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Research article

Mitochondrial reactive oxygen species mediate the lipopolysaccharide-induced pro-inflammatory response in human gingival fibroblasts

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ABSTRACT

Although periodontal diseases are initiated by bacteria that colonize the tooth surface and gingival sulcus, the host response is believed to play an essential role in the breakdown of connective tissue and bone. Mitochondrial reactive oxygen species (mtROS) have been proposed to regulate the activation of the inflammatory response by the innate immune system. However, the role of mtROS in modulating the response of human gingival fibroblasts (HGFs) to immune stimulation by lipopolysaccharides (LPS) has yet to be fully elucidated. Here, we showed that LPS from *Porphyromonas gingivalis* stimulated HGFs to increase mtROS production, which could be inhibited by treatment with a mitochondrial-targeted exogenous antioxidant (mito-TEMPO) or transfection with manganese superoxide dismutase (MnSOD). A time-course study revealed that an increase in the concentration of mtROS preceded the expression of inflammatory cytokines in HGFs. Mito-TEMPO treatment or MnSOD transfection also significantly prevented the LPS-induced increase of interleukin (IL)-1β, IL-6, and tumor necrosis factor-α. Furthermore, suppressing LPS-induced mtROS generation inhibited the activation of p38, c-Jun N-terminal kinase, and inhibitor of nuclear factor-κB kinase, as well as the nuclear localization of nuclear factor-κB. These results demonstrate that mtROS generation is a key signaling event in the LPS-induced pro-inflammatory response of HGFs.

1. Introduction

Periodontitis consists of a group of infections leading to the inflammation of gingival tissues and the destruction of periodontal tissues [1]. Human gingival fibroblasts (HGFs) are a major constituent of gingival connective tissue. The ability of these periodontal residential cells to recognize pathogens makes them crucial in dealing with microbial invasion. These cells release several inflammatory cytokines including interleukins (ILs), and thus play active roles in host defense. However, HGFs from diseased sites contribute to the pathogenesis of periodontitis [2].

Lipopolysaccharides (LPS) from gram-negative bacteria are potent inducers of pro-inflammatory mediators and can initiate a number of host-mediated destructive processes [3]. HGFs stimulated by LPS produce pro-inflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor-α (TNF-α), which are important in the initiation of periodontitis [4]. Increased IL-1β, IL-6, and TNF-α secretion by fibroblasts has been detected in periodontitis lesions, and their unrestricted production may contribute to chronic leukocyte recruitment and tissue destruction [5]. Regulating the inflammatory response of HGFs is one way of preventing and/or controlling the progression of periodontitis [6].

Reactive oxygen species (ROS) have historically been viewed as toxic metabolic by-products and causal agents in a myriad of human pathologies. The first evidence for a role of ROS in the periodontal tissues was the demonstration that these molecules were produced by neutrophils and caused the deterioration of surrounding tissues [7]. However, recent work indicated that ROS were critical intermediaries in cellular signaling pathways. For

Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility-shift assay; H_2DCFDA , 5,6-chloromethyl-2′,7′- dichlorodihydrofluorescein diacetate, acetyl ester; HGF, human gingival fibroblast; IKK, inhibitor of nuclear factor-κB kinase; IL, interleukin; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharides; MAPK, mitogen-activated protein kinase; MnSOD, manganese superoxide dismutase; mtROS, mitochondrial reactive oxygen species; NF, nuclear factor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDTC, pyrrolidine dithiocarbamate; qRT-PCR, quantitative reverse transcription polymerase chain reaction; ROS, reactive oxygen species; tMnSOD, transfected manganese superoxide dismutase; TNF, tumor necrosis factor

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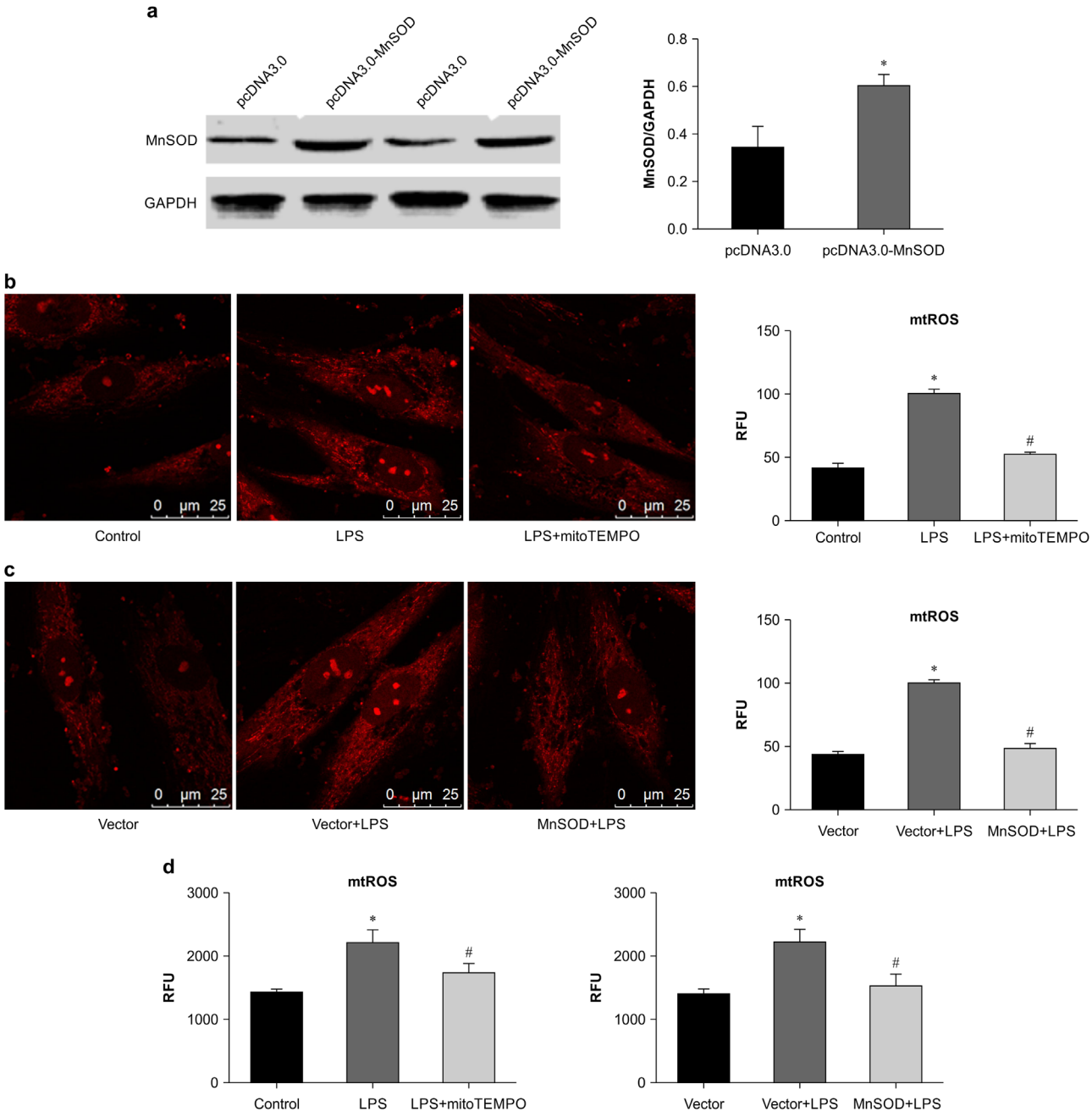

Fig. 1. Manganese superoxide dismutase (MnSOD) was overexpressed in human gingival fibroblasts (HGFs) by transfecting pcDNA3.0-MnSOD (tMnSOD) (a). Inhibitory effect of Mito-TEMPO or tMnSOD on the lipopolysaccharides (LPS)-stimulated augmentation of mtROS generation. HGFs were stimulated with LPS for 9 h in the absence or presence of the indicated concentration of Mito-TEMPO, or with the overexpression of MnSOD, and then incubated with a mitochondrial reactive oxygen species (mtROS) indicator, MitoSOX. Then, mtROS levels were analyzed by immunofluorescence microscopy (b, c) or using a multimode microplate reader (d). Data are presented as the mean ± standard deviation (SD) ($n=3$). $^{*}p<0.05$ (treated group compared to control group); $^{\#}p<0.05$ (blocked group compared to treated group).

example, ROS generated in the periodontal tissues upon stimulation by LPS from *Porphyromonas gingivalis* (*P. gingivalis*) contributed to the pathogenesis of periodontal disease by activating matrix-degrading metalloproteinases and up-regulating pro-inflammatory cytokines [8]. In addition, ROS modified pro-inflammatory gene expression by altering kinase cascades and activating transcription factors, including mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB [9–11].

Although mitochondria serve as the major intracellular source of ROS in most cells, few studies have examined the contribution of mitochondrial (mt)ROS to regulating the production of pro-inflammatory cytokines in HGFs stimulated by LPS. Recent publications have indicated that mtROS act as signaling molecules to trigger pro-inflammatory cytokines production [12–15]. In addition, Park et al. reported that the mitochondrion was the major source of LPS-stimulated ROS in microglial cells. Furthermore, they found that inhibiting ROS generation modulated the production of pro-inflammatory mediators by preventing LPS-induced MAPK and NF-κB activation [15].

These observations provide much-needed clarification regarding the cellular source of ROS that induces the production of certain pro-inflammatory cytokines. Based on these findings, it is possible that the suppression of mtROS might alleviate inflammation [15]. However, the role of mtROS production in HGFs has yet to be fully elucidated. Thus, in this study, we determined whether mtROS were associated with the generation of pro-

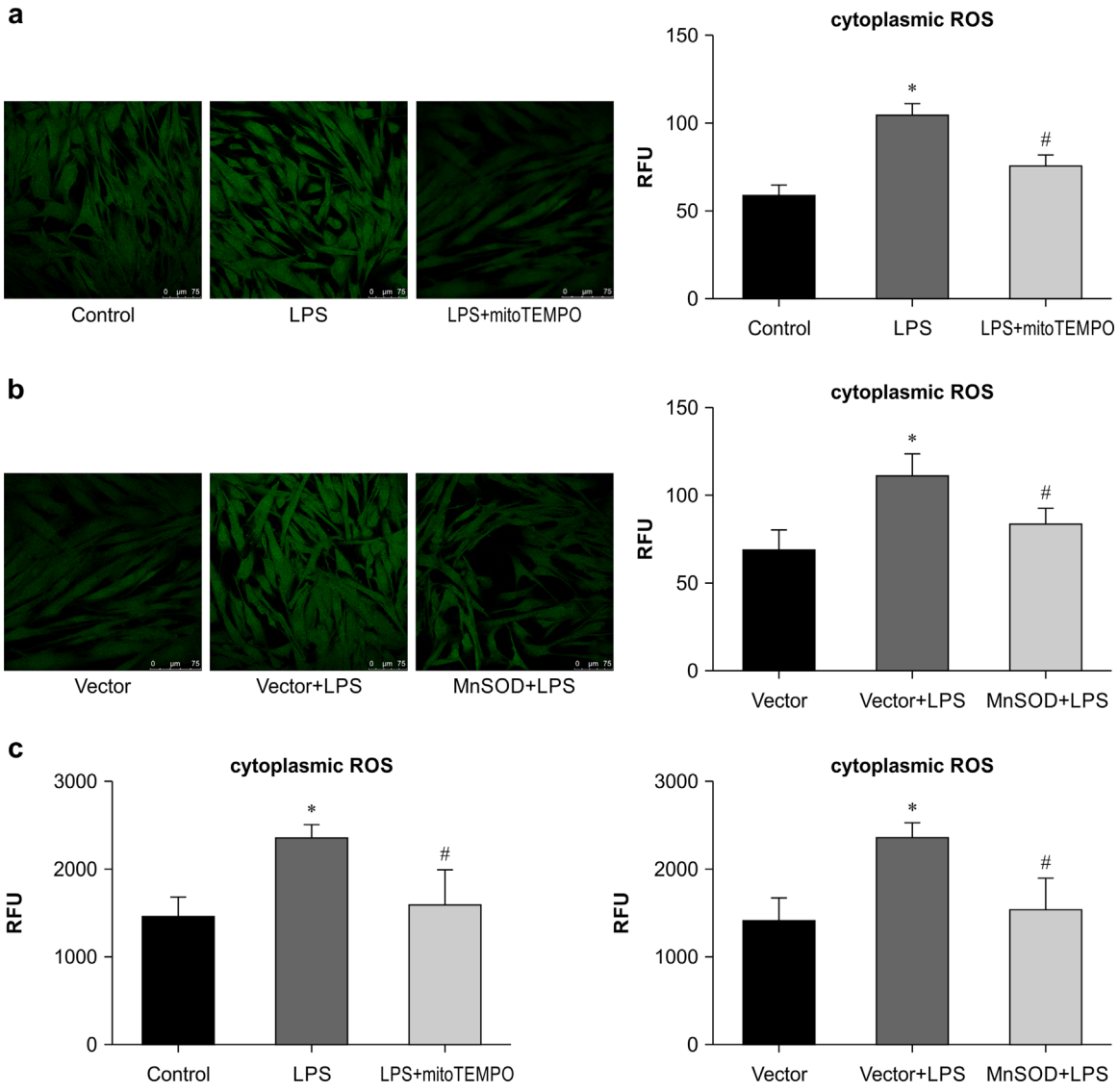

Fig. 2. Inhibitory effect of Mito-TEMPO or tMnSOD on the LPS-stimulated augmentation of cytoplasmic reactive oxygen species (ROS) generation. HGFs were stimulated with LPS for 9 h in the absence or presence of the indicated concentration of Mito-TEMPO, or with the overexpression of MnSOD, and then incubated with a cytoplasmic ROS indicator, H_2DCHFDA, after which cytoplasmic ROS levels were analyzed by immunofluorescence microscopy (a, b) or using a multimode microplate reader (c). Data are presented as the mean ± SD ($n=3$). $^{*}p < 0.05$ (treated group compared to control group); $^{\#}p < 0.05$ (blocked group compared to treated group).

inflammatory cytokines in HGFs stimulated with LPS. Moreover, we characterized the LPS-stimulated activation of MAPK and NF-κB in HGFs, and investigated whether these activities are altered by the inhibition of mtROS generation.

2. Materials and methods

This study was approved by the Review Board and Ethics Committee of Peking University Health Science Center. Written informed consent to use teeth was obtained from all subjects.

2.1. Cell culture, treatments, and transfection

After receiving the approval of the Ethics Committee (PKUSSIRB-2013017), HGFs were obtained from explants of human normal gingival tissues during crown lengthening surgery as described previously [16]. HGFs from 4 donors, who had no gingival or periodontal diseases, had no history or current signs of systemic diseases, and had received no medication within the previous 6 months were used. The HGFs were cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum, and 100 U/mL penicillin with 100 μg/mL streptomycin. HGFs were used between passages 3–8. The cells were treated with 1 μg/mL LPS derived from *P. gingivalis* (InvivoGen, Cayla, France; ATCC® 33,277) in the presence or absence of the mitochondrial-targeted antioxidant mito-TEMPO (50 μmol/L; Sigma-Aldrich, St. Louis, MO), or after transfection with the endogenous mitochondrial antioxidant enzyme manganese superoxide dismutase (tMnSOD). The following inhibitors were used: SB203580, a p38 inhibitor; SP600125, a c-Jun N-terminal kinase (JNK) inhibitor; and pyrrolidine dithiocarbamate (PDTC), an NF-κB inhibitor (Calbiochem, Darmstadt, Germany). HGFs were seeded onto 6-well plates the day before transfection to ensure 80% confluence at the time of transfection. Transfection with either empty vector (pcDNA3.0) or an MnSOD expression vector (pcDNA3.0-MnSOD) was performed using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. At the indicated time points, cells and culture supernatants were harvested and prepared for subsequent

experiments.

2.2. Measurement of mtROS production and cytoplasmic ROS levels

The production of mtROS in HGFs was assessed using MitoSOX™ Red (Invitrogen). MitoSOX Red is a novel fluorescent dye that was developed and validated for the highly selective detection of superoxide in the mitochondria of living cells. MitoSOX Red is live cell-permeant, and rapidly and selectively targets to mitochondria. Once in mitochondria, MitoSOX Red is oxidized by superoxide and exhibits red fluorescence (excitation at 510 nm and emission at 580 nm). Cytoplasmic ROS levels in HGFs were assessed by the fluorescence indicator 5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (H_2DCFDA, Molecular Probes, Eugene, OR). H_2DCFDA is de-esterified within cells releasing DCF which can be oxidized to the fluorescent dichlorofluorescein (excitation at 488 nm and emission at 525 nm).

2.3. Fluorescence detection

HGFs grown in confocal dishes were incubated with 5 μM MitoSOX Red for 30 min at 37 °C and washed twice in phosphate-buffered saline (PBS). Dishes were analyzed by immunofluorescence microscopy (TCS-SP82; Leica, Wetzlar, Germany). Fluorescence was also measured using a multimodal microplate reader (BioTek, Winooski, VT). HGFs were trypsinized and washed with cold PBS. The cells (1.8×10^5) were resuspended in 1 mL DMEM containing 5 μM MitoSOX Red and incubated in the dark in a CO_2 incubator for 30 min. The cells were centrifuged at $130 \times g$ for 5 min at room temperature, washed 3 times with PBS, and resuspended in 500 μL PBS. The mtROS content of cells was analyzed based on measurement of the fluorescence intensity of MitoSOX Red. The cytoplasmic ROS content of cells was analyzed based on measurement of the fluorescence intensity of H_2DCHFDA.

2.4. Cytokine measurements

HGFs were cultured in 96-well plates (1×10^4 per well). LPS was added in the presence or absence of mito-TEMPO or tMnSOD. The levels of IL-1β, IL-6, and TNF-α in cell culture supernatants were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.5. RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

HGFs were cultured in 6-well plates (1×10^5 cells per well). LPS was added in the presence or absence of mito-TEMPO or tMnSOD. The mRNA levels of IL-1β, IL-6, and TNF-α were measured according to the manufacturer's instructions. Total RNA was isolated using TRIzol® (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using Reverse Transcription Premix (Bioneer, Seoul, South Korea). qRT-PCR was performed using gene-specific primers and PCR premix (Kapa Biosystems, Wilmington, MA). The following primers were used for PCR amplification: 5′-*TNFA*, 5′-GCTCAGACATGTTTTCCGTGAA-3′; 3′-*TNFA*, ′ 3-GTCACCAAATCAGCATTGTTTAGA-5′;.

5′-*IL1B*, 5′-CTTCAGCCAATCTTCATTGCT-3′; 3′-*IL1B*,

3′-TCGGAGATTCGTAGCTGGAT-5′; 5′-*IL6*, 5′-GAGGGCTCTTCGGCAAATGTA-3′; 3′-*IL6*,

3′-CCCAGTGGACAGGTTTCTGAC-5′; 5′-*ACTB*, 5′-AGCACAATGAAGATCAAGATCAT-3′; and 3′-*ACTB*, 5′-ACTCGTCATACTCCTGCTTGC-3′. The PCR conditions were 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. All reactions were carried out in triplicate in 3 separate experiments. The relative expression of the targets in each sample was calculated using the comparative $2^{-\Delta\Delta Ct}$ method after normalization to the expression of *ACTB*.

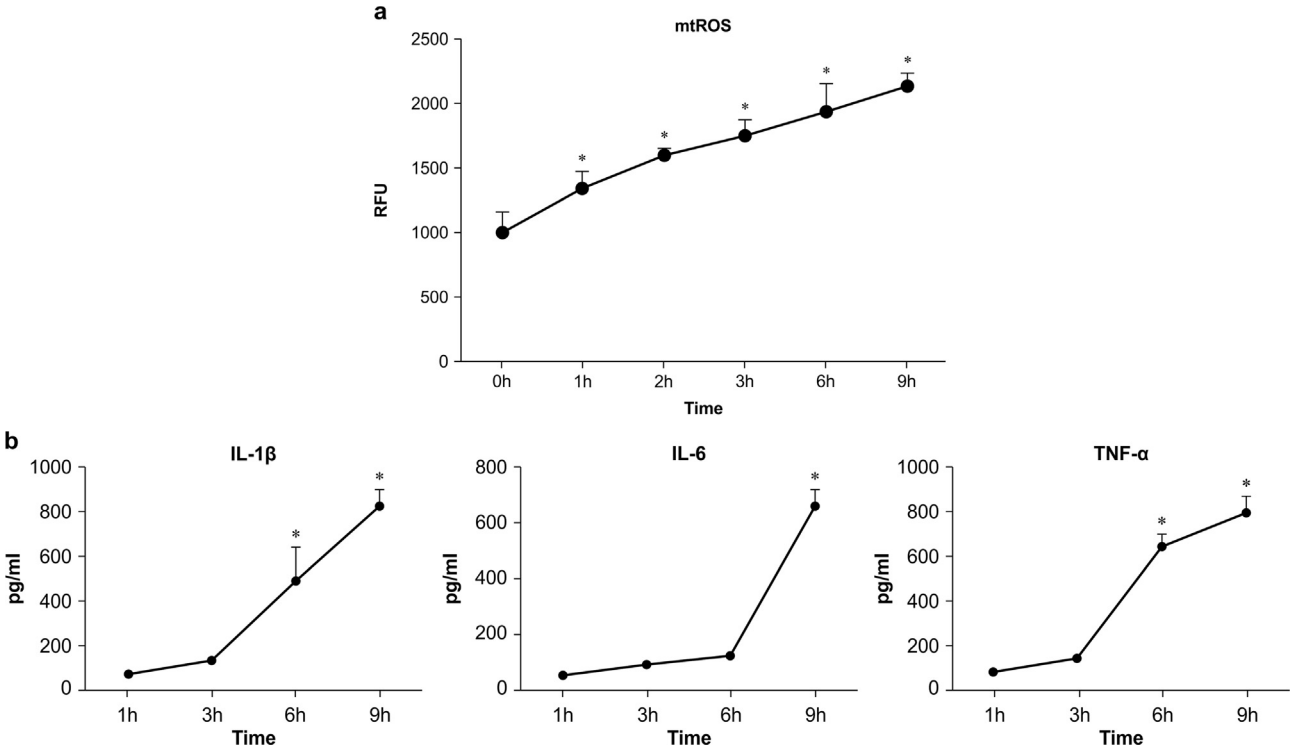

Fig. 3. Augmentation of mtROS generation by LPS occurred earlier than the production of pro-inflammatory cytokines in HGFs stimulated by LPS. The level of mtROS produced by HGFs was measured by microplate reader in cells treated with LPS for 1, 2, 3, 6, or 9 h (a). The abundances of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α were measured by enzyme-linked immunosorbent assay (ELISA) in HGFs treated with LPS for 1, 3, 6, or 9 h (b). Data are presented as the mean ± SD ($n=3$). $^*p < 0.05$ (treated group compared to control group); $^{\#}p < 0.05$ (blocked group compared to treated group).

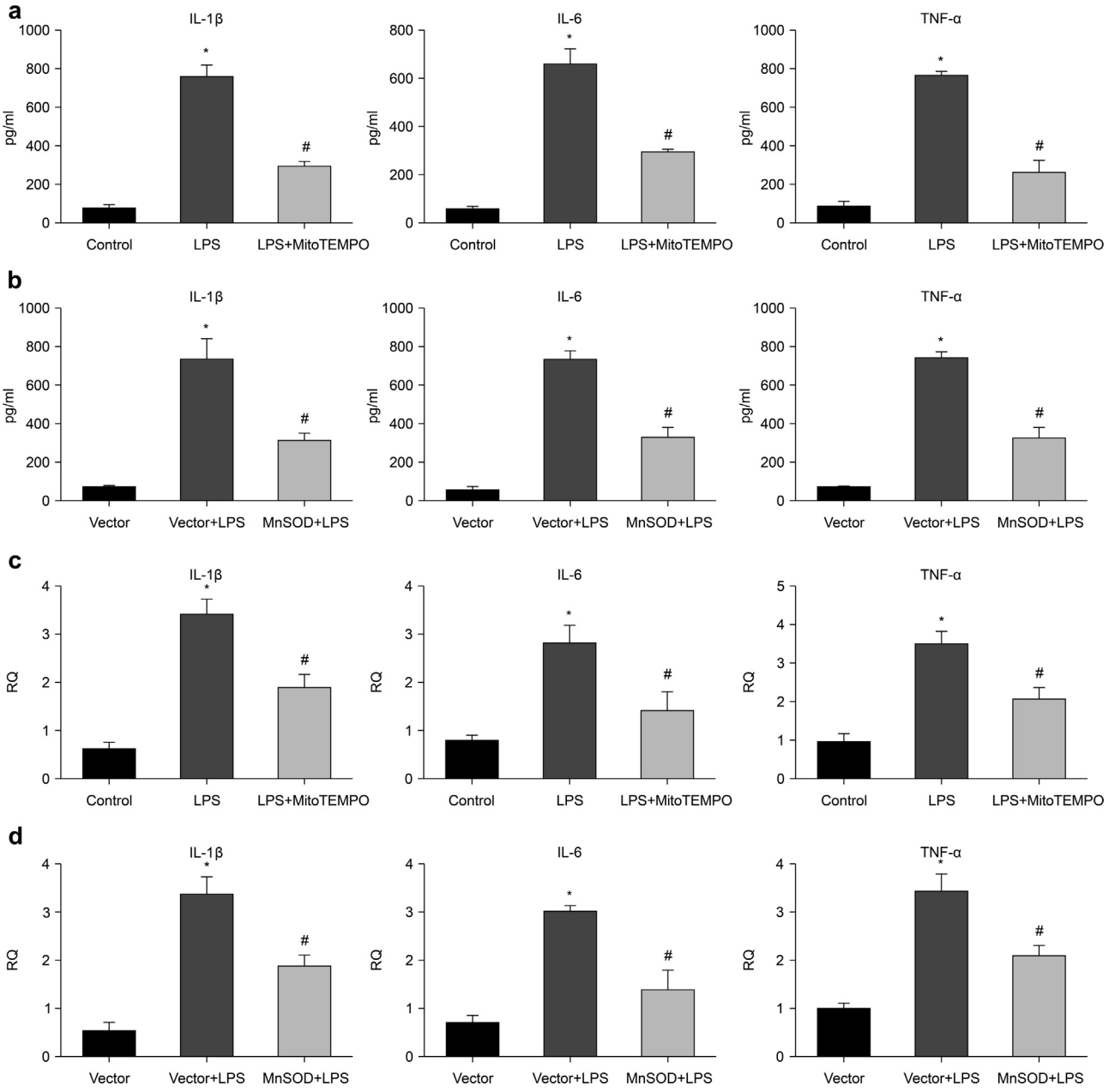

Fig. 4. Inhibitory effect of Mito-TEMPO or tMnSOD on the LPS-stimulated induction of pro-inflammatory cytokines. Protein levels of IL-1β, IL-6, and TNF-α in HGFs were established by ELISA (a, b). mRNA levels of IL-1β, IL-6, and TNF-α in HGFs were established by quantitative reverse transcription polymerase chain reaction (qRT-PCR) (c, d). Data are presented as the mean ± SD ($n=3$). $^{*}p<0.05$ (treated group compared to control group); $^{\#}p<0.05$ (blocked group compared to treated group).

2.6. Western blots

Whole protein lysates were prepared using PRO-PREP™ protein extraction solution (iNtRON Biotechnology, Seongnam, Korea). The nuclear and cytoplasmic fractions were isolated using an NE-PER® nuclear and cytoplasmic extraction reagent kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. Equal amounts of protein were separated by electrophoresis on 10–12% (v/v) sodium dodecyl sulfate–polyacrylamide gels and transferred onto nitrocellulose membranes (BD Biosciences, Franklin Lakes, NJ). The membranes were incubated overnight at 4 °C with anti-JNK, anti-p-JNK, anti-p38, anti-p-p38, and anti-GAPDH primary antibodies.

2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (5 mg) from HGFs were prepared using a Nuclear-Cytosol Extraction kit (Thermo Fisher Scientific) and subjected to EMSA. A horseradish peroxidase-labeled oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was synthesized to detect binding of the NF-κB protein to the probe. After incubation of the nuclear extracts and the probe, the binding activity of the NF-κB protein to DNA was analyzed using a LightShift™ Chemiluminescent EMSA kit (Pierce, Rockford, IL). The assay was conducted 3 times independently.

2.8. Data analysis and statistics

Data represent the means and standard deviation of 3 independent experiments. Differences in mean values among groups

were tested for statistical significance by one-way ANOVA using Prism 6 software (GraphPad, La Jolla, CA). A p value of < 0.05 was deemed to be statistically significant.

3. Results

3.1. LPS increases mtROS generation, while mito-TEMPO or tMnSOD reduces the level of LPS-induced mtROS in HGFs

LPS markedly elevated the concentration of mtROS. The elevated mtROS level was effectively reduced by mito-TEMPO ($p < 0.05$) or tMnSOD ($p < 0.05$) (Fig. 1). These results showed that mito-TEMPO and tMnSOD could both significantly reduce the LPS-induced increase in the concentration of mtROS.

3.2. LPS increases cytoplasmic ROS generation, while mito-TEMPO or tMnSOD reduce the level of LPS-induced cytoplasmic ROS in HGFs

LPS markedly elevated the concentration of cytoplasmic ROS. The elevated cytoplasmic ROS level induced by LPS was reduced by mito-TEMPO ($p < 0.05$) or tMnSOD ($p < 0.05$) (Fig. 2). These results showed that mito-TEMPO and tMnSOD could reduce the LPS-induced concentrations of cytoplasmic ROS significantly.

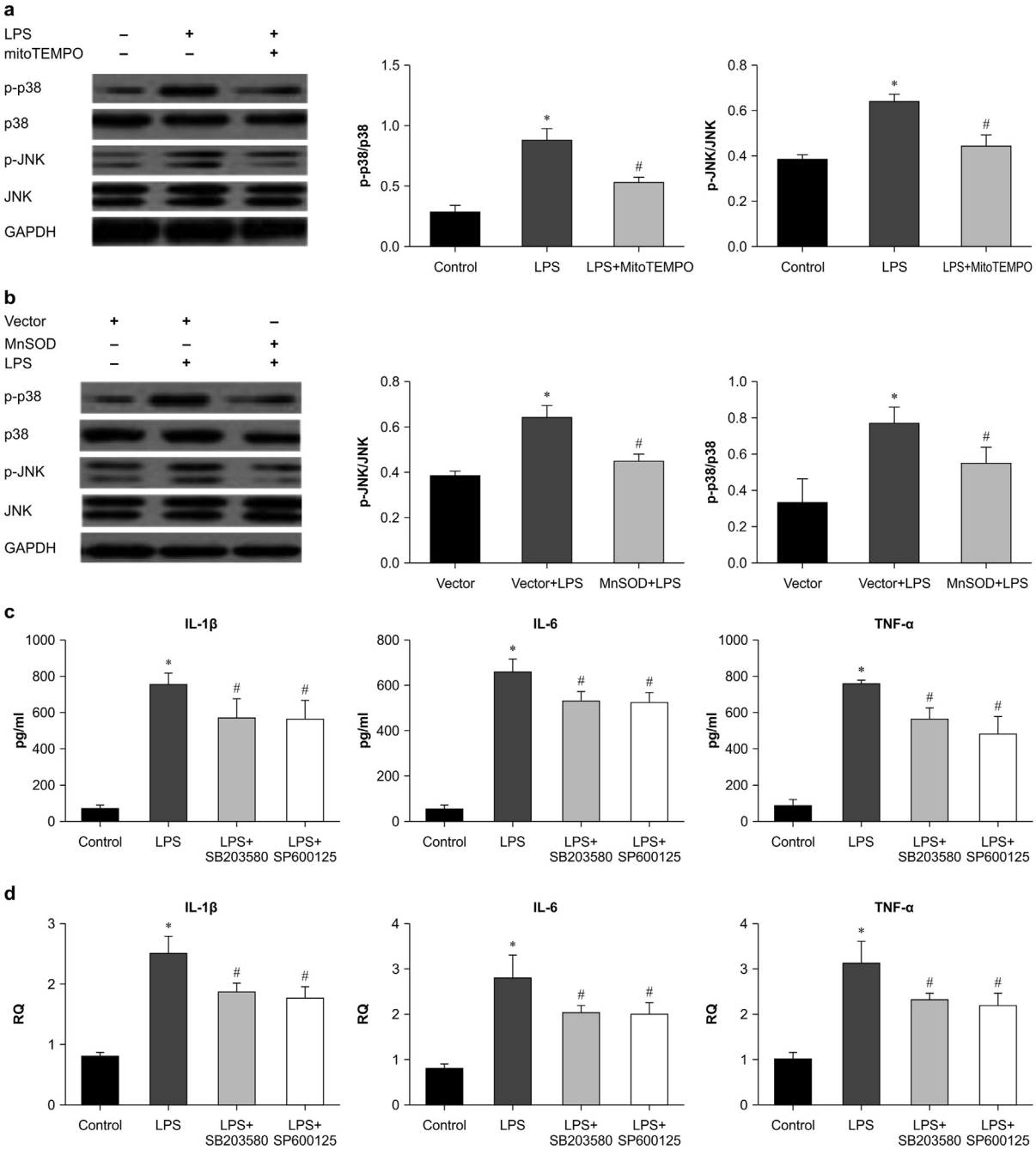

Fig. 5. Effect of Mito-TEMPO or tMnSOD on LPS-induced mitogen-activated protein kinases (MAPKs) activation. HGFs were treated with LPS for 9 h in the presence or absence of Mito-TEMPO or tMnSOD. Western blot analysis was performed using antibodies against p-p38 or p-JNK. Antibodies against p38, JNK, and GAPDH were used to generate reference values for the normalization of the phosphorylated protein levels (a, b). Treatment of the cells with p38 inhibitor and JNK inhibitor suppressed the synthesis of pro-inflammation cytokines, as determined by ELISA (c). p38 inhibitor and JNK inhibitor suppressed the transcription of pro-inflammatory cytokines, as shown by qRT-PCR (d). Data are presented as the mean ± SD ($n=3$). $^{*}p < 0.05$ (treated group compared to control group); $^{\#}p < 0.05$ (blocked group compared to treated group).

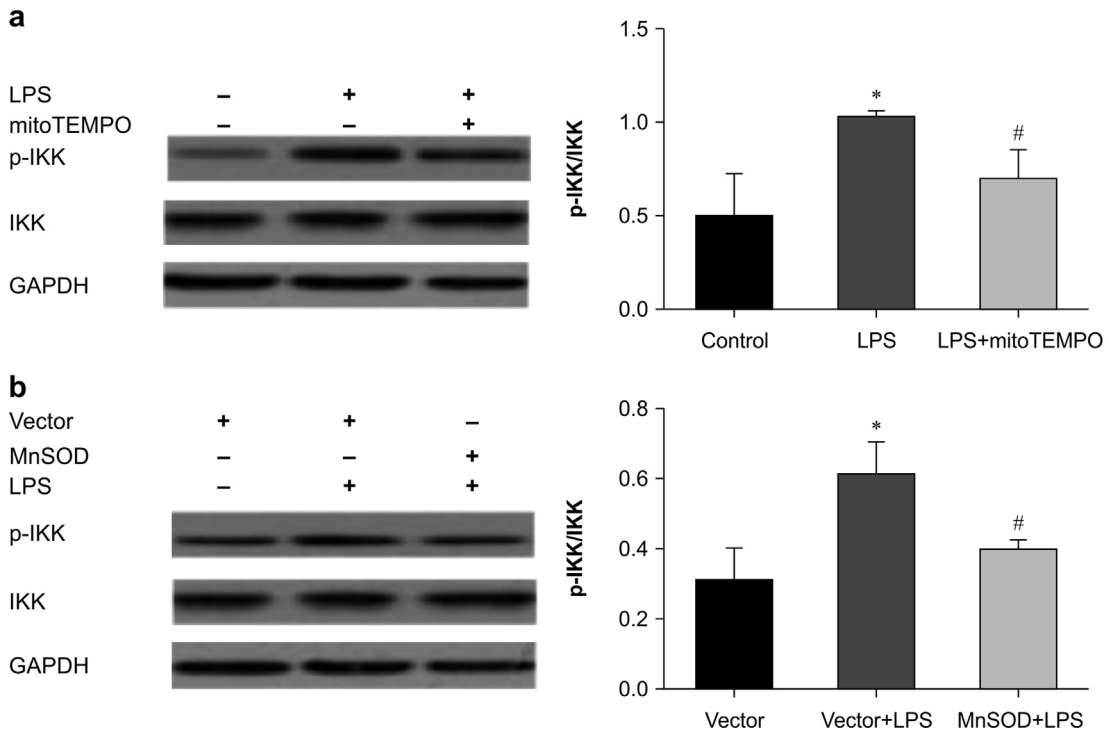

Fig. 6. Effect of Mito-TEMPO or tMnSOD on LPS-induced inhibitor of nuclear factor (NF)-κB kinase (IKK) activation. HGFs were treated with LPS for 9 h in the presence or absence of Mito-TEMPO, or with the overexpression of MnSOD. Western blot analysis was performed using antibodies against p-IKK, IKK, and GAPDH as control (a, b). Data are presented as the mean ± SD ($n=3$). $^{*}p<0.05$ (treated group compared to control group); $^{\#}p<0.05$ (blocked group compared to treated group).

3.3. Augmentation of mtROS generation by LPS occurred earlier than the production of pro-inflammatory cytokines in HGFs stimulated by LPS

ELISA experiments demonstrated that LPS stimulated secretion of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α, as well as the generation of mtROS, in a time-dependent manner (Fig. 3). Upon LPS stimulation, the generation of mtROS was induced prior to the production of pro-inflammatory cytokines in HGFs.

3.4. Inhibition of mtROS decreases the production of pro-inflammatory cytokines in HGFs stimulated by LPS

Using ELISA and qRT-PCR, we showed that mtROS were involved in the increased protein and mRNA expression of pro-inflammatory cytokines in HGFs following LPS challenge. Interestingly, our results showed that the up-regulated protein and mRNA expression of IL-1β, IL-6, and TNF-α were decreased by pre-treatment with mito-TEMPO ($p<0.05$) or tMnSOD ($p<0.05$) (Fig. 4). Consequently, our data suggest that mtROS contribute to regulating the production of pro-inflammatory mediators by HGFs challenged with LPS.

3.5. mtROS mediate the LPS-induced pro-inflammatory response of HGFs via regulating MAPK signaling

To investigate the mechanism by which mtROS influence inflammation, we focused on the MAPK signaling pathway. Inhibition of mtROS by pre-treatment with mito-TEMPO or tMnSOD dramatically reduced the LPS-induced activation of p38 and JNK (Fig. 5a, b), but not the activation of extracellular-regulated kinase (ERK) (data not shown). To further explore the roles of p38 and JNK in the production of pro-inflammatory cytokines in HGFs, we used an inhibitor of p38 (SB203580) and an inhibitor of JNK (SP600125). The results of ELISA and qRT-PCR showed that both inhibitors decreased the production of IL-1β, IL-6, and TNF-α (Fig. 5c, d). Taken together, our data indicate that the inhibition of LPS-induced mtROS production suppresses the activation of HGFs by modulating p38 and JNK signaling.

3.6. mtROS control the LPS-induced pro-inflammatory response of HGFs through regulating inhibitor of nuclear factor-κB kinase (IKK)

Stimulation of NF-κB signaling leads to the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α [8]. We next determined whether LPS-induced mtROS were involved in the activation of IKK, which positively regulates NF-κB. Our results demonstrated that the LPS-stimulated activation of IKK was reduced significantly by inhibiting LPS-induced mtROS generation (Fig. 6). These results suggest that LPS-induced mtROS production promotes the pro-inflammatory response in HGFs by regulating the activation of IKK.

3.7. mtROS modulate the activation of NF-κB in HGFs stimulated by LPS

Analysis by EMSA demonstrated that the DNA-binding activity of NF-κB was reduced significantly by inhibiting LPS-induced mtROS generation (Fig. 7a). To further explore the role of NF-κB in the production of pro-inflammatory cytokines, we used the NF-κB inhibitor, PDTC. ELISA and qRT-PCR demonstrated that PDTC inhibited the production of pro-inflammatory cytokines (Fig. 7b, c). Taken together, our data show that the inhibition of LPS-induced mtROS production suppresses the inflammatory response of HGFs by inhibiting the NF-κB signaling pathway.

3.8. The p38 and JNK signaling pathways are involved in the activation of NF-κB as a result of mtROS production

Through analysis of the activity of NF-κB by EMSA, we demonstrated that inhibitors of p38 and JNK partially prevented the activation of NF-κB signaling by LPS. These results show that p38

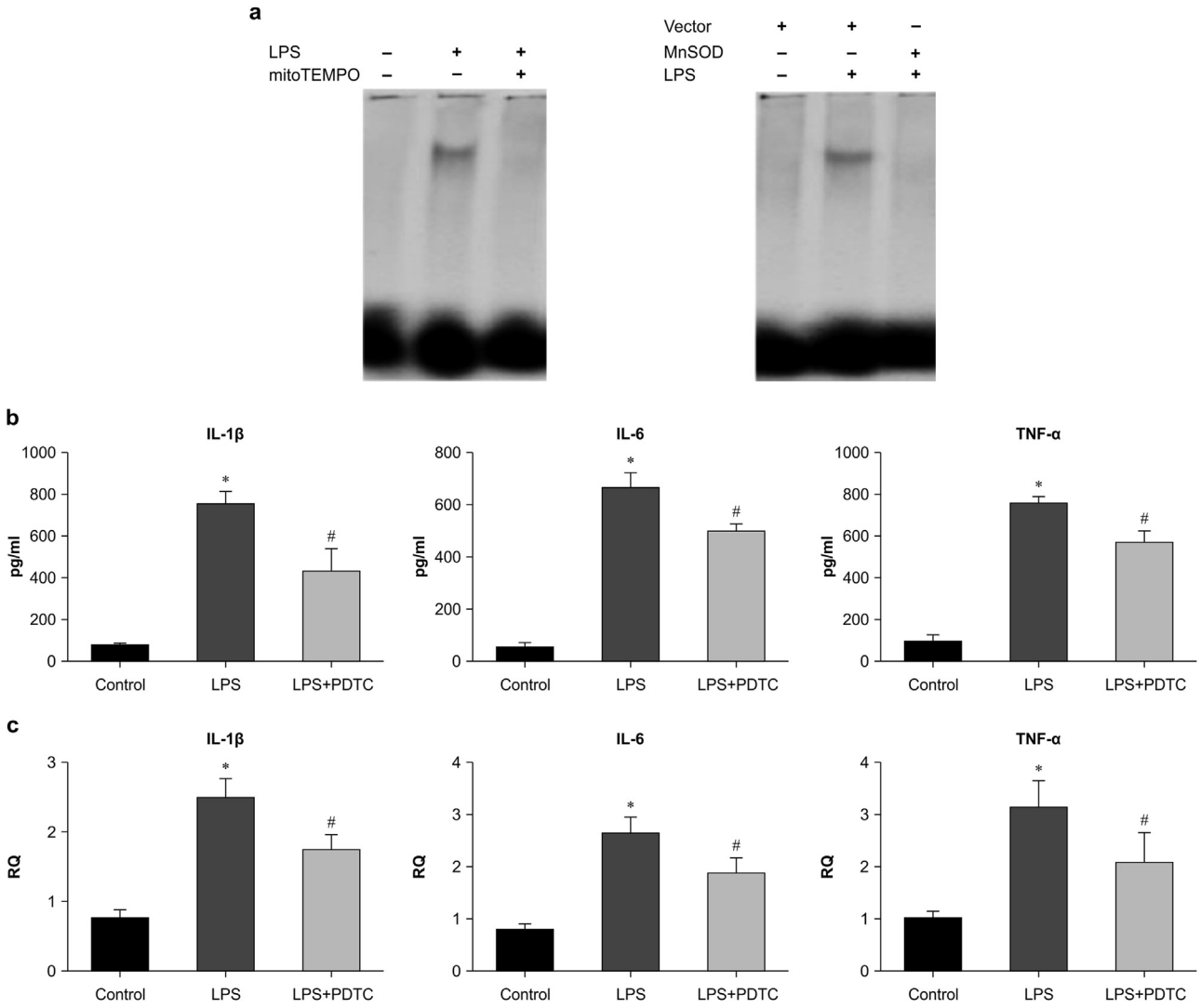

Fig. 7. The activity of nuclear factor (NF)-κB signaling was assessed by electrophoretic mobility shift assay (EMSA). NF-κB signaling was activated by LPS, and the signaling activity was lower when HGFs were treated with Mito-TEMPO or tMnSOD (a). Protein expression levels of IL-1β, IL-6, and TNF-α in HGFs treated with LPS for 9 h in the presence or absence of an inhibitor of NF-κB, PDTC (b). mRNA expression levels of IL-1β, IL-6, and TNF-α in HGFs treated with LPS for 9 h in the presence or absence of PDTC (c). Data are presented as the mean ± SD ($n=3$). $^{*}p<0.05$ (treated group compared to control group); $^{\#}p<0.05$ (blocked group compared to treated group).

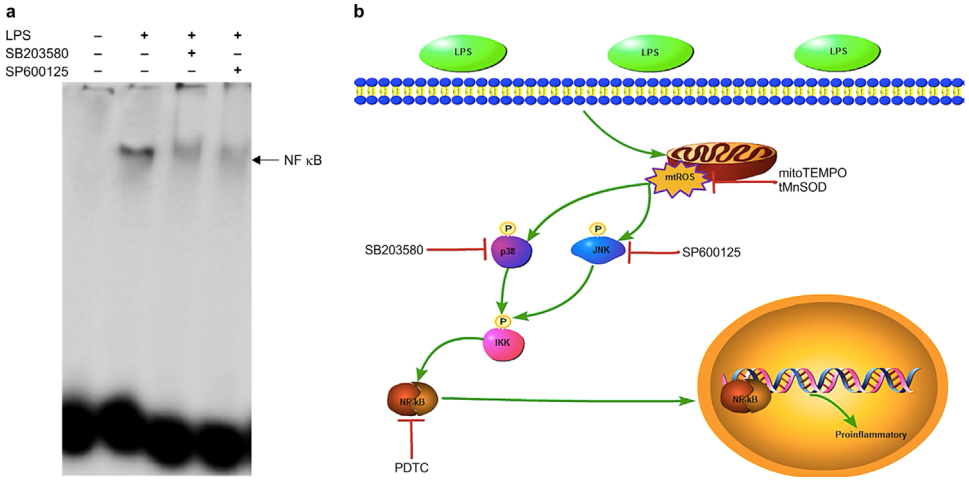

Fig. 8. The activity of NF-κB signaling was assessed by EMSA. Inhibitors of p38 and JNK partially prevented the activity of NF-κB signaling (a). Therefore, p38 and JNK are upstream of NF-κB in the signaling pathway. Data are presented as the mean ± SD ($n=3$). $^{*}p<0.05$ (treated group compared to control group); $^{\#}p<0.05$ (blocked group compared to treated group). Schematic diagram of the signaling pathways involved in mtROS-induced IL-1β, IL-6, and TNF-α production in gingival fibroblasts (b).

and JNK act upstream of NF-κB in the LPS-response pathway in HGFs. The proposed signaling events are detailed in Fig. 8.

4. Discussion

ROS are produced in cells following stimulation by numerous physiological and environmental stresses including infection, ultraviolet radiation, and pollutants. In addition, ROS have been implicated in the pathogenesis of inflammatory diseases including rheumatoid arthritis [17], multiple sclerosis [18], and thyroiditis [19]. Periodontal disease is no exception to the potential influence of ROS.

The respiratory chain in mitochondria is a primary source of ROS production [20], and mitochondria are the major source of ROS in most cell types. Therefore, we investigated the role of mtROS in regulating the production of pro-inflammatory cytokines in HGFs following LPS challenge, a pathway that had not been fully elucidated. A key finding was that the concentration of mtROS increased after LPS challenge but before IL-1β, IL-6, and TNF-α secretion, suggesting that the elevation of mtROS led to the induction of periodontal inflammation. The induction of pro-inflammatory cytokines by LPS in HGFs is well-established. However, the induction of mtROS in HGFs by LPS has not been reported previously.

ROS are considered to be generated as by-products of energy production, and the induction of mtROS by LPS is dependent on the normal structure and function of mitochondria [21]. Moreover, recent evidence has suggested that the mitochondrial production of ROS is a tightly controlled process [22]. Toll-like receptor (TLR) signaling was reported to enhance mtROS production in murine macrophages stimulated with LPS, which is a TLR4 agonist [22]. With respect to mtROS production, the results of our study are in accordance with previous reports in other cell types.

In our study, we used 2 antioxidants, mito-TEMPO (an exogenous small molecule) and tMnSOD (an endogenous enzyme), both of which suppressed enhanced mtROS and cytoplasmic ROS production in LPS-stimulated HGFs. Therefore, mtROS are the major source of cytoplasmic ROS. Then, we demonstrated that the suppression of mtROS generation reduced LPS-induced cytokine production. When the increase of mtROS was inhibited, the production of pro-inflammatory cytokines in response to LPS was blunted.

To confirm the importance of mtROS as mediators of the pro-inflammatory response in LPS-treated HGFs, we assessed the mRNA and protein expression of IL-1β, IL-6, and TNF-α. We found that the transcription and secretion of these cytokines were markedly suppressed by pre-treatment with mito-TEMPO or overexpression of MnSOD.

MAPKs are important downstream signal transducers of ROS, and their signaling is critical in modulating the expression of pro-inflammatory cytokines [23]. We found that blocking the elevation of mtROS following LPS challenge also suppressed LPS-induced p38 and JNK signaling, implicating this pathway as a link between mtROS and the inflammatory response.

ROS overproduction can lead to cellular stress, at least partly via activating the NF-κB signaling pathway [24]. ROS are thought to also regulate the activation of HGFs through MAPKs signaling independently of NF-κB. Here, we confirmed that NF-κB signaling is induced by mtROS production, since blocking the elevation of mtROS using antioxidants reversed the LPS-induced activation of IKK and NF-κB.

A recent report demonstrated that the activation of inflammasomes by NLRP3, which is involved in IL-1β secretion, was regulated by mtROS in macrophages [25,26]. The results of our study confirmed that mitochondria were the major source of LPS-stimulated ROS generation in HGFs. In addition, the results showed that inhibiting mtROS production suppressed the production of pro-inflammatory mediators by preventing the LPS-induced activation of MAPKs and NF-κB in HGFs. These findings indicate that the targeted suppression of ROS generation in mitochondria could be a useful tool for investigating the effects of preventing the inflammatory response in LPS-induced HGFs.

ROS in mitochondria are always modulated by mitochondrial dysfunction. However, in the current study, we did not examine mitochondrial function. Future studies should elucidate the influence of LPS on the function of mitochondria and assess the benefits of preserving mitochondrial function with mitoTEMPO or tMnSOD.

Mitochondrial-targeted drugs enable the manipulation of specific aspects of cellular metabolism. Nevertheless, mitoTEMPO or tMnSOD did not block the inflammatory response completely, and we hypothesize that other pathways participate in the pro-inflammatory response of HGFs to LPS challenge. The mitochondria are the promising intracellular targets for drug delivery, because damage to these organelles contributes to periodontitis [27–29]. Delivering drugs to mitochondria to limit such damage may therefore improve the function of mitochondria and delay the progression of periodontitis. Further exploration of these processes may lead to the development of targeted antioxidants as adjuvant therapies for periodontitis.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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