	This study
	R	TGCTCCATGCCTTTGTTGC			

*TAP: Tracheal Antimicrobial Peptide; LAP: Lingual Antimicrobial Peptide; DEFB1: Defensin Beta 1; BNBD4: Bovine Neutrophil Beta Defensin 4; BNBD5: Bovine Neutrophil Beta Defensin 5; BNDB10: Bovine Neutrophil Beta Defensin 10; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; TLR2: Toll-Like Receptor 2; TNF-α: Tumor Necrosis Factor-α; IL-1β: Interleukin 1-β; IL-6: Interleukin-6; IL-8: Interleukin-8; IL-10: Interleukin-10; S100A7: S100 Calcium-binding protein A7; ERα: Estrogen Receptor α; ERβ: Estrogen Receptor β.

conditioned media from bMECs treated with E2 (50 pg/mL) and/or *S. aureus* were collected. The concentrations of TNF-α were measured using the Duoset ELISA development kit (R&D Systems) to quantify the bovine TNF-α levels according to the manufacturer's instructions, and the concentrations of IL-1β and IL-6 were assessed using the bovine IL-1β and IL-6 screening kits, respectively (Thermo Scientific). The measurement of bovine DEFB1 in bMECs culture medium was analyzed using the ELISA kit for DEFB1 (MyBiosource).

2.10. Nitric Oxide Production Assay. The nitric oxide (NO) secreted by bMECs into culture medium was evaluated by measuring the nitrite concentration (NO^{2-}) in cell-free media using the Griess reaction [16]. After the invasion

assays, the medium was filtered through 0.22 μM membranes (Millipore) to eliminate bacteria.

2.11. Data Analysis. The data were obtained from three independent experiments, each of which was performed in triplicate (except for the ELISA of DEFB1, which was performed in duplicate) and compared by analysis of variance (ANOVA). The results are reported as the means ± the standard errors (SE) and the significance level was set at $P < 0.05$, except for RT-qPCR analysis where standard deviations are shown and fold-change values greater than 2 or less than 0.5 were considered as significantly differentially expressed mRNAs according to Morey et al. [17]. The results from internalization assays, gene expression analyses, and receptor membrane abundance experiments are shown normalized to the cells treated with vehicle (1% ethanol).

3. Results

3.1. Estradiol Does Not Affect Bacterial Growth and bMEC Viability. To evaluate the effect of E2 on *S. aureus* growth and bMEC viability, we incubated bacteria or bMECs in the presence of different concentrations of this hormone (1–500 pg/mL). Bacterial viability was analyzed by counting the CFUs and by the MTT assay. The results showed that E2 did not have effect on bMEC viability after 24 h of culture (Figures 1(a) and 1(b)), which was determined by a trypan blue exclusion analysis and the MTT assays. In the same way, we showed that E2 did not affect the bacterial viability and growth (Figures 1(c) and 1(d)).

3.2. Estradiol Reduces S. aureus Internalization into the bMECs. To determine the effect of E2 on *S. aureus* internalization into the bMECs, we carried out gentamicin protection assays. To achieve this, bMECs were treated with different concentrations of the hormone (1–500 pg/mL) 24 h before the challenge with the bacteria. According to the number of CFUs recovered, a concentration of 50 pg/mL of E2 significantly inhibited *S. aureus* internalization into the bMECs by 50% (Figure 2). The concentrations of 1, 10, and 300 pg/mL also reduced internalization by 20%. The other concentrations did not affect the internalization rate of *S. aureus* into the bMECs. Considering these results, in the following experiments related to the regulation of the innate immune response of bMECs by E2, we analyzed only the concentration of 50 pg/mL.

3.3. $\alpha5\beta1$ Integrin Membrane Abundance Is Not Required for the Internalization of S. aureus Inhibited by E2. Because $\alpha5\beta1$ integrin is one of the main extracellular receptors of epithelial cells employed during *S. aureus* internalization, we wondered whether the specific functional blocking of $\alpha5\beta1$ integrin would modify the number of bacteria internalized into the bMECs. To test this, primary cultures of bMECs treated for 24 h with E2 were incubated with a specific antibody against $\alpha5\beta1$ integrin (Figure 3(a)). E2 treatment (50 pg/mL) reduced the number of CFUs internalized into the bMECs, but the blockade of $\alpha5\beta1$ integrin slightly reduced this number (~20%). In addition, the bMECs treated with vehicle reduced ~60% of the *S. aureus* internalization when $\alpha5\beta1$ integrin was blocked. This finding suggests that, during the internalization of *S. aureus* into the E2-treated bMECs, integrin $\alpha5\beta1$ does not participate.

To determine whether this effect is correlated with $\alpha5\beta1$ integrin gene expression, we evaluated the gene expression of both subunits by RT-qPCR. *S. aureus* induced the expression of the $\alpha5$ subunit (~8-fold), and E2 upregulated the expression of both subunits. However, the effect of both treatments was similar to the effect of *S. aureus* (Figure 3(b)). To analyze whether this effect is correlated with the $\alpha5\beta1$ integrin MA in the bMECs, we performed a flow cytometry analysis. The mean fluorescence intensity was used as an indicator of the level of integrin expression per cell in the population. This analysis is shown in Figure 3(c). E2 significantly reduces the level of $\alpha5\beta1$ integrin MA (~40%); however, after 2 h of challenge with *S. aureus*, the level of $\alpha5\beta1$ integrin MA was reduced in a similar manner as that of the E2-treated cells. A similar effect was also observed when the E2-treated bMECs were infected, indicating that integrin is taken up together with bacteria. Typical population profiles from the flow cytometry analysis are also shown in Figure 3(c). We additionally evaluated the level of $\alpha5\beta1$ integrin MA at different times of challenge with *S. aureus* in the E2-treated bMECs. The results from this evaluation are shown in Figure 3(d). The presence of $\alpha5\beta1$ integrin at the membrane of the bMECs was reduced starting from 30 min of *S. aureus* challenge, showing a similar behavior both in the control and E2-treated cells. Despite the data showing that E2 reduces integrin MA (24 h treatment), these results suggest that bacteria are able to induce integrin trafficking. Thus, $\alpha5\beta1$ integrin MA does not contribute to the reduction in internalization detected in the E2-treated bMECs (Figure 2).

3.4. Regulation of TLR2 Expression by E2 in the bMECs. To explore whether TLR2 participates in the reduction of *S. aureus* internalization into the bMECs induced by E2, we investigated whether the blockade of this receptor with a specific antibody would modify bacterial endocytosis. As shown in Figure 4(a), the blockade of TLR2 results in a significant reduction in the amount of *S. aureus* internalized (~50%); however, a slight reduction was detected in the E2-treated cells (~15%). Surprisingly, E2 induces TLR2 gene expression (~6-fold) (Figure 4(b)). As we previously reported [10], *S. aureus* induces TLR2 mRNA expression (~3-fold); however, the effect of both treatments was similar to the effect of *S. aureus* alone. The analysis of the mean fluorescence intensity (Figure 4(c)) shows that only *S. aureus* (~1.4-fold) increased the TLR2 MA, but there is no difference in the TLR2 MA in the presence of E2 and bacteria compared with that obtained with infection alone. Thus, TLR2 could not be involved in *S. aureus* internalization, and it is probably inactivated in E2-treated bMECs.

3.5. Activation of TLR2 Signaling Pathways by E2 and S. aureus. Next, we evaluated whether E2 (50 pg/mL) induces the activation of MAPKs (p38, JNK, or ERK1/2), which are activated by TLR2. Initially, we investigated whether these kinases participate in *S. aureus* internalization into bMECs

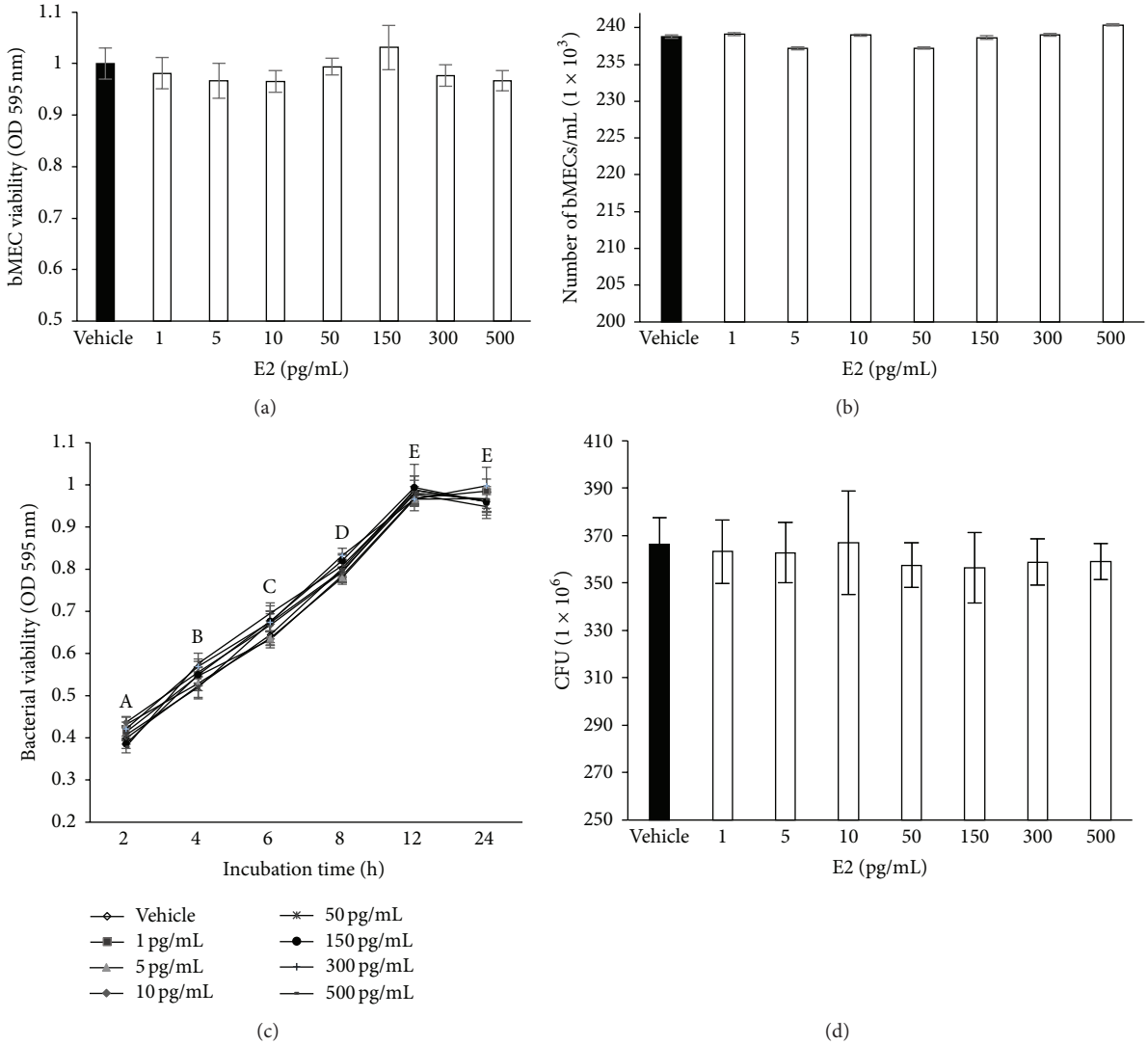

Figure 1: Bovine mammary epithelial cell viability and *S. aureus* growth in the presence of E2. (a) bMECs were cultured with E2 and viability was determined by MTT assay at 24 h. (b) bMECs were cultured with the hormone and viability was calculated by a trypan blue exclusion assay. The number of viable bMECs/mL is shown. In all cases, concentrations evaluated were 1, 5, 10, 50, 150, 300, and 500 pg/mL. (c) *S. aureus* was cultured in the presence of E2 at different times during 24 h and bacteria viability was analyzed by MTT assay. (d) Bacterial growth was determined counting the CFUs of *S. aureus* treated with different concentrations of E2 during 24 h. Each point in (a), (b), (c), and (d) shows the mean of triplicates ± SE of three independent experiments. The vehicle corresponds to bMECs treated with 1% ethanol. Different letters indicate significant changes among the different incubation times ($P < 0.05$).

using pharmacological inhibitors prior to bacterial invasion. bMECs that were incubated (for 30 min or 1 or 2 h) with pharmacological inhibitors of p38 (5 μM, SB203580), JNK (20 μM, SP600125), or ERK1/2 (2.5 μM, U0126) showed a considerable reduction in *S. aureus* internalization (~50–60% reduction), indicating that these kinases are involved in this process (Figure 5(a)). *S. aureus* internalization was also analyzed by CFU counting and flow cytometry, which obtained similar results (Figure 5(b)). Interestingly, these kinases do not participate in the reduction of bacterial internalization induced by E2 because in the presence of inhibitors this reduction persists.

Furthermore, we evaluated p38, JNK, and ERK1/2 phosphorylation to relate their activation states with the E2-mediated reduction of *S. aureus* internalization into bMECs. When the bMECs were *S. aureus*-challenged (2 h), the basal activation of p38 was not modified, phosphorylated JNK1/2 was augmented, and ERK1/2 activation was reduced (Figure 5(c)), as we previously reported [18]. The vehicle employed for the MAPK dilutions was 0.1% DMSO, which did not have an effect on internalization assays (data not showed), as was previously reported [18]. Interestingly, the bMECs that were treated with E2 demonstrated reduced p38 phosphorylation (~5-fold), and the ERK1/2 activation

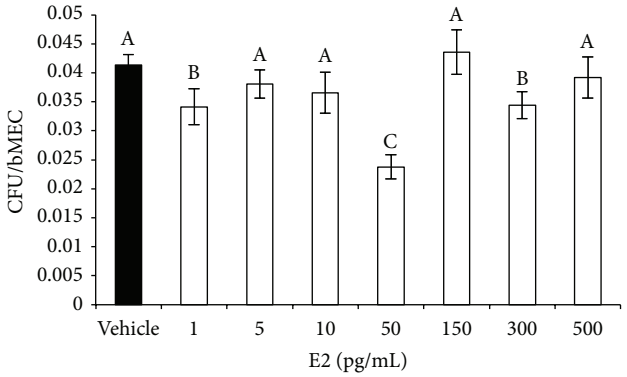

Figure 2: E2 decreases *S. aureus* internalization into bMECs. bMECs were treated with the concentrations indicated of E2 (24 h), and then were challenged with *S. aureus* during 2 h. Data are showed as the percentage of CFU recovered after bMEC lysis. Values were determined considering the control (bMECs cultured with the vehicle 1% ethanol) as 100% internalization. Each bar shows the mean of triplicates ± SE of three independent experiments. Different letters indicate significant changes among treatments ($P < 0.05$).

levels were also reduced by ~1-fold, but JNK1/2 remained unchanged in relation to the control cells. When the E2-treated cells were *S. aureus*-challenged, the p38 activation level was augmented; however, the JNK1/2 and ERK1/2 activation levels were essentially not changed. The MAPK results indicated that E2, prior to bacterial invasion, inhibits the bMEC inflammatory response via p38 and ERK1/2 inactivation.

3.6. E2 Induces the Activation of ERα in bMECs. Estrogens act through the estrogen receptors ERα and ERβ, which belong to the nuclear receptor superfamily of transcription factors. To determine whether these receptors are activated in bMECs treated with E2 and/or infected with *S. aureus*, we performed a flow cytometry analysis. We used an anti-ERα antibody, which recognizes its phosphorylated isoform, as an indicator of its activation. To perform these analyses, we isolated bMEC nuclei. In Figure 6(a), we show that E2 slightly induces the activation of ERα (~20%), which was returned to basal levels during *S. aureus* infection. Regarding the expression of ERα mRNA, we observed that E2 also induces the expression of its gene (~5-fold), but after infection this induction is higher (~25-fold) (Figure 6(b)).

With regard to ERβ activation, we performed flow cytometry analysis on the bMEC nuclei. In Figure 6(c), we show that only the bMECs infected with *S. aureus* induce the activation of this receptor in both vehicle- or E2-treated cells. However, E2 treatment does not induce ERβ activation. In addition, the expression of ERβ mRNA was induced both in the E2-treated and the *S. aureus*-challenged bMECs (~3.5-fold) (Figure 6(d)). The infection of the bMECs treated with the hormone maintained the upregulated levels of ERβ (~2.5-fold). According to these results, we hypothesized that 50 pg/mL E2 activates the bMECs via ERα.

3.7. E2 Maintains an Anti-Inflammatory Profile in the bMECs. Next, we explored whether E2 modified the inflammatory response of the bMECs before and during *S. aureus* infection. We focused on (i) proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6; (ii) an anti-inflammatory cytokine (IL-10); and (iii) a chemokine, IL-8 (Figure 7). Regarding the proinflammatory cytokines, we performed RT-qPCR for mRNA expression and ELISAs for the evaluation of cytokine secretion. The bMECs treated with E2 (50 pg/mL, 24 h) significantly increased TNF-α and IL-1β mRNA expression (~7- and 3-fold, resp.) (Figure 7(a)). *S. aureus* infection alone also induced the expression of both cytokines (~5-fold for TNF-α and ~3-fold for IL-1β). In the presence of *S. aureus*, the E2-treated bMECs reduced the expression of TNF-α and IL-1β mRNAs. The expression of IL-6 remained unchanged in all of the treatments. Interestingly, in the E2-treated cells the secretion of these proinflammatory cytokines was diminished in relation to bMECs treated with vehicle (Figure 7(c)). Furthermore, 50 pg/mL of E2 also reduced the secretion of TNF-α and IL-6 in the infected bMECs. Only the secretion of IL-1β was increased when the E2-treated cells were infected with *S. aureus*. In Figure 7(b), we show that E2 also induces the expression of IL-10 and IL-8 mRNAs (~2.5 and 4.5-fold, resp.). The infection induced the expression of IL-10 but together with the hormone increased its expression (~8-fold). The expression of IL-8 was not modified in the presence of infection both in the vehicle- and E2-treated cells. Altogether, these results point to the anti-inflammatory effects of E2 on the bMECs infected with *S. aureus*.

3.8. Effects of E2 on the Expression and Secretion of Antimicrobial Molecules in the bMECs. To explore if some antimicrobial mediators are being regulated by E2, which could explain the reduction in *S. aureus* internalization, we measured the viability of *S. aureus* in the infection assays. In Figure 8(a), we show the viability of *S. aureus* from the supernatants obtained from the invasion assays (2 h). *S. aureus* employed in the invasion assays from the E2-treated bMEC cultures showed a reduced viability rate (60% of reduction) compared to the bacteria recovered from the supernatants of the vehicle-treated bMECs. This result suggests that antimicrobial components are present in the culture media from the bMECs treated with 50 pg/mL of E2. With the purpose of reinforcing these data, we incubated *S. aureus* for 2 h with 1 mL of conditioned media obtained from the vehicle- or E2-treated bMECs. According to Figure 8(b), the conditioned medium from the E2-treated bMECs reduces the number of CFU of *S. aureus* (a 60% of reduction). These results suggest that E2-treated bMECs secrete antimicrobial components. Next, we explored whether the gene expression of some antimicrobial elements was induced in the E2-treated bMECs. Figure 8(c) shows the RT-qPCR analysis of different antimicrobial peptides, including several β-defensins and 1 psoriasin (S100A7). E2 upregulates the expression of the β-defensins bovine defensin β1 (DEFB1, ~5-fold) and Bovine Neutrophil Beta Defensin 5 (BNBD5, ~3-fold). In addition, E2 induces the expression of psoriasin S100A7 (~6.5-fold). In the presence of infection, only psoriasin S100A7 mRNA was induced (~13-fold). The other peptides analyzed remained unchanged in

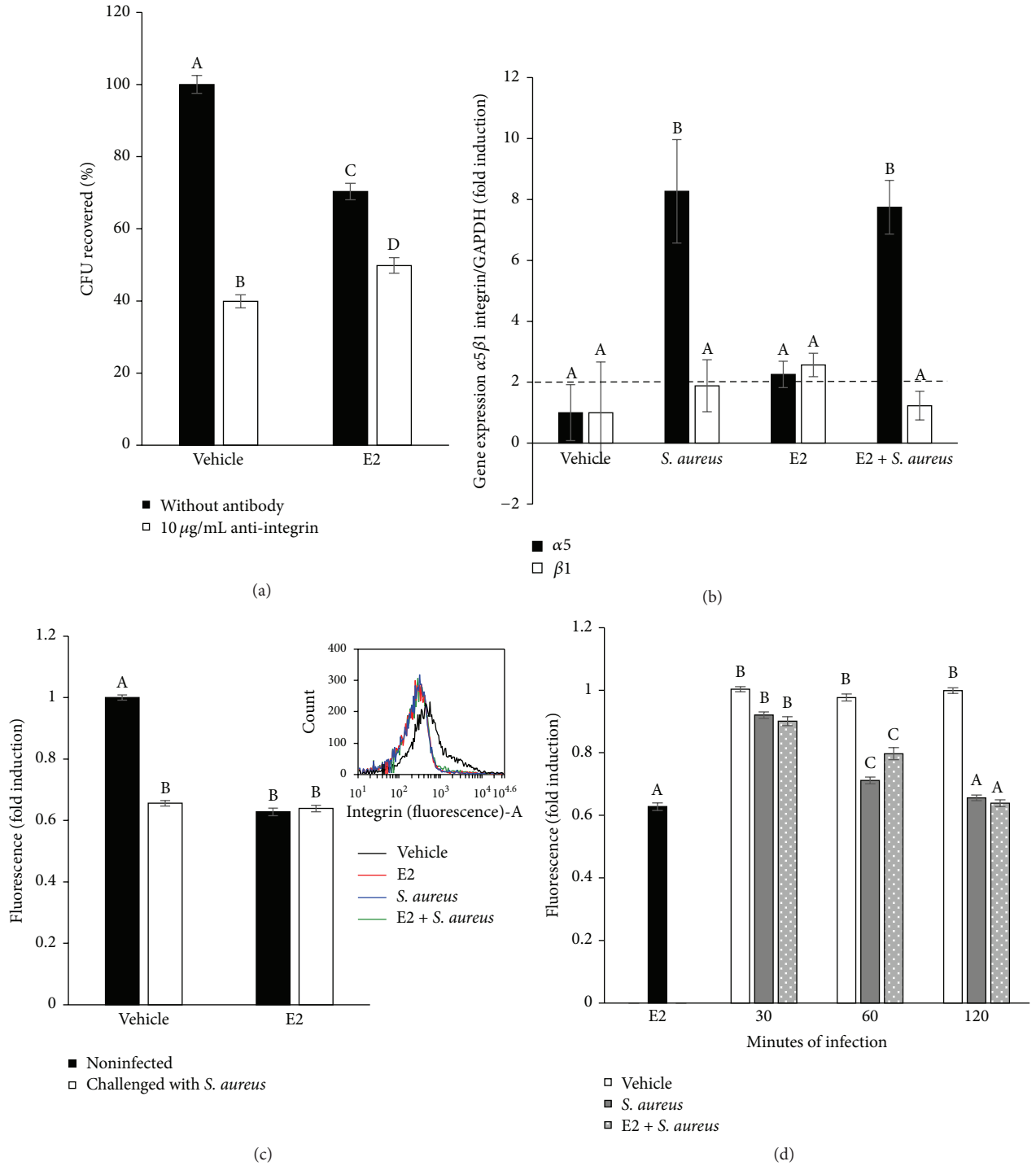

Figure 3: Role of $\alpha5\beta1$ integrin during the internalization of *S. aureus* into bMECs inhibited with E2. (a) Primary cultures of bMECs treated for 24 h with E2 (50 pg/mL) were incubated with 10 μg/mL of a specific blocking antibody against $\alpha5\beta1$ integrin for 1 h and then challenged with *S. aureus* (MOI 30 : 1 bacteria per cell). The number of internalized bacteria is represented by the ratio of CFU/bMEC recovered after lysis of bMECs. (b) The $\alpha5\beta1$ mRNA expression was analyzed by RT-qPCR and GAPDH was used as endogenous gene in all conditions. Fold-change values greater than 2 or less than 0.5 were considered as significant differentially expressed mRNAs. Each bar shows the mean of triplicates ± SD of three independent experiments. (c) The relative fluorescence intensities of $\alpha5\beta1$ membrane abundance in bMECs treated with E2 and infected with *S. aureus* are shown. Fluorescence intensity was estimated from 10,000 events. An inserted histogram plot that shows $\alpha5\beta1$ staining data in vehicle-treated bMECs (black line), cells that were challenged with *S. aureus* (blue line), cells that were stimulated with E2 (red line), and E2-treated bMECs infected with *S. aureus* (green line). The cells were fixed and stained extracellularly with an anti-$\alpha5\beta1$ antibody overnight and analyzed by flow cytometry. (d) The abundance of $\alpha5\beta1$ integrin in the bMEC membrane determined as the mean of the fluorescence of challenged bMECs at different times of infection with the same MOI (30 : 1 bacteria per cell). A total of 10,000 events were measured. Different letters indicate significant changes among treatments ($P < 0.05$), except for (b), where different letters above the bars indicate significant changes among the four treatments within the same gene evaluated ($P < 0.05$). Vehicle: 1% ethanol.

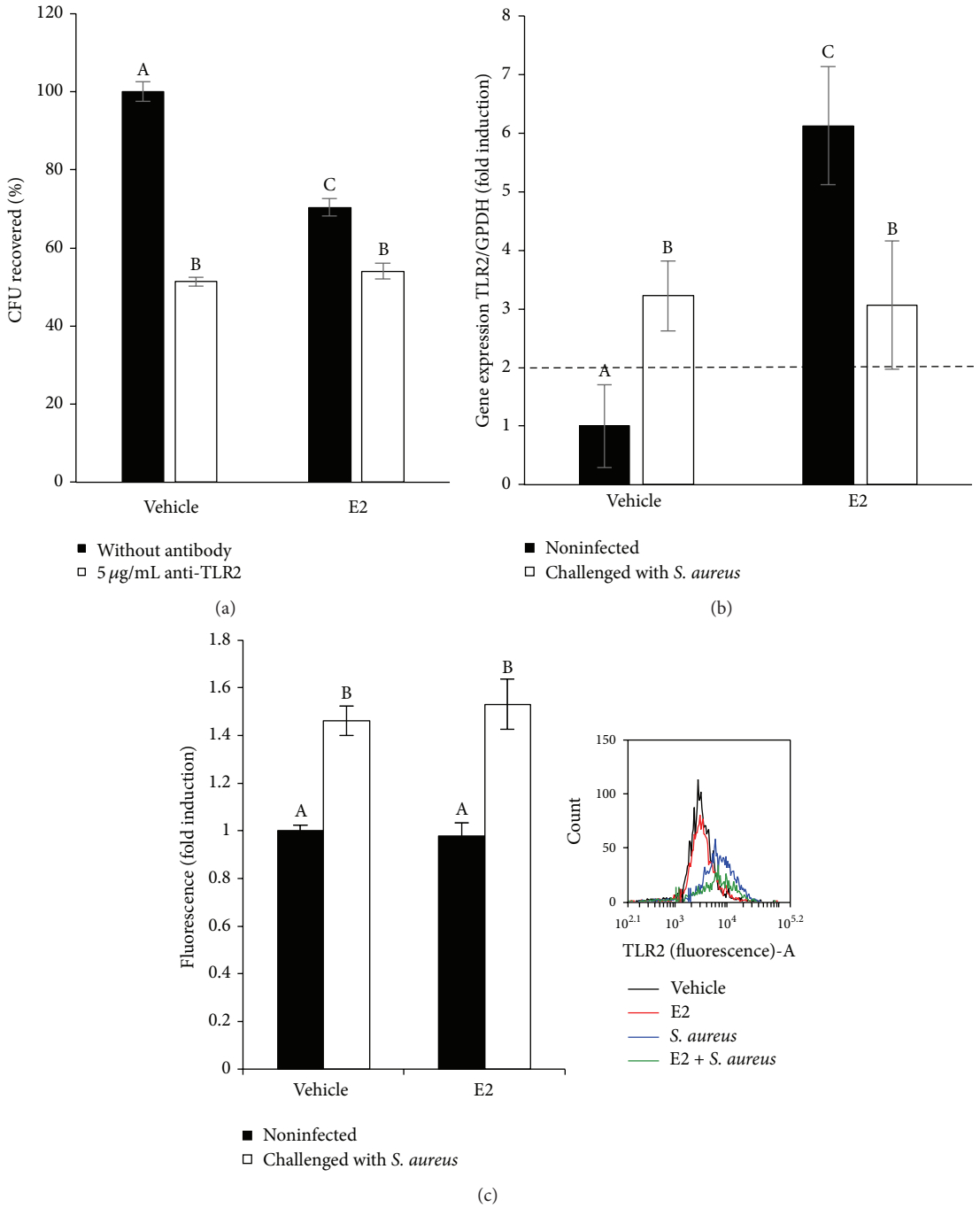

Figure 4: Participation of TLR2 in the internalization of *S. aureus* by bMECs inhibited with E2. (a) Primary cultures of bMECs treated for 24 h with E2 (50 pg/mL) were incubated with 5 μg/mL of a specific blocking antibody against TLR2 for 1 h and then challenged with *S. aureus* (MOI 30 : 1 bacteria per cell). The number of internalized bacteria is represented by the ratio of CFU/bMEC recovered after lysis of bMECs. (b) The TLR2 mRNA expression was analyzed by RT-qPCR and GAPDH was used as endogenous gene in all conditions. Fold-change values greater than 2 or less than 0.5 were considered as significant differentially expressed mRNAs. Each bar shows the mean of triplicates ± SD of three independent experiments. (c) The relative fluorescence intensities of TLR2 membrane abundance in bMECs treated with E2 and infected with *S. aureus* are shown. Fluorescence intensity was estimated from 10,000 events. An inserted histogram plot that shows TLR2 staining data in vehicle-treated bMECs (black line), cells that were challenged with *S. aureus* (blue line), cells that were stimulated with E2 (red line), and E2-treated bMECs infected with *S. aureus* (green line). The cells were fixed and stained extracellularly with an anti-TLR2 antibody overnight and analyzed with flow cytometry. Different letters indicate significant changes among treatments ($P < 0.05$). Vehicle: 1% ethanol.

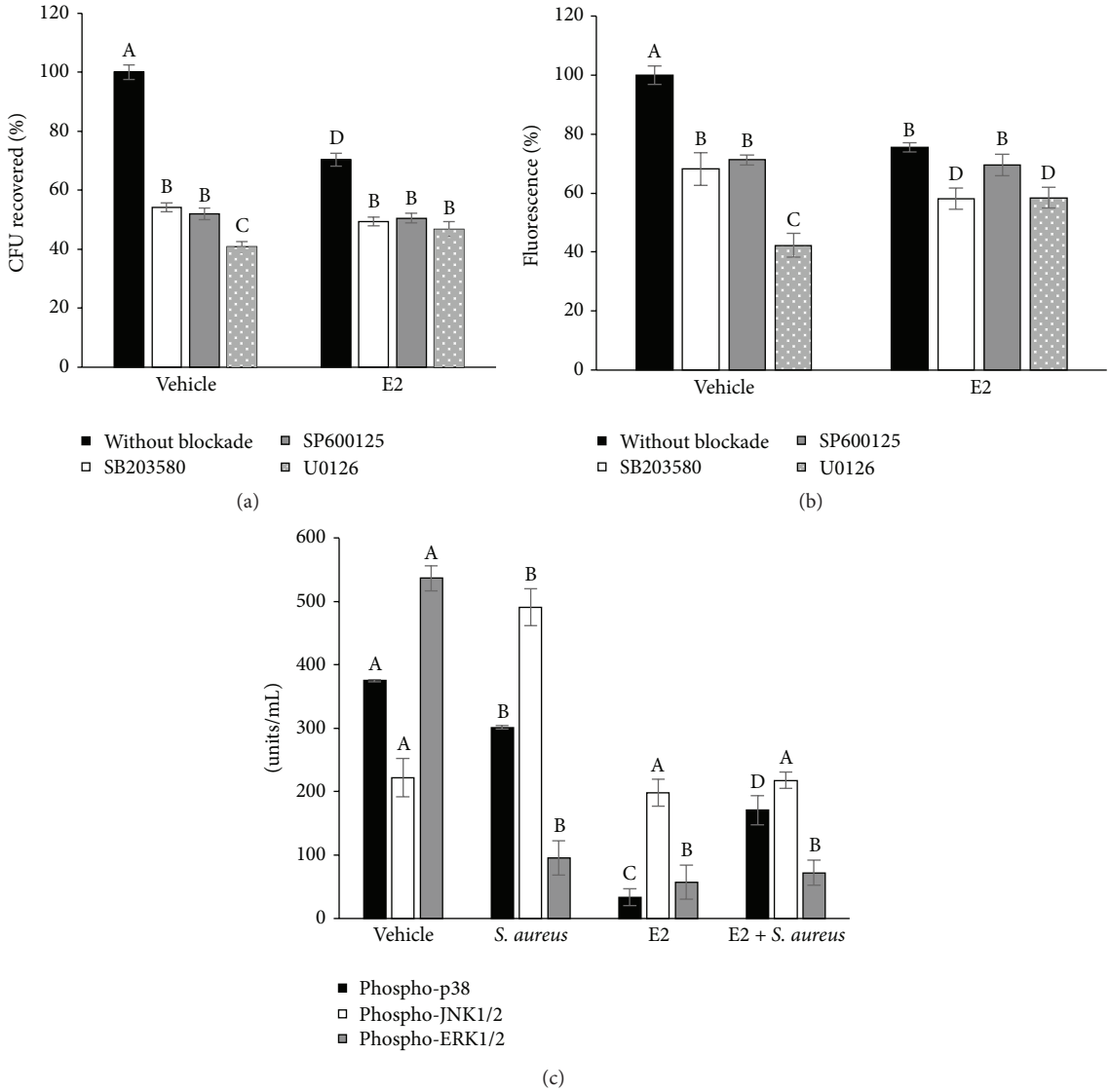

Figure 5: Role of MAPKs p38, JNK, and ERK1/2 in E2-treated bMECs during *S. aureus* internalization. (a) Participation of MAPKs in *S. aureus* internalization determined by CFU units. bMECs were incubated (30 min, 1 or 2 h) with pharmacological inhibitors of p38 (5 μM, SB203580), JNK (20 μM, SP600125), or ERK1/2 (2.5 μM, U0126) prior to infection with *S. aureus* (2 h). Values were determined considering the control (bMECs cultured with the vehicle 1% ethanol) as 100% internalization. (b) The same procedure was used to determine *S. aureus* internalization by flow cytometry using the LIVE/DEAD BacLight Bacterial viability kit. Fluorescence intensity was estimated from 10,000 events. (c) Phosphorylation was measured in bMECs that were treated with 50 pg/mL E2 and/or challenged with *S. aureus* by flow cytometry. The phosphorylated MAPK concentrations (U/mL) are represented: (a) pp38, (b) pJNK, and (c) pERK1/2. SB203580: p38 inhibitor. SP600125: JNK1/2 inhibitor. U0126: ERK1/2 inhibitor. According to manufacturer's protocol 300 events were obtained. In (a) and (b) different letters indicate significant changes among treatments ($P < 0.05$), and in (c) different letters above the bars indicate significant changes among the four treatments within the same MAPK evaluated ($P < 0.05$). Vehicle: 1% ethanol.

relation to the control cells. A similar behavior was observed in the cells treated with E2 and infected with *S. aureus*, where the expression of psoriasin was upregulated by ~9-fold. Considering these results, we measured the accumulation of DEFB1 in the bMEC medium by ELISA. In Figure 8(d), we show that E2 does not induce the secretion of DEFB1 in the culture medium, which is stimulated by infection. *S. aureus* infection in the E2-treated cells reduces the concentration of DEFB1 secreted in relation to infection alone. Thus, this is not the mechanism by which bacterial internalization is reduced in the E2-treated cells. In addition, the secretion of NO was not modified in the presence of E2 in the bMECs (Figure 8(e)), and thus it is not involved in the reduced internalization detected in the E2-treated bMECs.

4. Discussion

It is well known that, during the postpartum period, dairy cows experience an increased incidence of mammary infectious diseases, such as mastitis. This augmented frequency

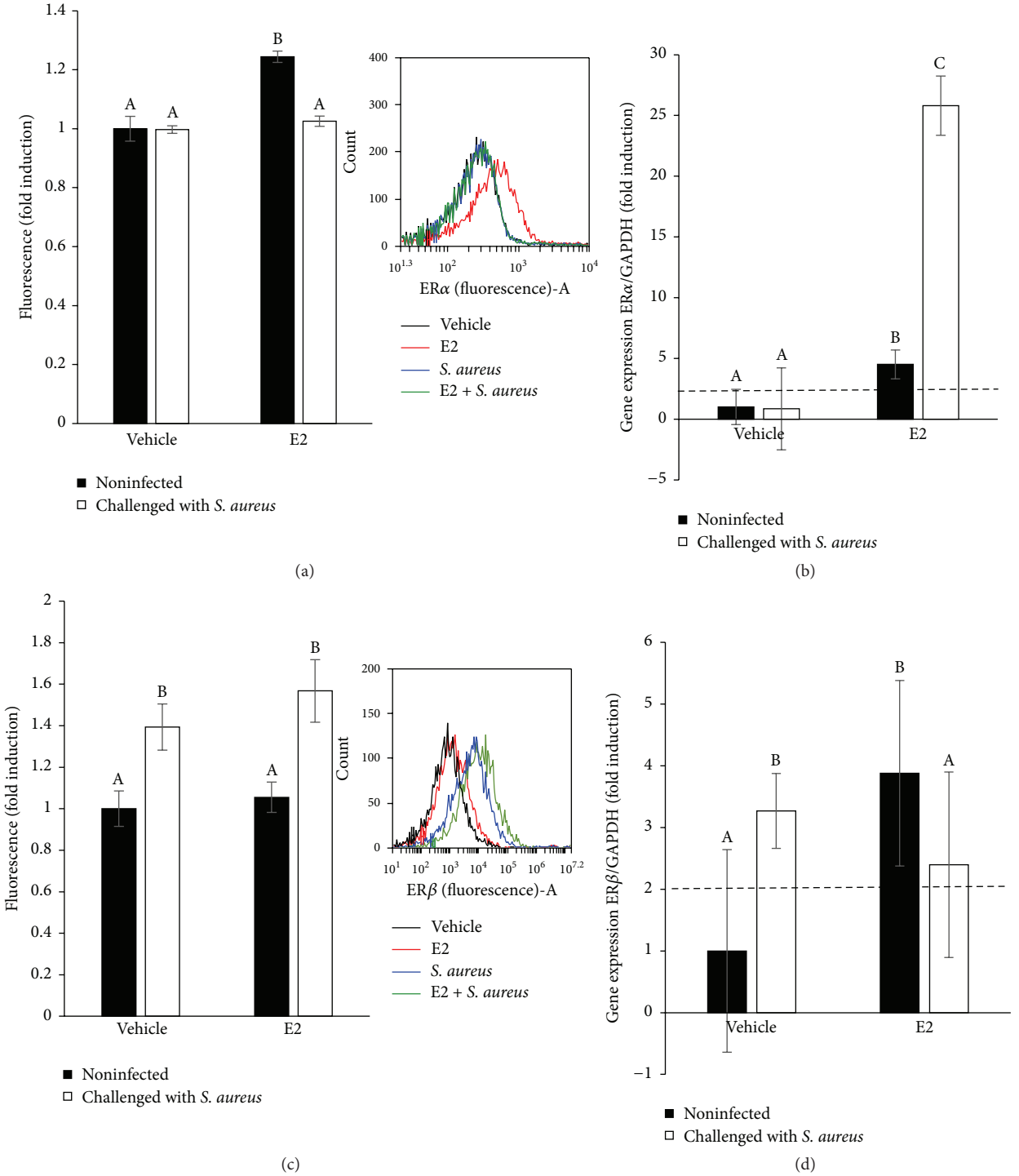

Figure 6: Participation of ERs in E2-treated bMECs. (a) The relative fluorescence intensities of ERα activation in bMECs treated with E2 and infected with *S. aureus* are shown. Fluorescence intensity was estimated from 10,000 events. An inserted histogram plot that shows ERα staining data in vehicle-treated bMECs (black line), cells that were challenged with *S. aureus* (blue line), cells that were stimulated with E2 (red line), and E2-treated bMECs infected with *S. aureus* (green line). The bMEC nuclei were fixed and stained with an anti-pERα antibody overnight and analyzed with flow cytometry. (b) The ERα mRNA expression was analyzed by RT-qPCR and GAPDH was used as endogenous gene in all conditions. Fold-change values greater than 2 or less than 0.5 were considered as significant differentially expressed mRNAs. Each bar shows the mean of triplicates ± SD of three independent experiments. (c) The relative fluorescence intensities of ERβ activation in bMECs treated with E2 and infected with *S. aureus* are shown. Fluorescence intensity was estimated from 10,000 events. An inserted histogram plot that shows ERβ staining data in vehicle-treated bMECs (black line), cells that were challenged with *S. aureus* (blue line), cells that were stimulated with E2 (red line), and E2-treated bMECs infected with *S. aureus* (green line). The bMEC nuclei were fixed and stained with an anti-ERβ antibody overnight and analyzed with flow cytometry. (d) The ERβ mRNA expression was analyzed by RT-qPCR and GAPDH was used as endogenous gene in all conditions. Fold-change values greater than 2 or less than 0.5 were considered as significant differentially expressed mRNAs. Each bar shows the mean of triplicates ± SD of three independent experiments. Different letters indicate significant changes among treatments ($P < 0.05$). Vehicle: 1% ethanol.

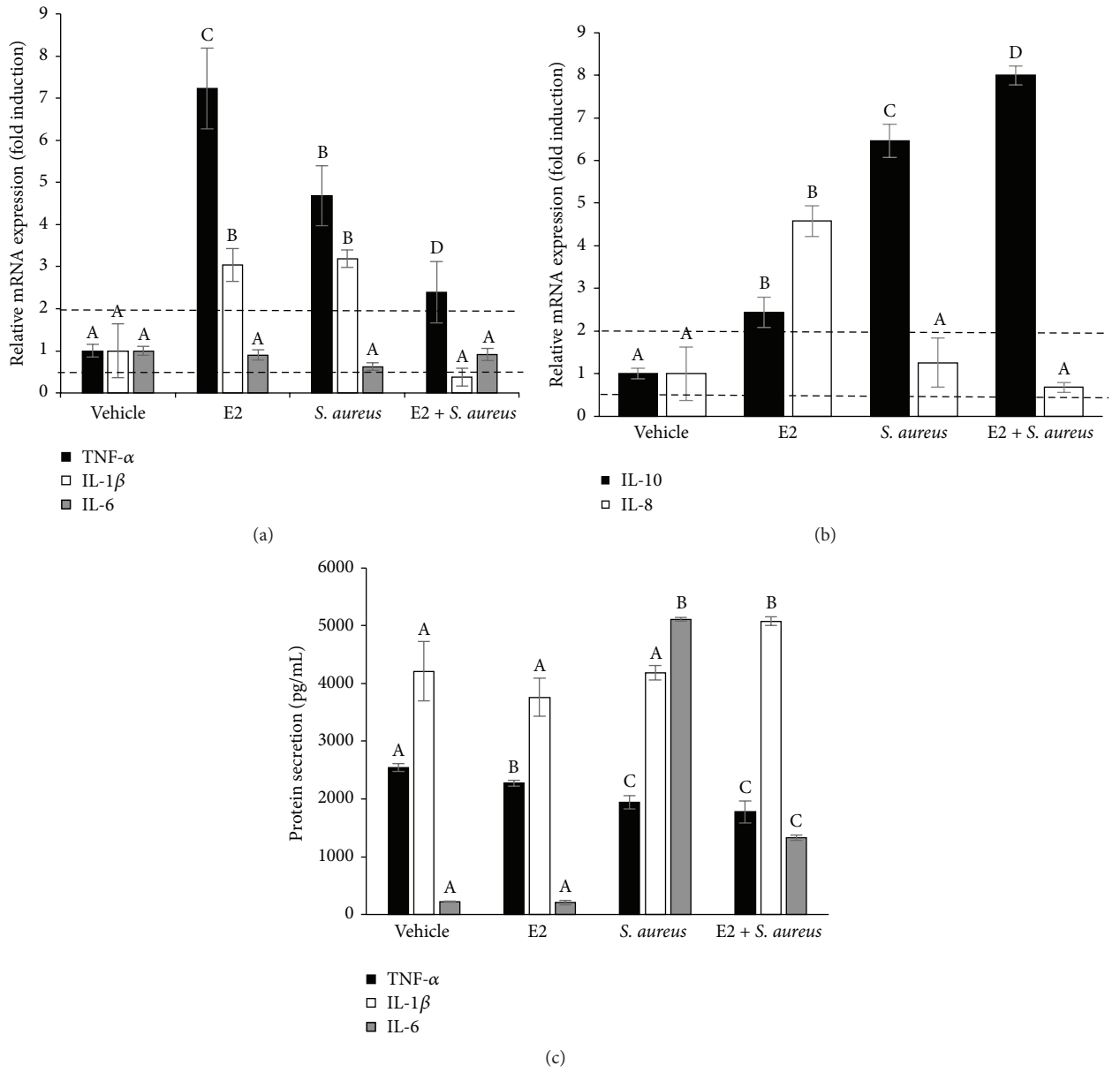

Figure 7: Expression of pro- and anti-inflammatory genes in bMECs treated with E2 and infected with *S. aureus*. (a) RT-qPCR analysis showing the mRNA expression levels of TNF-α, IL-1β, and IL-6. The bMECs were treated with 0.5 pg/mL of E2 for 24 h and then challenged with *S. aureus* for 2 h. (b) RT-qPCR analysis showing the mRNA expression levels of IL-10 and IL-8. The bMECs were treated with 50 pg/mL of E2 for 24 h and then challenged with *S. aureus* for 2 h. The data are presented as the ratio of the target gene expression compared with the expression level of GAPDH. Each bar shows the mean of triplicates ± SD of three independent experiments. Fold-change values greater than 2 or less than 0.5 were considered as significant differentially expressed mRNAs. (c) Concentrations of TNF-α, IL-1β, and IL-6 in culture medium of bMECs treated with 50 pg/mL of E2 for 24 h and then challenged with *S. aureus* for 2 h. The protein concentrations were determined by ELISA. Different letters above the bars indicate significant changes among the four treatments within the same gene or cytokine evaluated ($P < 0.05$). Vehicle: 1% ethanol.

has been correlated with a decreased functionality of the innate immune system [3]. During this period, E2 levels rise abruptly in the last week before parturition, peak in the last 3 days before delivery, and fall rapidly after calving and regain basal values [3, 4]. It has been described that estrogens have both anti-inflammatory and proinflammatory functions, and their role in the modulation of the innate immune response of epithelial cells during infection has been documented [19]. It has been shown that E2 is required for mammary epithelial cell proliferation and ductal development in the growing animal [20]. In addition, the presence of ERs has been shown in this tissue [20]. The goal of this work was to study the immunomodulatory functions of E2 on bMECs during *S. aureus* infection, which have not been explored to date.

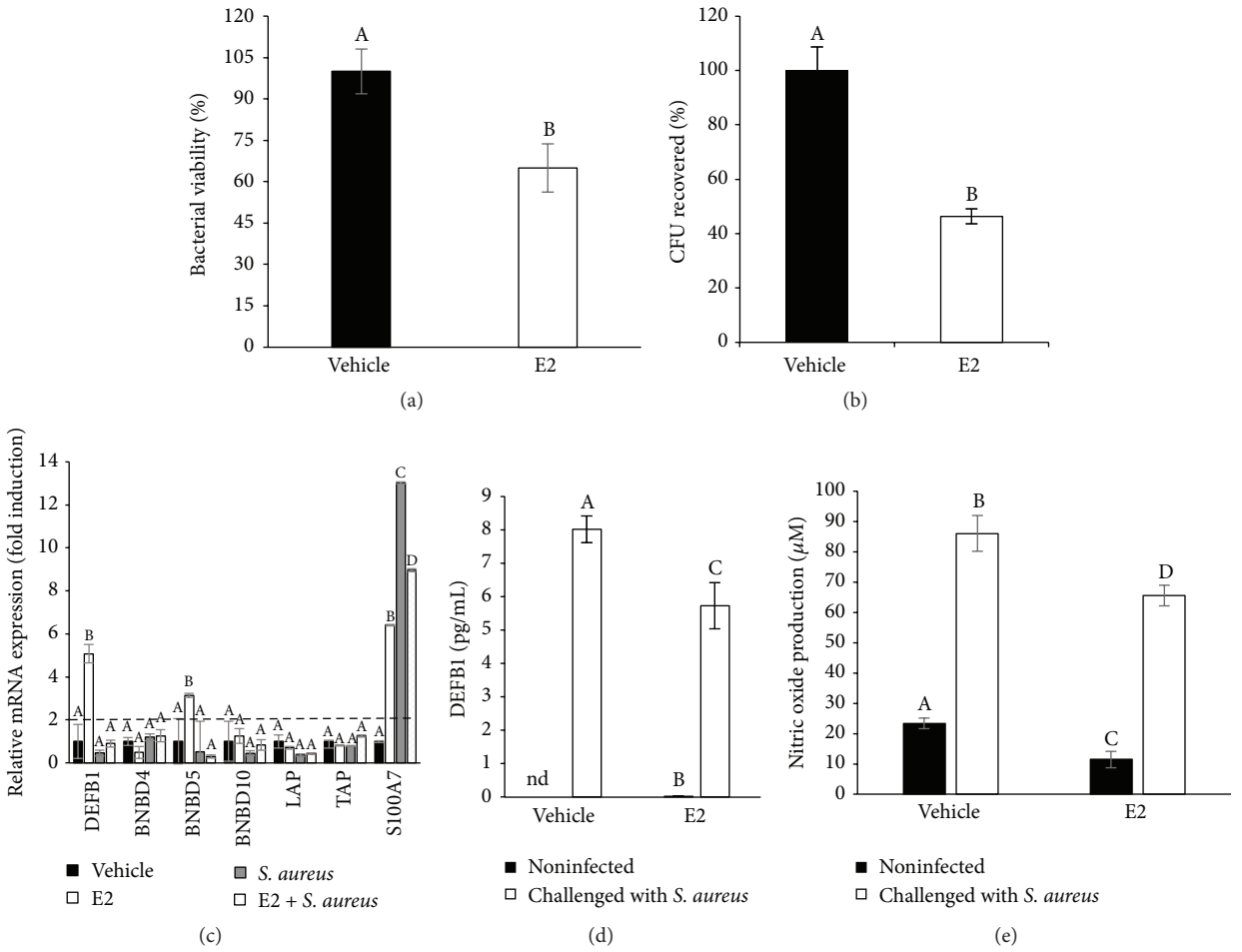

Figure 8: Expression and production of antimicrobial molecules in bMECs treated with E2 and infected with *S. aureus*. (a) *S. aureus* viability from supernatants of infection assays performed in the absence of gentamicin. Noninternalized bacteria viability was analyzed by flow cytometry using the LIVE/DEAD BacLight Bacterial viability kit. Fluorescence intensity was estimated from 10,000 events. Values were determined considering the effect of control (bMECs cultured with the vehicle 1% ethanol) as 100% of viability. (b) Effect of conditioned medium from bMECs treated with vehicle (1% ethanol) or E2 (50 pg/mL) for 24 h on *S. aureus* viability. Bacteria were incubated 2 h with conditioned media and viability was analyzed by flow cytometry using the LIVE/DEAD BacLight Bacterial viability kit. Fluorescence intensity was estimated from 10,000 events. Values were determined considering the effect of control-conditioned medium (bMECs cultured with the vehicle 1% ethanol) as 100% of viability. (c) RT-qPCR analysis showing the mRNA expression levels of DEFB1, BNBD4, BNBD5, BNBD10, LAP, TAP, and S100A7. The bMECs were treated with 50 pg/mL of E2 for 24 h and then challenged with *S. aureus* for 2 h. The data are presented as the ratio of the target gene expression compared with the expression level of GAPDH. Each bar shows the mean of triplicates ± SD of three independent experiments. Fold-change values greater than 2 or less than 0.5 were considered as significant differentially expressed mRNAs. (d) Concentration of DEFB1 in culture medium of bMECs treated with 50 pg/mL of E2 for 24 h and then challenged with *S. aureus* for 2 h. The protein concentrations were determined by ELISA. Each bar shows the mean of duplicates ± SD from different experiments, nd: nondetermined. (e) Nitric oxide production in bMECs treated with 50 pg/mL of E2 for 24 h and then challenged with *S. aureus* for 2 h, measured as NO_2 concentration in culture medium. Each bar shows the mean of triplicates ± SE of three independent experiments. Different letters indicate significant changes among treatments ($P < 0.05$), except for (c), where different letters above the bars indicate significant changes among the four treatments within the same gene evaluated ($P < 0.05$). Vehicle: 1% ethanol.

According to previous reports showing immunomodulatory functions of E2 on bovine neutrophils, we evaluated E2 in a range of concentrations between 1 and 500 pg/mL [6]. Bacteria are capable of metabolizing sex steroid hormones, such as estradiol [21]. Thus, it is important to analyze whether E2 alters *S. aureus* viability or growth. At the incubation times and the range of E2 concentrations analyzed we did not detect a bacteriostatic or a bactericidal effect of E2 on *S. aureus* (Figure 1). In agreement, Hosoda et al. [22] showed that different steroid hormones were not bactericidal for *S. aureus*. In addition, at the range of concentrations tested, E2 was not toxic to the bovine mammary epithelial cells. Sobolewska et al. [23] have reported that similar concentrations of E2 and progesterone exert stimulatory effects on autophagy in bMECs after 48 h, which is implied during the involution of the bovine mammary gland in the dry period. In the present study, we cannot discard the possibility that longer incubation times with E2 could be cytotoxic for these cells.

In this work, we demonstrated that 50 pg/mL of E2 significantly inhibits ~50% *S. aureus* internalization into the bMECs (Figure 2). Because E2 does not have antibacterial activity, this reduction might be the result of the immunomodulatory effects that have been associated with this hormone. According to our results, the effect of E2 on *S. aureus* internalization shows a nonmonotonic dose-response curve. This behavior requires further investigation, however; nonmonotonic effects of E2 have been described on the development of mammary gland in *in vivo* models, as well as in human breast cancer cells. A possible explanation could be related to the versatility of E2 actions, which include the classic genomic functions, as well as nongenomic or membrane-initiated estrogen-signaling pathway [24, 25]. The inhibitory effect of E2 on *S. aureus* internalization cannot be attributable to the reduction in integrin $\alpha5\beta1$ MA because this receptor does not participate in the internalization of *S. aureus* in E2-treated bMECs. In addition, although E2 reduces $\alpha5\beta1$ MA, the presence of bacteria promotes its traffic at early times of infection (Figure 3), as we reported previously [10]. Considering this, the inhibition on *S. aureus* internalization in the E2-treated bMECs could be a consequence of the activation of immunomodulatory pathways. To explore this possibility, we analyzed the participation of the receptor TLR2 in this process. Accordingly, the results shown in Figure 4 suggest that E2 does not induce the MA of this receptor despite the fact that it upregulates the expression of its gene. Mammary epithelial cells play an essential role in the surveillance of mammary tissue during infections because they help in immune cell recruitment and bacterial recognition via the TLR signaling pathways [26, 27]. TLR2 is the major receptor for *S. aureus* detection and also mediates immunomodulatory functions [28]. We have previously demonstrated that *S. aureus* induces TLR2 MA and activation in bMECs in the same way that the lactogenic hormone prolactin stimulates *S. aureus* internalization in the bMECs [10]. E2 shows differential effects on TLR2 expression and/or activation; for example, in THP-1 cells, it increases the expression of TLR2 mRNA [29], but, in a bovine oviduct epithelial cell culture, it reduces the LPS-induced TLR2 mRNA expression [30]. Thus, the activation of the TLR2 pathways by E2 depends on the tissue and the physiological condition of the organism. In agreement with the absence of TLR2 activation in E2-treated bMECs, we detected that the MAPK p38 and ERK1/2 are inactivated in these cells (Figure 5). In addition, these kinases are not employed during the inhibition of *S. aureus* internalization induced by E2 (Figure 5). We have previously reported that these three MAPKs are required during *S. aureus* internalization into the bMECs [18].

We next explored whether E2 or infection regulates ERs activation because the direct effects of this hormone are exerted via intracellular ERs. In Figure 6, we showed that E2 induces the intracellular activation of ERα, as well as upregulating the expression of its gene. Likewise, *S. aureus* infection increases ERα mRNA expression in the E2-treated bMECs, but this induction is not related with ERα activation. In agreement, Yart et al. [31] reported that in a cell line of bMECs (MAC-T) the expression of ERα protein increases in response to E2 treatment. However, the activation of ERα by infection has not been extensively explored to date. Accordingly, Holm et al. [32] described that LPS from Gram-negative bacteria reduces ERα protein in endothelial cells. The presence of ERβ in the nuclei from infected bMECs (Figure 6(c)) indicates the participation of this receptor during *S. aureus* infection, which has not been reported. Nevertheless, flow cytometry analysis reveals that E2 does not activate ERβ. Taken together, these results suggest that E2 activates ERα in bMECs, which might be directly or indirectly implicated in the reduction of *S. aureus* internalization. Because we did not detect the activation of MAPKs in the E2-treated bMECs (Figure 5(c)) it is probable that E2 triggers the direct activation of ERα in bMECs, which up- and/or downregulates the transcription of various genes by binding to the estrogen response element of the genes or by interacting with other transcription factors [33]. To address this hypothesis, we analyzed some inflammatory genes in the E2-treated bMECs in the absence or presence of *S. aureus* infection. We previously reported that, with the exception of TNF-α, *S. aureus* inhibits the innate immune response of bMECs, as it is unable to induce the gene expression of some proinflammatory cytokines, such as IL-1β or IL-6 [10]. However, we also reported that, in the presence of ethanol as vehicle (2%), *S. aureus* slightly induces IL-1β mRNA expression [15]. The results from this work strengthen previous reports. Interestingly, E2 at 50 pg/mL significantly increases proinflammatory cytokine mRNA expression such as TNF-α and IL-1β, prior to *S. aureus* infection. However, upon infection, the levels of both cytokine mRNAs were downregulated in E2-treated bMECs. The differential influence of E2 on the expression of innate immune response genes depends on multiple factors, such as the hormone concentration or the physiological condition of the individuals [34]. These results coincide with other reports using urinary epithelial cells, where E2 reduces the LPS-induced cytokine expression [19]. Steroids have the ability to suppress (and/or resolve) an inflammatory response. In agreement with the anti-inflammatory function of E2 on bMECs, we detected a significant reduction in the secretion of proinflammatory cytokines (Figure 7(c)) in the E2-treated cells. This effect was maintained in the presence of *S. aureus*, with exception of IL-1β, which was induced in the infected E2-treated cells. This effect can be the consequence of the activation of other mechanisms, such as inflammasome activation, which requires further research [35]. Interestingly, and in agreement with the anti-inflammatory actions of the hormone, E2 also induces the mRNA expression of IL-10, an anti-inflammatory cytokine, which was upregulated by the hormone in the E2-treated cells challenged with *S. aureus*.

In addition to the immunomodulatory role of E2, it has been reported that this hormone also induces the production of antibacterial compounds in uterine epithelial cells [19]; a similar effect in the present model could explain the reduction in the internalization of *S. aureus*. Our data suggest that E2-treated bMECs produce an antibacterial compound, which is secreted into the culture medium (Figures 8(a) and 8(b)). To determine the origin of this activity, we analyzed the mRNA expression of different antimicrobial peptides (β-defensins and psoriasin). E2 induces the expression of DEFB1,

BNBD5, and psoriasin S100A7 mRNAs. We also analyzed the secretion of DEFB1 into the culture medium, which was only induced by bacteria and not by E2. It is necessary to analyze the concentrations of BNBD5 or psoriasin in the culture medium to resolve if they are implicated in the reduction of *S. aureus* internalization in the E2-treated bMECs. Accordingly, Fahey et al. [19] determined that in uterine epithelial cells E2 exerts anti-inflammatory effects and enhances the production of antimicrobial peptides, which display direct defense actions during the infection of this tissue. It is possible that a similar effect could be occurring in bMECs, but further research is necessary to corroborate this possibility. In addition, the induction of psoriasin expression by E2 has been demonstrated in the human mammary epithelial cell line MCF-7 [36]. According to our results, E2 is not favoring the production of NO as a possible antimicrobial compound. Altogether, these results indicate that E2 at 50 pg/mL reduces *S. aureus* internalization in the bMECs by modulating the innate immune response through the activation of ERα. Because we did not detect ERα activation in *S. aureus*-challenged bMECs, we can hypothesize that 50 pg/mL of E2 during 24 h activates ERα in bMECs, which promotes a slightly anti-inflammatory response that is enhanced during infection. It is possible that during infection other mechanisms (i.e., transcription factors) are participating because the activation of ERα is turned off. In this process, the TLR2/MAPK pathway is not activated.

5. Conclusions

E2 (50 pg/mL) induces the anti-inflammatory response of the bMECs during *S. aureus* internalization through the participation of ERα. This leads to the increased production of antimicrobial molecules, favoring *S. aureus* elimination.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Research Article

Sex Hormones Enhance Gingival Inflammation without Affecting IL-1β and TNF-α in Periodontally Healthy Women during Pregnancy

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Hormones (progesterone and estradiol) change greatly during pregnancy; however, the mechanism of hormonal changes on gingival inflammation is still unclear. This study is to evaluate the effects of hormonal changes during pregnancy on gingival inflammation and interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in gingival crevicular fluid (GCF). 30 periodontally healthy pregnant women were evaluated in the first, second, and third trimesters. 20 periodontally healthy nonpregnant women were evaluated twice (once per subsequent month). Clinical parameters including probing pocket depth (PPD), bleeding index (BI), gingival index (GI), clinical attachment level (CAL), and plaque index (PLI) were recorded. GCF levels of IL-1β and TNF-α and serum levels of progesterone and estradiol were measured. From the data, despite low PLI, BI and GI increased significantly during pregnancy; however, no significant changes in PLI, CAL, IL-1β, or TNF-α GCF levels were observed. Although IL-1β, not TNF-α, was higher in pregnant group than in nonpregnant group, they showed no correlation with serum hormone levels during pregnancy. GI and BI showed significant positive correlation with serum hormone levels during pregnancy. This study suggests that sex hormone increase during pregnancy might have an effect on inflammatory status of gingiva, independent of IL-1β and TNF-α in GCF.

1. Introduction

Since the 1960s, it has been proposed that periodontal health is associated with pregnancy [1, 2]. It is widely accepted that preexisting gingivitis or periodontitis in women would be worsening dramatically during pregnancy. Taani et al. have summarized that the prevalence of gingivitis during pregnancy ranged widely from 35 to 100% [3]. Though the exact mechanisms of exacerbating gingival inflammation during pregnancy have not yet been completely elucidated, it was supposed in the 1970s that the increase in serum estrogen and progesterone had a dramatic effect on the periodontium throughout pregnancy, which was correlated with clinical signs [4, 5]. However, some studies demonstrated no obvious gingival changes during pregnancy compared with nonpregnant controls [6, 7]. Thus, the correlation between hormone levels during pregnancy and gingival inflammation remains controversial.

Investigators have reported that increased female sex hormones may modulate the function of immune cells [8, 9]. Immunological changes during pregnancy have been considered to be, at least in part, responsible for periodontal

conditions [10]. Meanwhile, proinflammatory cytokines play a major role in the progression of gingival inflammation [11]. Interleukin- (IL-) 1β and tumor necrosis factor- (TNF-) α regulate the initial stages of inflammation by increasing the recruitment of neutrophils and monocytic phagocyte [12].

The effects of hormones on these cytokines in periodontium have been studied extensively in vitro. Morishita et al. reported that estradiol at 0.04 ng/mL or more inhibited IL-1 secretion, and progesterone at 0.1 ng/mL or more and 0.02 ng/mL or more, respectively, suppressed the production of IL-1α and IL-1β induced by lipopolysaccharides (LPS) in human monocytes [13], which indicates that high levels of estradiol and progesterone inhibited IL-1 secretion in human peripheral monocytes stimulated by LPS. In vitro study showed that sex hormones at physiological concentrations (estradiol of 10^{-9} to 10^{-7} M) had an inhibitory effect on the secretion of IL-1β and TNF-α by human periodontal ligament cells treated with *E. coli* LPS [14]. Also, Smith et al. found that TNF-α level in blood neutrophils decreased when estrogen and progesterone concentration were elevated [15]. These in vitro studies mentioned above focused on the effect of sexual hormones on cytokines under the challenge of bacteria.

As for human studies, many researchers investigated the change of inflammatory cytokines in pregnant women with gingivitis or periodontitis. A significant impact of periodontal therapy such as scaling and planning on the levels of IL-1β in gingival crevicular fluid was observed in pregnant women with periodontitis [16, 17]. Also, it is well known that gingival inflammation associated with pregnancy has been initiated by dental plaque and exacerbated by endogenous steroid hormones [18]. These studies did not exclude the effects of previously existing periodontal inflammation and dental plaque. It has been already reported that good oral hygiene in pregnancy was able to partially neutralize hormonal effect [19]. In early reports, some authors stated that healthy gingiva was not affected by pregnancy and the incidence of gingivitis was only 0.03% if a plaque-free state was maintained [4, 20]. Nevertheless, the sole effect of sex hormones on gingival inflammation is still unclear. Meanwhile, the research evaluating the change of periodontal status and local inflammatory responses in periodontally healthy women during pregnant is scarce. Thus, in this study, we collected women with healthy periodontium and excellent oral hygiene, to evaluate the effect of hormonal changes occurring during pregnancy on gingival inflammation and GCF levels of IL-1β and TNF-α.

2. Material and Methods

2.1. Subjects. Ethical approval was obtained from the Research and Ethics Committee of Shenzhen Maternity and Child Healthcare Hospital (China, approval number: 19) in full accordance with the World Medical Association Declaration of Helsinki (version 2002). With informed consent, the volunteers with excellent oral hygiene, periodontal health, and no smoking were recruited from June 2010 to June 2012 in Shenzhen Maternity and Child Healthcare Hospital. Exclusion criteria were systemic or topical antimicrobial/anti-inflammatory therapy within the previous 3 months, chronic systemic disease (e.g., diabetes, hypertension, epilepsy, cardiac disease, lung disease, and renal disease), and positive test for human immunodeficiency virus (HIV), multifetal gestation.

Table 1: Social characteristics of the groups studied.

Social variable	Nonpregnant women	Pregnant women
Age (years)	29.250 ± 2.314	28.333 ± 1.971
Ethnicity	Han	Han
Occupation		
Housewife	0	3
Employee	20	27
Level of education		
<Technical	6	10
Bachelor	9	13
Master	5	7
Economic status	Regular	Regular

During the observation period, subjects who had average PI scores >1 were excluded from the study. In pregnant group (Pr group), thirty pregnant women (aged 25 to 35) with gestational age at 12–14 weeks were recruited. Gestational age was determined according to information of sequential physical exams, data from menstrual cycles, and ultrasound test [18]. In nonpregnant control group (N-Pr group), 20 volunteers were selected in the same dental department. There was no difference in social-economical situation between two groups (Table 1).

The women in Pr group were examined three times during pregnancy (Pr I: 12–14 weeks; Pr II: 23–25 weeks; and Pr III: 33–36 weeks (gestational age)) [21]. The women in N-Pr group were examined twice (N-Pr I and N-Pr II) around the luteal period of the menstrual cycle, once per subsequent month [22]. At each examination, serum and gingival crevicular fluid (GCF) samples and clinical data were collected. Meantime, oral hygiene instructions were performed.

2.2. Clinical Measurements. Periodontal examination was performed by the same periodontists, using a manual periodontal probe (Kangqiao, Shanghai, China). The following clinical parameters were recorded at three sites (mesiobuccal, buccal, and distobuccal) of each tooth, excluding the third molar: plaque index (PLI): "0" = an absence of plaque on the clinical crown, "1" = the presence of soft deposits covering gingival margin, "2" = the presence of soft deposits covering between one-third and two-thirds of the crown, and "3" = the presence of soft deposits covering more than two-thirds of the crown [23]; gingival index (GI): "0" = normal gingival, "1" = mild inflammation, "2" = moderate inflammation, and "3" = severe inflammation [23]; bleeding index (BI): "0" = no bleeding, "1" = the presence of bleeding as a single point, "2" = the presence of bleeding as a thin line, and "3" = the presence of profuse bleeding as an immediate flow [22]; probing pocket depth (PPD): defined as the distance from the free gingival margin to the bottom of the sulcus; clinical attachment level (CAL): defined as the distance from the cemento-enamel junction to the bottom of the sulcus.

2.3. GCF Sampling. At each visit, GCF samples were collected from the mesiobuccal sites of upper premolars (14, 15, 24, and 25) before clinical measurements, using Waterman III filter-paper (Whatman International Ltd., Maidstone, England) (four samples per patient and per visit). Prior to collecting GCF, the sampling sites were gently air-dried. One filter-paper strip per each sampling site was used. The strip was inserted into gingival sulcus until slight resistance was felt and left there for 30 seconds. The samples contaminated with blood were discarded and a substituted GCF sample was taken from the mesiobuccal sulcus of the adjacent upper canine (13 or 23). The strips of each subject were immediately placed into two sterilized Eppendorf tubes and kept at −70°C.

2.4. Serum Sampling. Blood samples were collected in the morning at the same examination. 5 mL of blood was drawn from every subject into a free-anticoagulant vacuum tube. Serum was obtained after immediate centrifugation at 3000 g for 5 min and stored at −70°C until further evaluation.

2.5. IL-1β and TNF-α Assessment. GCF samples were extracted from the paper strips by eluting with 200 μL of phosphate-buffered saline and 2 μL of phenylmethanesulfonyl fluoride (20 mM) and incubated for 30 min at 4°C. Then, the tubes were centrifuged at 19,000 g for 5 min. The supernatants were collected and used to measure IL-1β and TNF-α level. The concentrations of IL-1β and TNF-α level were determined by using commercially enzyme-linked immunosorbent assay (ELISA) kits (R&D, MN, USA) according to the protocol.

2.6. Estradiol and Progesterone Assays. After serum samples thawed at room temperature and centrifuged at 4000 g for 5 min, supernatants were extracted for measurement of estradiol and progesterone by means of chemiluminescence method (Beckman Coulter Inc., MN, USA).

2.7. Statistical Analyses. Data were presented as mean and standard deviations. t-test or Analysis of Variance (ANOVA) was used for data. Correlations among the clinical parameters, GCF cytokines, and serum hormones were evaluated with Pearson's test. P values < 0.05 were considered statistically significant.

3. Results

3.1. Periodontal Parameters. At the first visit, all subjects had 28–32 teeth and the periodontal examination was in Table 2, which showed that there were no differences in PLI, PPD, GI, BI, and CAL (CAL = 0). During the pregnancy, PLI did not change compared to N-Pr group ($F = 0.64$, $P = 0.6373$), which indicated that all subjects kept good hygiene (Table 3). Although PPD had the increasing tendency, the difference was not significant ($F = 2.40$, $P = 0.0536$) (Table 3). GI and BI increased significantly ($F = 19.76$, $P < 0.05$; $F = 19.98$, $P < 0.001$) during pregnancy, which was higher than in the N-Pr group (Table 3). No changes in CAL were detected during the follow-ups (CAL = 0).

Table 2: Clinical periodontal parameters ($\overline{x} \pm s$) in the groups.

Variable	N-Pr group ($n = 20$)	Pr group ($n = 30$)	t value	P
PLI	0.570 ± 0.060	0.598 ± 0.098	0.67	0.5087
GI	1.250 ± 0.126	1.205 ± 0.109	1.33	0.1907
BI	1.145 ± 0.079	1.217 ± 0.135	−1.27	0.2092
PPD (mm)	2.235 ± 0.253	2.282 ± 0.267	0.79	0.4343
CAL (mm)	0	0	—	—

Pr group: pregnancy group; N-Pr group: nonpregnancy group; PLI: plaque index; GI: gingival index; BI: bleeding index; PPD: periodontal pocket depth; CAL: clinical attachment loss; n: number.

3.2. Inflammatory Cytokines in GCF. Table 4 showed the concentrations of IL-1β and TNF-α in the Pr group and the N-Pr group. Compared with the N-Pr group, there were no significant changes in GCF TNF-α level in Pr group ($F = 0.45$, $P = 0.7726$); however, GCF IL-1β level increased obviously ($F = 7.41$, $P < 0.0001$). During pregnancy, the GCF IL-1β and TNF-α levels in three trimesters did not show significant difference.

3.3. Hormonal Levels in Serum and Correlation with Gingival Inflammation during Pregnancy. Serum estradiol and progesterone concentrations in the Pr group were higher than in the N-Pr group. Furthermore, serum estradiol and progesterone levels increased gradually during pregnancy ($F = 73.87$, $P < 0.0001$; $F = 64.23$, $P < 0.0001$) (Table 5).

In the Pr group, positive correlation was found between gingival inflammation (GI and BI) and serum estradiol level during pregnancy ($r = 0.695$, $P < 0.0001$; $r = 0.683$, $P < 0.0001$), while no obvious correlation was found between PPD and serum estradiol level ($r = 0.23$, $P = 0.222$). Positive correlation was found between gingival inflammation (GI and BI) and serum progesterone level during pregnancy ($r = 0.694$, $P < 0.0001$; $r = 0.683$, $P < 0.0001$), while no obvious correlation was found between PPD and serum progesterone level ($r = 0.23$, $P = 0.222$) (Table 5). Because no changes were observed in GCF IL-1β and TNF-α level during pregnancy, they could not be correlated with the increase in gingival inflammation or in serum hormones.

4. Discussion

This study describes the changes in periodontal parameters and GCF inflammatory cytokines during pregnancy in periodontally healthy women and the correlation among serum hormonal levels, periodontal parameters, and inflammatory cytokines.

As some investigations showed, repetition and reinforcement of oral hygiene instructions were critical in improving oral hygiene and were able to reduce clinical signs of gingivitis in pregnant women and other nonpregnant subjects [24, 25]. In this study, participants persisted in plaque control and kept low plaque index, which eliminated the effects of bacteria on periodontal inflammation as much as possible. From our data, even though the PLI in pregnancy women was similar to in nonpregnancy women, pregnant women had elevated gingival inflammation, which further

Table 3: Periodontal parameters in pregnant group and nonpregnant group ($\overline{x} \pm s$).

Group	PPD (mm)	GI	BI	PLI
Pr group ($n = 30$)				
First trimester	2.282 ± 0.267^{a}	1.205 ± 0.109^{a}	1.217 ± 0.135^{a}	0.598 ± 0.098^{a}
Second trimester	2.336 ± 0.250^{a}	1.357 ± 0.152^{b}	1.360 ± 0.194^{b}	0.603 ± 0.110^{a}
Third trimester	2.411 ± 0.249^{a}	1.485 ± 0.169^{c}	1.510 ± 0.223^{c}	0.610 ± 0.097^{a}
N-Pr group ($n = 20$)				
First month	2.235 ± 0.253^{a}	1.250 ± 0.126^{a}	1.145 ± 0.079^{a}	0.570 ± 0.060^{a}
Second month	2.225 ± 0.233^{a}	1.235 ± 0.108^{a}	1.237 ± 0.113^{a}	0.590 ± 0.076^{a}
F	2.40	19.76	19.98	0.64
P	0.0536	<0.0001	<0.0001	0.6373

Pr group: pregnancy group; N-Pr group: nonpregnancy group; PPD: periodontal pocket depth; GI: gingival index; BI: bleeding index; PLI: plaque index. Different letters represent significant difference.

Table 4: IL-1β and TNF-α level in pregnant group and nonpregnant group ($\overline{x} \pm s$).

Group	IL-1β (ng/L)	TNF-α (ng/L)
Pr group ($n = 30$)		
First trimester	11.192 ± 3.186^{b}	167.111 ± 68.733^{a}
Second trimester	11.033 ± 2.647^{b}	158.124 ± 46.857^{a}
Third trimester	11.368 ± 2.632^{b}	173.455 ± 52.738^{a}
N-Pr group ($n = 20$)		
First month	8.031 ± 3.509^{a}	166.072 ± 39.098^{a}
Second month	7.972 ± 3.758^{a}	156.427 ± 51.091^{a}
F	7.41	0.45
P	<0.0001	0.7726

Pr group: pregnancy group; N-Pr group: nonpregnancy group; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor-α; Different letters represent significant difference.

Table 5: Serum estradiol and progesterone in pregnant group and nonpregnant group ($\overline{x} \pm s$).

Group	Estradiol (pg/mL)	Progesterone (ng/mL)
Pr group ($n = 30$)		
First trimester	$24609.67 \pm 18176.32^{d}$	76.10 ± 30.59^{d}
Second trimester	$62142.00 \pm 23346.25^{c}$	123.84 ± 37.36^{c}
Third trimester	$81307.00 \pm 32481.23^{b}$	210.16 ± 92.27^{b}
N-Pr group ($n = 20$)		
First month	1438 ± 413.4018^{a}	18.85 ± 9.95^{a}
Second month	1470 ± 369.3237^{a}	20.47 ± 12.60^{a}
F	73.87	64.23
P	<0.0001	<0.0001

Pr group: pregnancy group; N-Pr group: nonpregnancy group. Different letters represent significant difference.

confirmed the hypothesis that pregnancy may be associated with inflammatory changes in gingival tissues. Also, our data were consistent with the previous researches [21, 22]. In these two researches, gingival inflammation during pregnancy was examined, in which healthy periodontium and good oral hygiene were included in the subject criteria. Figuero et al. [21] found that GI increased, despite of fairly low Pl values, and maintained high levels in the third trimester in 48 pregnant Spanish women. However, the data about PPD was not reported [21]. In the other longitudinal study, gingival inflammation in 30 periodontally healthy pregnant women with good oral hygiene in Finland was evaluated by bleeding on probing values and proportion of periodontal pockets (≥4 mm) which increased without relation to plaque between the first and second trimesters [22]. The data about PPD were not consistent with our data. In this present study, PPD showed an increasing tendency in these three trimesters; however, there was no significant difference among three stages. It is possibly due to the different race and different reaction to hormonal levels. Meanwhile, although in some previous cross-sectional and longitudinal studies on subjects with gingivitis and periodontitis, PPD significantly increased during pregnancy [1–4], it was explained that the PPD change was induced by bacteria and increased hormones simultaneously.

Pregnant women showed significantly increased gingival inflammation (GI, BI), which reached high levels in the third trimester with little change in attachment level, although plaque scores were nearly unchanged and kept fairly low (PLI < 1). The levels of GI and BI in early pregnancy were already higher than that of nonpregnant women. It suggested that the early pregnancy had already affected the gingiva. However, gingival pockets (>3 mm) and GI values (>2) were not found in the study, indicating that pregnancy has limited influence on gingival inflammation, when good plaque control was maintained. Additionally, no change in CAL was detected during the follow-ups, which was in agreement with most previous reports [3, 26]. Miyazaki et al. [7] suggested that the increase of periodontal pocket during pregnancy is caused by enlargement of gingival tissue rather than periodontal destruction, which was confirmed in our study. It could be speculated that a chronic and lasting inflammatory state of the gingiva is essential to cause attachment loss.

Since the localization of estrogen receptor and progesterone receptor has been reported in the human periodontium, Preshaw [27] considered that the obvious increase in circulating levels of estrogen and progesterone was supposed to have a dramatic effect on the periodontium throughout pregnancy and be correlated with clinical phenomenon. In our results, serum estradiol and progesterone levels increased

greatly during the pregnancy as expected and were much higher than those of nonpregnant group. Positive correlation was found between the increased inflammation in gingiva and the increase in serum estradiol and progesterone levels during pregnancy. Sexual hormones levels could be the factors that are responsible for the increase of gingival inflammation. Vogt et al. [28] mentioned that sexual hormones would influence the inflammatory status of healthy gingiva in a limited degree when good plaque control was maintained, which is consistent with our study. GI and BI, not PPD, changed significantly during pregnancy, which might be explained by the impact of sexual hormones on vascular permeability [12]. However, in the aforementioned study from Spain, no significant correlation was found between GI increase and salivary hormone levels. It is possible that salivary hormone levels are different from serum hormone levels, resulting in different results.

Furthermore, we explored the changes of inflammatory cytokines in GCF during pregnancy. One longitudinal study, on 18 premenopausal women, observed that the GCF levels of IL-1β and TNF-α remained stable between ovulation and progesterone peak [11]. Thus, in our study, the nonpregnant women during the luteal period of the menstrual cycle according to Gürsoy et al. [22] represented the comparable women before gestation. In the present study, there were no remarkable differences in the GCF IL-1β levels among three trimesters in pregnant women, although their concentrations were significantly higher than found in nonpregnant women, which is in agreement with the results of Figuero et al. [21]. At the first visit, GI and BI had no significant difference between N-Pr and Pr group; however, GCF IL-1β level had increased in Pr group. Afterward, although GCF IL-1β level kept stable during pregnancy, its level was higher than in N-Pr group. As Boronat-Catalá et al. [29] mentioned that IL-1β in GCF could be used as a reliable marker of the degree of inflammation in gingivitis, we considered that increased IL-1β was involved in the gingival inflammation in pregnant women. Under the strict control of dental plaque during the observation period, an elevated level of GCF IL-1β in the first trimester suggested that the early pregnancy had already affected GCF IL-1β. However, whether the increase of GCF IL-1β was induced by sex hormone was unknown. Shu et al. [14] demonstrated that sex hormones at physiological concentrations (Estradiol of 10^{-9} to 10^{-7} M) had an inhibitory effect on the secretion of IL-1β by human periodontal ligament (hPDL) cell. Meanwhile, from some studies, estrogen had no effect on IL-1β or negatively regulated its secretion [30, 31]. Thus, we inferred that IL-1β increase was not induced by sex hormone. The mechanism needs further to be explored. The reason for stable GCF IL-1β level during pregnancy could be explained as following. First, immunosuppression, to some extents, occurs in pregnancy [32] and suppresses the further increase in IL-1β level. Second, high levels of estradiol and progesterone inhibited further IL-1 secretion, which is consistent with previous research [13, 14].

TNF-α stimulates collagenase production and bone resorption and impairs the repair capacity of the periodontium. In samples from nonpregnant women, GCF TNF-α level could not be classified as a marker of inflammation in gingivitis [30]. GCF TNF-α level is more associated with alveolar bone resorption and attachment loss. In our study, GCF TNF-α level did not change during pregnancy, which could explain the reason that no attachment loss occurred. Furthermore, no positive correlation between serum hormones and GCF TNF-α level was found. It is possible that elevating estrogen and progesterone inhibited TNF-α secretion, which was described in blood neutrophils [14].

5. Conclusion

Sexual hormones estradiol and progesterone would influence the inflammatory status of gingiva even under good oral hygiene control during pregnancy, independent of GCF levels of IL-1β and TNF-α.

Conflict of Interests

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

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Research Article

Genetic Investigation of Complement Pathway Genes in Type 2 Diabetic Retinopathy: An Inflammatory Perspective

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Diabetic retinopathy (DR) has complex multifactorial pathogenesis. This study aimed to investigate the association of complement pathway genes with susceptibility to DR. Eight haplotype-tagging SNPs of *SERPING1* and *C5* were genotyped in 570 subjects with type 2 diabetes: 295 DR patients (138 nonproliferative DR [NPDR] and 157 proliferative DR [PDR]) and 275 diabetic controls. Among the six *C5* SNPs, a marginal association was first detected between rs17611 and total DR patients ($P = 0.009$, OR = 0.53 for recessive model). In stratification analysis, a significant decrease in the frequencies of G allele and GG homozygosity for rs17611 was observed in PDR patients compared with diabetic controls ($P_{corr} = 0.032$, OR = 0.65 and $P_{corr} = 0.016$, OR = 0.37, resp.); it was linked with a disease progression. A haplotype AA defined by the major alleles of rs17611 and rs1548782 was significantly predisposed to PDR with increased risk of 1.54 ($P_{corr} = 0.023$). Regarding other variants in *C5* and *SERPING1*, none of the tagging SNPs had a significant association with DR and its subgroups (all $P > 0.05$). Our study revealed an association between DR and *C5* polymorphisms with clinical significance, whereas *SERPING1* is not a major genetic component of DR. Our data suggest a link of complement pathway with DR pathogenesis.

1. Introduction

Diabetes mellitus (DM) is reaching an alarming proportion worldwide, as is known that DM has a complex multifactorial pathogenesis. The devastating complications of diabetes are the macro- and microvascular diseases [1, 2]. Of them, diabetic retinopathy (DR) is the most common microvascular complication and is a leading cause of blindness across the globe [3]. To date, many environmental and clinical factors have been proposed to confer risk of DR development, such as prolonged duration of diabetes, alteration of glucose metabolism, and poor glycemic control [4]. Additionally, genetic predisposition, independent of the above-mentioned factors, has been found to contribute to DR pathology; the evidence comes from the observation of disease aggregation among family members and multiple DR-associated genes identifications [5–7]. So far, the exact pathogenesis of DR is still unclear and is known to be involved in several physiopathologic pathways, such as angiogenesis factors, oxidative stress, apoptosis, and protein kinase C (PKC) [8–10]. More recent evidence depicting DR as a retinal disease associated with inflammation has drawn special attention and garnered great research interests [11–13].

Complement system is an important component of innate immunity and involved in the modulation of several immune and inflammatory responses. The complement system can be divided into classical, lectin, and alternative pathway; activation of the system is tightly regulated by complement

factors; disruption of complement regulation can lead to several distinct downstream inflammatory actions *en route to* the pathogenesis of DR [14, 15]. Evidence for the link comes from the observation of increased expression of several complement factors in DR patients; these factors included C1 inhibitor (C1INH, also known as serpin peptidase inhibitor, clade G, *SERPING1*), C5, factor H (CFH), and factor B (CFB) [16, 17]. In our previous studies, genetic variants in the *CFH* and *CFB* genes, both involved in complement alternative pathway, have been evaluated and identified as susceptibility genes for DR [18]. Moreover, *CFH* and *CFB*, as well as other complement pathway genes, have also been found to be associated with a range of inflammatory diseases [19].

Therefore, a genetic study focused on other complement genes was designed with a view to elucidating the involvement of complement system in DR development. Two complement genes, *SERPING1* and *C5*, involved in the classical pathway and in the central part of complement cascade, respectively, were selected for evaluation. Furthermore, stratification by DR stage and genotype-phenotype correlation analysis were also performed to identify these factors associated with prognosis and clinical features.

2. Materials and Methods

2.1. Study Participants. The study protocol was approved by the Ethics Committee on Human Research, Harbin Medical University. The study procedures were conducted in accordance with the tenets of the Declaration of Helsinki. Written informed consent was obtained from all study subjects after explanation of the nature of the study. All study subjects were Han Chinese recruited from the First Affiliated Hospital of Harbin Medical University.

All patients received complete ophthalmic examinations and clinical information collection, including corrected visual acuity, slit-lamp biomicroscopy, fundoscopic examination, age, gender, progression time from diabetes to DR, body mass index (BMI), HbA1c level, smoking status, and presence of hypertension and hyperlipidemia, as well as insulin application. The study involved 570 unrelated individuals with type 2 diabetes mellitus (T2DM); patients with type 1 diabetes, gestational diabetes, or maturity-onset diabetes were excluded from the study. The diagnosis of T2DM was based on World Health Organization criteria [20]. Of the group, 295 patients were diagnosed with DR (156 [52.9%] PDR and 139 [47.1%] NPDR); 275 subjects without DR but with type 2 diabetes duration of more than 10 years were considered as DM controls. The stage of DR was determined according to the Early Treatment Diabetic Retinopathy Study (ETDRS) criteria [21]. People with any systemic inflammation diseases, or any other ocular disorders such as age-related macular degeneration (AMD), glaucoma, or retinal venous occlusion, were also excluded.

2.2. SNP Selection and Genotyping. We adopted a haplotype-tagging SNP approach and obtained the tagging SNPs across the targeted regions, from the International HapMap Project for the Chinese Han Beijing (CHB) population (http://hapmap.ncbi.nlm.nih.gov/, HapMap Genome Browser). Two SNPs (rs1005511 and rs3824988) from *SERPING1* and six from *C5* (rs12237774, rs2269066, rs17611, rs1548782, rs10985126, and rs1017119) were selected by the tagger-pairwise method with r^2 and MAF (minor allele frequency) values greater than 0.8 and 0.10, respectively. Genomic DNA was extracted from whole blood using a QIAamp Blood Kit (Qiagen, Hilden, Germany) according to the protocol. All the SNPs were genotyped by *Taq*Man SNP Genotyping Assays (Applied Biosystems Inc., Foster City, CA) in the LightCycler® 480 Real-Time PCR System (Roche, Switzerland) according to the manufacturer's instructions.

2.3. Statistical Analysis. Hardy-Weinberg equilibrium (HWE) of individual SNP was tested by χ^2 test. Allelic and genotypic association of each SNP was calculated by using χ^2 test or Fisher exact test. Dominant and recessive models were also applied to investigate the disease association with regard to the minor allele. The odds ratios (OR) and 95% confidence intervals (CI) were calculated. Pairwise linkage disequilibrium (LD, D') between polymorphisms and expectation-maximization- (EM-) based haplotype association analysis were assessed using the Haploview software. Student's t-test and χ^2 test were used to compare continuous clinical data and categorical variables, respectively. Stratification analysis based on DR stage (NPDR and PDR) was also performed. $P < 0.05$ was considered as statistically significant. P values were corrected by Bonferroni test (n = total number of SNPs) or permutation test in Haploview software.

3. Results

In our study, a total number of 570 unrelated individuals with T2DM were recruited, comprising 295 DR patients and 275 DM controls. Since we aimed to recruit T2DM patients without DR as controls, the mean duration of disease was longer than that of the DR group so as to largely rule out late-onset DR ($P < 0.01$). The proportions of hyperlipidemia and insulin application were higher in DR group than that in DM controls ($P = 0.042$ and $P < 0.001$, resp.). No significant differences in other clinical features were observed between two groups (Table 1).

Two haplotype-tagging SNPs in *SERPING1* and six haplotype-tagging SNPs in *C5* were selected, which capture over 90% of all alleles across their corresponding locus with a MAF larger than 0.10 and a mean r^2 of 0.80 in the HapMap Chinese Han population. The genotype frequencies of the eight selected SNPs followed the HWE in all subjects. For *C5*-rs17611, there was an obvious trend towards lower proportions of G allele and GG homozygosity in DR patients than DM controls ($P = 0.056$, OR = 0.79, 95% CI = 0.62–1.0; $P = 0.009$, $P_{corr} = 0.072$, OR = 0.53, 95% CI = 0.33–0.86, resp.), but the associations either were marginal or could not remain after adjustment for multiple testing. For other SNPs, no significant associations were detected with DR in any genetic models (Table 2).

Table 1: Characteristics of the study subjects.

Characteristic	DR (n = 295)	DM (n = 275)	P value
Age (years)	55.9 ± 13.2	56.3 ± 7.6	0.66
Gender (F/M)	151/144	153/122	0.29
Duration of diabetes (years)	13.1 ± 9.4	18.1 ± 6.7	<0.01
Duration of DR (years)	5.3 ± 3.9	—	N/A
Progression time from diabetes to DR (years)	7.9 ± 5.8	—	N/A
HbA1C (%)	8.0 ± 1.6	7.9 ± 1.9	0.50
BMI (kg/m^2)	24.0 ± 5.7	24.1 ± 4.4	0.82
Hypertension (%)	73.2	66.9	0.10
Hyperlipidemia (%)	30.8	23.3	0.042
Smoking (%)	13.9	16.0	0.48
Insulin therapy (%)	46.8	26.2	<0.001
Family history of diabetes (%)	26.1	21.5	0.19

P values were compared by χ^2 or Student's t-test, and $P < 0.05$ was considered statistically significant. DR: diabetic retinopathy; DM: diabetes mellitus; HbA1c: glycosylated hemoglobin; BMI: body mass index.

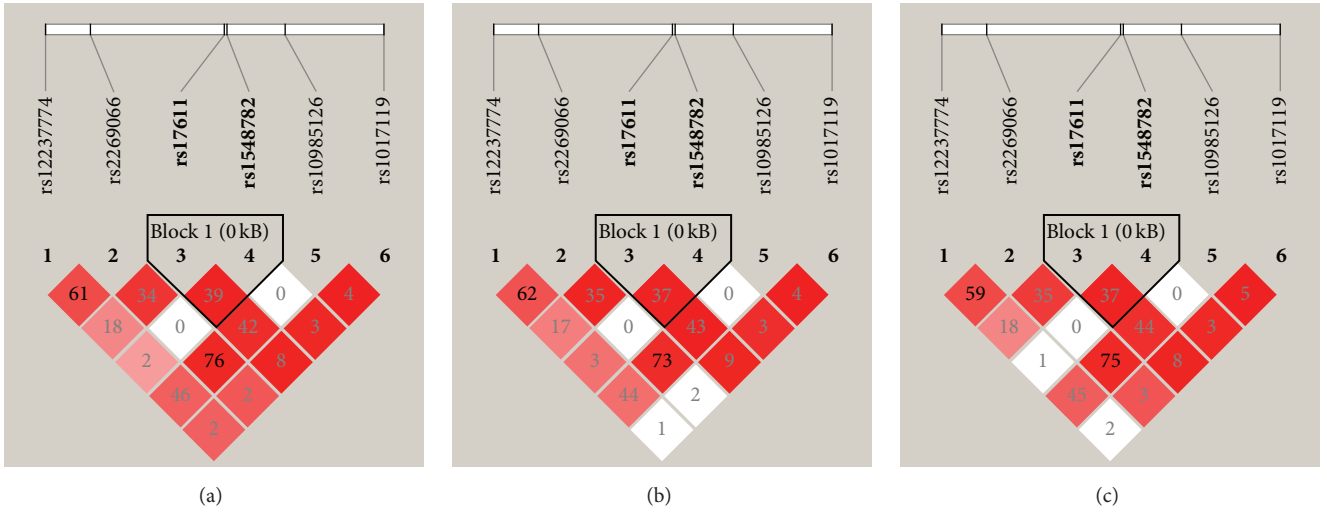

Figure 1: Linkage disequilibrium (LD) structure of the *C5* locus for DR (a), NPDR (b), and PDR (c). LD was measured using data from all controls and total DR and its subtypes. The haplotype block was defined by the confidence interval method implemented in the Haploview software. The LD (r^2) between any two SNPs is listed in the cross cells. DR: diabetic retinopathy; NPDR: nonproliferative diabetic retinopathy; PDR: proliferative diabetic retinopathy.

Among the 295 DR patients, 139 (47.1%) were NPDR and 156 (52.9%) were PDR; stratification analysis by the DR stage was performed. In PDR patients, significant lower frequencies of G allele and GG homozygosity for *C5*-rs17611 were found compared to that in DM group even after multiple testing correction (P_{corr} = 0.032, OR = 0.65, 95% CI = 0.48–0.87; P_{corr} = 0.016, OR = 0.37, 95% CI = 0.19–0.71, resp.), implying a protective effect; such difference was not observed in NPDR patients (Table 3). For other SNPs, no significant differences in the allelic or genotypic frequencies were found in either NPDR or PDR subtypes compared with DM controls.

Pairwise LD analysis showed that two *SERPING1* tagging SNPs were included in one haplotype block in NPDR, PDR, and total DR patients. Three groups showed a similar distribution of haplotype. No haplotype was significantly associated with any group (all $P > 0.1$, Table 4). Regarding *C5*, LD analysis revealed one haplotype block in three groups including SNPs rs17611 (the most significant finding) and rs1548782 (Figure 1). The haplotype AA, defined by the two SNPs, showed a significant risk for PDR patients (P = 0.004, permutation P = 0.023; OR = 1.54, 95% CI = 1.15–2.06). No significant haplotype association was detected among the other two comparisons (Table 5).

Considering the significance of *C5*-rs17611 in this study, correlations of the specific genotype with clinical features were evaluated in total DR patients. The results showed that DR patients carrying protective rs17611 GG genotype

Table 2: Comparison of genotype and allele frequencies of *SERPING1* and *C5* polymorphisms in DR patients and DM controls.

SNP ID	Minor allele	Allele distribution (%)			*P* value	Odds ratio (95% CI)	Genotype distribution (%)		*P* value	Odds ratio (95% CI)
			DR ($n = 590$)	DM ($n = 550$)			DR ($n = 295$)	DM ($n = 275$)		
C1INH (*SERPING1*)										
rs1005511	G	G	137 (23.2)	125 (22.7)	0.84		173/107/15	165/95/15	0.74†	
		A	453 (76.8)	425 (77.3)					0.84‡	
rs3824988	C	C	71 (12.0)	57 (10.4)	0.37		228/63/4	220/53/2	0.43†	
		T	519 (88.0)	493 (89.6)					0.68‡*	
C5										
rs12237774	T	T	107 (18.1)	109 (19.8)	0.47		199/85/11	175/91/9	0.34†	
		C	483 (81.9)	441 (80.2)					0.82‡*	
rs2269066	T	T	121 (20.5)	120 (21.8)	0.59		187/95/13	165/100/10	0.41†	
		C	469 (79.5)	430 (78.2)					0.64‡	
rs17611	G	G	210 (35.6)	226 (41.1)	0.056	0.79 (0.62–1.0)	117/146/32	100/124/51	0.42†	0.53 (0.33–0.86)
		A	380 (64.4)	324 (58.9)					0.009‡ (0.072)	
rs1548782	T	T	119 (20.2)	112 (20.4)	0.94		191/89/15	172/94/9	0.59†	
		A	471 (79.8)	438 (79.6)					0.28‡	
rs10985126	C	C	136 (23.1)	134 (24.4)	0.60		170/114/11	160/96/19	0.89†	
		T	454 (76.9)	416 (75.6)					0.089‡	
rs1017119	C	C	73 (12.4)	78 (14.2)	0.37		225/67/3	202/68/5	0.44†	
		T	517 (87.6)	472 (85.8)					0.49‡*	

Data are the number of subjects (% of the total group). DR: diabetic retinopathy; DM: diabetes mellitus; *Fisher exact test; †*P* value for dominant model; ‡*P* value for recessive model. The genotype distribution was showed as major allele homozygous/heterozygous/minor allele homozygous.

Table 3: Comparison of genotype and allele frequency of *C5*-rs17611 in DR and DM stratified by disease severity.

SNP ID	Genotype/allele	NPDR (n = 139)	PDR (n = 156)	DM (n = 275)	NPDR versus DM		PDR versus DM	
					P value	Odds ratio (95% CI)	P value	Odds ratio (95% CI)
rs17611	GG	20 (14.4)	12 (7.7)	51 (18.5)	0.51[†]	1.15 (0.75–1.78)	0.062[†]	0.68 (0.46–1.02)
	AG	73 (52.5)	73 (46.8)	124 (45.1)	0.29[‡]	0.74 (0.42–1.30)	0.002[‡] (0.016)	0.37 (0.19–0.71)
	AA	46 (33.1)	71 (45.5)	100 (36.4)	0.62[#]	0.85 (0.46–1.59)	0.001[#] (0.008)	0.33 (0.17–0.67)
	G	113 (40.6)	97 (31.1)	226 (41.1)	0.90	0.98 (0.73–1.32)	0.004 (0.032)	0.65 (0.48–0.87)
	A	165 (59.4)	215 (68.9)	324 (58.9)				

Data are the number of subjects (% of the total group). NPDR: nonproliferative diabetic retinopathy; PDR: proliferative diabetic retinopathy; DM: diabetes mellitus. [†]P value for dominant model; [‡]P value for recessive model; [#]P value for codominant model.

Table 4: Haplotype association of *SERPING1* gene with DR and its subtypes.

Haplotype rs1005511-rs3824988	Frequency				Association (P value)		
	Total DR	NPDR	PDR	DM	DR versus DM	NPDR versus DM	PDR versus DM
A-T	0.76	0.79	0.74	0.77	0.79	0.51	0.31
G-T	0.12	0.11	0.13	0.13	0.60	0.38	0.99
G-C	0.12	0.10	0.13	0.10	0.42	0.96	0.19

would present a delayed progression from DM to DR onset compared with patient carrying AA genotype (9.3 ± 6.4 versus 7.0 ± 5.5, $P = 0.045$; Figure 2); no significant difference for other clinical features was detected among different genotype carriers.

4. Discussion

In this study, we performed a haplotype-tagging SNP analysis of two complement pathway genes, *SERPING1* and *C5*, in T2DM and DR patients. Our results demonstrated that *C5*-rs17611 was significantly associated with DR, particularly conferred to the PDR susceptibility; this functional variant also linked with certain clinical significance. Moreover, a haplotype conferring an increased risk for PDR was also detected. In contrast, none of the SNPs in *SERPING1* were significantly associated with DR and its subtypes. These findings together suggest that *SERPING1* is not a disease gene for DR, but *C5* is likely to be a susceptibility gene for DR in Chinese patients. To our knowledge, this is the first genetic study to investigate *SERPING1* and *C5* genes in DR patients. Over the past decade, great achievements have been made in elucidating the genetic background of the disease; so far, more than 30 DR-associated genes involved in different metabolic mechanisms and functional pathways have been reported [22, 23]. Our previous study has successfully identified two complement alternative pathway genes, *CFH* and *CFB*, which were associated with DR development [18]. Results of this study enrich our knowledge of the genetic architecture of DR and the involvement of each complement pathway in DR pathogenesis. In addition, we also found that the proportions of insulin application were higher in DR group than that in DM controls; it was supposed that these DM patients without complications have a relatively good metabolic control, which may explain the lower frequency of individuals with insulin therapy.

As described above, the complement system is a key component of innate immunity, consisting of a large family of membrane-bound proteins that are critical for protection against bacterial infection and immune complex deposition. Uncontrolled complement activation is considered an important contributor in the pathogenesis of DR [15]. C5, being the first of many components of the terminal pathway, mediates many potent inflammatory events and plays a major role in the complement system. In the cascade, a critical event is the cleavage of C5 into fragments of C5a and C5b, as well as the subsequent formation of MAC (C5b-9) which is involved in cytolysis, cell activation, and production of inflammatory mediators [24]. *In vitro* study has revealed that C5a treatment induced increased production of several inflammatory cytokines, such as MCP-1, IL-6, IL-8, and VEGF, from retinal pigment epithelial cells [25, 26]. In clinical study, C5b-9 deposition was detected on the endothelial surface of retinal vessels in eye donors with diabetes; vitreous concentration of C5a increased significantly in PDR patients compared with controls [14, 27]. Furthermore, *C5* gene has been found to affect susceptibility to several inflammatory conditions, including AMD, rheumatoid arthritis, and renal allograft outcomes [28–30]. In the present study, *C5*-rs17611 was found to be associated with PDR patients; meanwhile, rs17611 was also found to be associated with periodontitis and the GG genotype was linked with increased C5 levels

Table 5: Haplotype association of *C5* gene with DR and its subtypes.

Haplotype rs17611-rs1548782	Frequency				Association (P value) (permutation test)		
	Total DR	NPDR	PDR	DM	DR versus DM	NPDR versus DM	PDR versus DM
A-A	0.64	0.59	0.69	0.59	0.064	0.94	0.004 (0.023)
G-T	0.20	0.23	0.17	0.20	0.88	0.29	0.21
G-A	0.16	0.17	0.15	0.21	0.025 (0.11)	0.21	0.019 (0.09)

NPDR: nonproliferative diabetic retinopathy; PDR: proliferative diabetic retinopathy; DM: diabetes mellitus. P_{corr} association analysis results from permutation test (iterations 10,000).

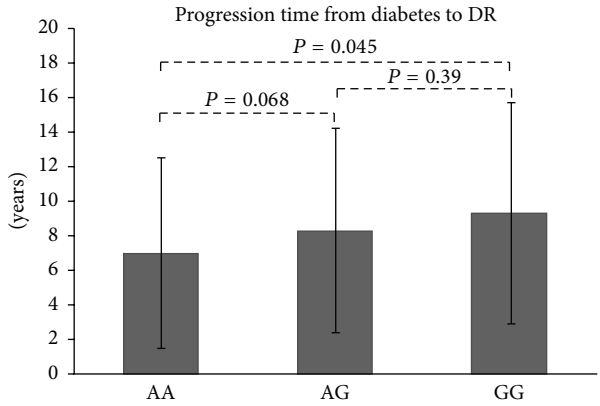

Figure 2: The average time of progression from diabetes to DR according to genotype. AA: 7.0 ± 5.5; AG: 8.3 ± 5.9; GG: 9.3 ± 6.4 (years).

in patients with rheumatoid arthritis [29, 31]. The change of rs17611 A>G nucleotide results in the synthesis of Valine instead of Isoleucine; a functional analysis on rs17611 showed that individuals homozygously expressing the risk s17611 allele exhibit increased C5a and decreased C5 in plasma, evidence of increased C5 turnover; this structural change might alter the rate of C5 cleavage and explain its association with inflammatory diseases [29].

Component 1 inhibitor gene (*SERPING1*) encoding C1INH is a key regulator in classic and lectin complement pathway and involved in the development of several immune-related diseases. In addition, C1INH was also found to be expressed in both retinal and retinal pigment epithelium (RPE) layers [32–34]. Based on the evidence, *SERPING1* was considered as a candidate gene for DR. But, in our study, no association between *SERPING1* polymorphisms and DR was found, even stratified by DR stage or considered clinical features. The results suggested that *SERPING1*, and the gene-involved classical pathway, might not contribute significantly to the risk of DR. Further studies to determine the biologic roles of these polymorphisms and the haplotype in DR are still warranted; additionally, it would be better to include healthy controls in this study to fully reflect the disease association.

In summary, this study first demonstrated that *C5* rs17611 is a susceptibility locus for DR and particularly predisposes to PDR subtype with clinical significance. The complement classical pathway gene, *SERPING1*, may confer no or limited risk for DR development. Together with our previous findings, our results help to further enrich the growing understanding of genetic spectrum of DR and clarify the involvement of each complement pathway in DR pathogenesis from molecular perspectives.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Ming Ming Yang, Jiao Jie Fan, and Yan Teng designed the experiments. Jun Wang and Jiao Jie Fan performed the experiments. Ming Ming Yang, Yun Duan Sun, and Jun Wang performed the analysis and wrote the paper. Yan Bo Li and Hong Ren revised the paper. All authors contributed to the editing of the paper and to scientific discussions. Ming Ming Yang and Jun Wang contributed equally to this work. Dr. Ming Ming Yang and Dr. Jiao Jie Fan contributed equally to the work in this paper and can be considered as co-corresponding authors.

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