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Telmisartan, an angiotensin II receptor blocker, attenuates *Prevotella intermedia* lipopolysaccharide-induced production of nitric oxide and interleukin-1β in murine macrophages

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ABSTRACT

Telmisartan, widely prescribed for the treatment of hypertension, has an anti-inflammatory property in addition to being an angiotensin II type 1 receptor antagonist. This study was carried out to explore the influence of telmisartan upon the elaboration of inflammatory mediators in murine macrophages stimulated with lipopolysaccharide (LPS) prepared from *Prevotella intermedia*, a periodontal pathogen, as well as its molecular mechanisms. Telmisartan significantly inhibited LPS-induced generation of inducible nitric oxide (NO) synthase-derived NO and interleukin-1β (IL-1β) as well as their gene expressions in RAW264.7 cells. Telmisartan treatment of LPS-activated cells significantly up-regulated arginase 1 (Arg-1) and chitinase-like 3 (Ym-1), which are specific markers of M2 macrophages. Telmisartan caused a significant increase in heme oxygenase-1 (HO-1) expression in cells stimulated with LPS, and its inhibitory action against NO production was reversed by treatment with SnPP, an HO-1 inhibitor. Phosphorylation of STAT1 and STAT3 induced by LPS was attenuated by telmisartan. Telmisartan inhibited LPS-induced generation of NO and IL-1β independently of PPAR-γ activation. In addition, activation of NF-κB as well as JNK and p38 signaling induced by LPS was not modulated by telmisartan. In summary, telmisartan is a potent inhibitor of *P. intermedia* LPS-induced generation of NO and IL-1β in RAW264.7 cells and promotes macrophage phenotype switching toward the M2 phenotype. Telmisartan may have potential to be developed into host modulatory agent for inflammatory periodontal disease, although additional studies are needed to confirm the therapeutic effect.

1. Introduction

Periodontal disease, which is a highly prevalent inflammatory condition caused by certain groups of bacterial species that present in subgingival bacterial biofilm, leads to breakdown of tissues supporting the tooth and is a major cause of tooth loss in adults [1]. Accumulating scientific evidence strongly suggests that the periodontal disease may predispose individuals to a number of other diseases such as heart diseases, diabetes, stroke and adverse pregnancy outcomes [2].

Prevotella intermedia is a pathogenic bacterium associated with various forms of periodontal disease [3–5]. Besides, this organism is also known to be implicated in endodontic infections [6] and systemic conditions such as cystic fibrosis [7], chronic bronchitis [8] and atherosclerosis [9]. *P. intermedia* has been reported to possess a number of virulence components that may be significant in the pathogenicity of this bacterium [10–12].

Bacterial lipopolysaccharide (LPS) is a major virulence factor found in the outer membrane of gram-negative microbes. LPS activates macrophages and mononuclear cells, leading to liberation of various proinflammatory mediators [13]. We have reported that *P. intermedia* LPS induces a significantly higher generation of nitric oxide (NO) and specific proinflammatory cytokines in macrophages [14–17]. *P. intermedia* LPS inhibited osteogenesis and caused the liberation of osteolytic mediators from murine osteoblasts as well [18].

Excess generation of proinflammatory mediators occurs in periodontal disease [19–21], and evidence from studies suggests that these

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soluble mediators are central in driving the tissue breakdown processes seen in periodontal disease [18,22–25]. Thus, modulation of these destructive mediators is considered as one of therapeutic strategies against periodontal disease [26,27].

Telmisartan is an angiotensin II receptor blocker (ARB) widely prescribed for the treatment of hypertension [28]. It was reported that telmisartan has an anti-inflammatory property in addition to being an angiotensin II type 1 receptor (AT_1R) antagonist [29–33]. In addition, recent studies in animal models showed that this ARB significantly attenuated the inflammatory response and bone loss associated with periodontitis [34,35]. However, informations are very limited with respect to possible mechanistic basis of telmisartan upon the progress of periodontal disease.

Macrophages can be polarized either to classically activated M1 phenotype or to an alternatively activated M2 phenotype depending on the local tissue microenvironment [36,37]. M1 macrophages are mainly induced by proinflammatory stimuli including LPS and secret high levels of various proinflammatory mediators like iNOS-derived NO and IL-1β, thereby promoting inflammation and tissue damage, whereas M2 phenotypes secrete anti-inflammatory cytokines and facilitate tissue repair [38,39]. The well-characterized RAW264.7 murine macrophage cell line has frequently been used to study the compounds for potential anti-inflammatory effects [40–42]. This study was carried out to explore the influence of telmisartan upon the elaboration of inflammatory mediators in RAW264.7 macrophage cells activated with LPS prepared from *P. intermedia*, a periodontal pathogen, as well as its molecular mechanisms.

2. Materials and methods

2.1. Reagents and antibodies

We purchased telmisartan from Tokyo Chemical Industry Co. (Tokyo, Japan). Such antibodies against iNOS, HO-1 and β-actin were the products of Santa Cruz Biotechnology (Santa Cruz, CA, USA), while other antibodies utilized were all purchased from Cell Signaling Technology (Beverly, MA, USA). Ciglitazone, pioglitazone and GW9662 were the products of Cayman Chemical (Ann Arbor, MI, USA). Tin protoporphyrin IX (SnPP) (Frontier Scientific, Logan, UT, USA) and T0070907 (Selleckchem, Houston, TX, USA) were also utilized. Unless otherwise mentioned specifically, other reagents used in this work were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Isolation of LPS

P. intermedia ATCC strain 25611 was anaerobically grown at 37 °C in general anaerobic medium (GAM) broth (Nissui, Tokyo, Japan) supplemented with hemin (5 μg/ml) and menadione (1 μg/ml). LPS was prepared from harvested bacterial cells by hot phenol-water extraction procedure according to a previously published protocol [16].

2.3. Cell culture and viability analysis

RAW264.7 murine macrophages (ATCC TIB-71) were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultivated with Dulbecco's modified Eagle's medium (DMEM) as supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% heat-inactivated fetal bovine serum at 37 °C in an atmosphere containing 5% CO_2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out to assess the influence of telmisartan upon cell viability.

2.4. Determination of NO level

Nitrite (NO_2^-) level in culture supernatants was assessed as an indicator of NO generation using Griess reagent assay [43]. To put it briefly, cells were cultured with increasing doses of telmisartan (20, 40, 60 and 80 μM) for 24 h in the presence or absence of *P. intermedia* LPS, and a same volume (100 μl) of culture supernatant and Griess reagent were mixed. After 10 min, the absorbance readings at the wavelength of 540 nm were determined with a Spectra Max 250 ELISA Reader (Molecular Devices, Sunnyvale, CA, USA). Nitrite level was calculated using the calibration curve generated from standard solutions of sodium nitrite.

2.5. Determination of IL-1β level

Cells were cultured with indicated doses of telmisartan in the presence or absence of *P. intermedia* LPS for 48 h, and the IL-1β level in culture medium was measured by using an ELISA kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.6. RNA preparation and real-time polymerase chain reaction analysis

Cells were challenged with indicated doses of telmisartan in the presence or absence of *P. intermedia* LPS for the time periods indicated in figure legends. Then, real-time PCR analysis was carried out for the measurement of the target gene induction according to a previously published protocol [16]. In brief, we used an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) to isolate total RNA from cell cultures. Isolated RNA was reverse transcribed into cDNA with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), and cDNA was amplified utilizing an SsoFast EvaGreen Supermix (Bio-Rad) and CFX96 real-time PCR detection system (Bio-Rad). The PCR conditions were as follows: Following denaturing at 98 °C for 30 s, PCR was conducted for 45 cycles, each consisted of denaturing at 95 °C for 1 s, annealing/extending at 60 °C for 5 s. The primer sequences used are shown in Table 1. The induction level of each gene was normalized to that of β-actin.

2.7. Immunoblot analysis

Cells were challenged with indicated doses of telmisartan in the presence or absence of *P. intermedia* LPS for the time periods indicated in figure legends. After which, immunoblotting was carried out according to our previously published protocol [16]. In brief, whole-cell proteins were extracted from cells collected following incubation using lysis buffer. Thirty micrograms of the total proteins were loaded and electrophoresed on a 10% SDS-polyacrylamide gel and subsequently transferred electrophoretically onto a nitrocellulose sheet. The membranes were then probed with the appropriate first antibodies for 1 h at room temperature, followed by incubation with the corresponding secondary antibodies. After which, reactive proteins were visualized with enhanced chemiluminescence detection kit (Cell Signaling Technology).

Table 1
The sequences for the specific oligonucleotide primers utilized in this study.

Genes	Sequences	
iNOS	Forward	5′-GCACCACCCTCCTCGTTCAG-3′
	Reverse	5′-TCCACAACTCGCTCCAAGATTCC-3′
IL-1β	Forward	5′-TTCAGGCAGGCAGTATCA-3′
	Reverse	5′-AGGATGGGCTCTTCTTCAA-3′
HO-1	Forward	5′-CAATGTGGCCTTCTCTCTGT-3′
	Reverse	5′-TTTTGGTGAGGGAACTGTGT-3′
PPAR-γ	Forward	5′-GCTGACCCAATGGTTGCTGATTAC-3′
	Reverse	5′-ACAGACTCGGCACTCAATGGC-3′
Arg-1	Forward	5′-GAACACGGCAGTGGCTTTAAC-3′
	Reverse	5′-TGCTTAGTTCTGTCTGCTTTGC-3′
Ym-1	Forward	5′-CATGAGCAAGACTTGCGTGAC-3′
	Reverse	5′-GGTCCAAACTTCCATCCTCCA-3′
β-Actin	Forward	5′-TGAGAGGGAAATCGTGCGTGAC-3′
	Reverse	5′-GCTCGTTGCCAATAGTGATGACC-3′

2.8. Secretory alkaline phosphatase assay

RAW-Blue cells (Invivogen, San Diego, CA, USA), stably expressing a secreted embryonic alkaline phosphatase (SEAP) gene inducible by NF-κB, were cultivated in DMEM under the selection of Zeocin (Invivogen). Cells were challenged with indicated doses of telmisartan for 24 h in the presence or absence of *P. intermedia* LPS. Then, SEAP reporter assay was conducted following the manufacturer's instructions. In brief, aliquots of 50 μl of culture supernatant were incubated with 150 μl of SEAP detection medium (Quanti-Blue; Invivogen) in a 96-well culture plate for 30 min at 37 °C. Then, the SEAP levels were determined by assessing the absorbance readings at 630 nm with the microplate reader (Molecular Devices).

2.9. Statistical analysis

Results are reported as means ± S.D. One-way analysis of variance (ANOVA) was carried out to appraise the significant differences within the groups. The comparison between two groups was assessed by Tukey's *post-hoc* comparisons. A P value of < 0.05 was regarded as statistically significant.

3. Results

3.1. Telmisartan suppresses generation of NO and IL-1β evoked by P. intermedia LPS

LPS prepared from *P. intermedia* induced a significantly higher release of NO and IL-1β in comparison with vehicle-treated cells (Fig. 1A, B). Exposure of cells to telmisartan significantly decreased the accumulation of these LPS-induced inflammatory mediators, and the inhibitory effect was enhanced with the increase of dose (Fig. 1A, B). It was found that telmisartan decreased the amount of NO and IL-1β production by about 68% and 94%, respectively, at the highest dose of 80 μM. The cell viability was not inhibited by telmisartan at the doses utilized in the present work as assessed by MTT test (Fig. S1). Thus,

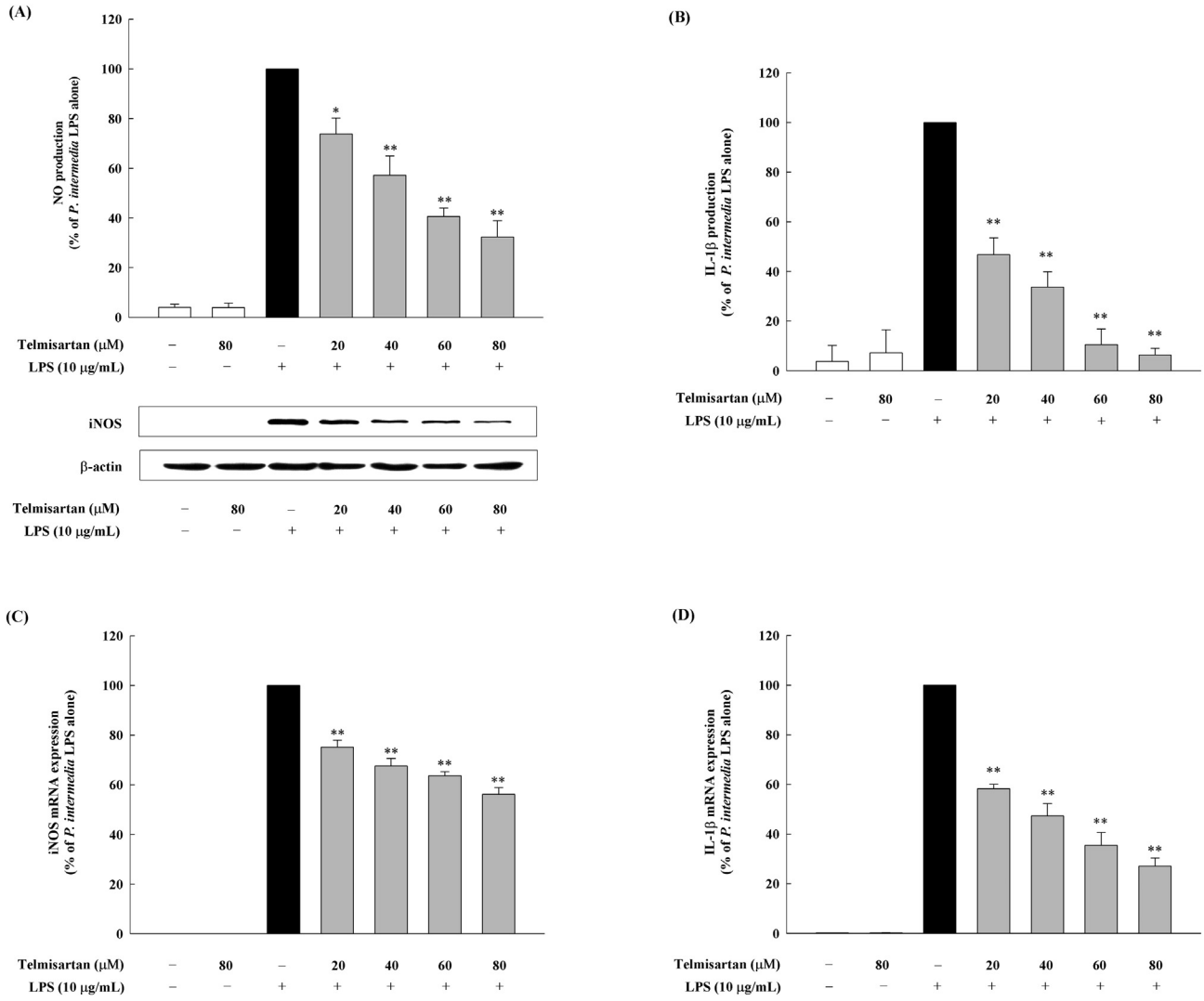

Fig. 1. Effect of telmisartan on iNOS-derived NO and IL-1β induction by *Prevotella intermedia* LPS in RAW264.7 cells. (A) Cells were treated with different doses of telmisartan in the absence or presence of *P. intermedia* LPS (10 μg/ml) for 24 h, after which the culture supernatant were assayed for NO. The results are means ± S.D. of three independent experiments. *$P < 0.05$ versus *P. intermedia* LPS alone. **$P < 0.01$ versus *P. intermedia* LPS alone. iNOS protein synthesis was measured by immunoblot analysis of cell lysates. A representative immunoblot from three separate experiments with similar results is shown. (B) Cells were treated with different doses of telmisartan in the absence or presence of *P. intermedia* LPS (10 μg/ml) for 48 h, after which the culture supernatant were assayed for IL-1β. The results are means ± S.D. of three independent experiments. **$P < 0.01$ versus *P. intermedia* LPS alone. (C, D) Cells were treated with telmisartan in the absence or presence of *P. intermedia* LPS for 6 h, after which real-time PCR was carried out for the measurement of iNOS and IL-1β mRNA expression. The results are means ± S.D. of three independent experiments. **$P < 0.01$ versus *P. intermedia* LPS alone.

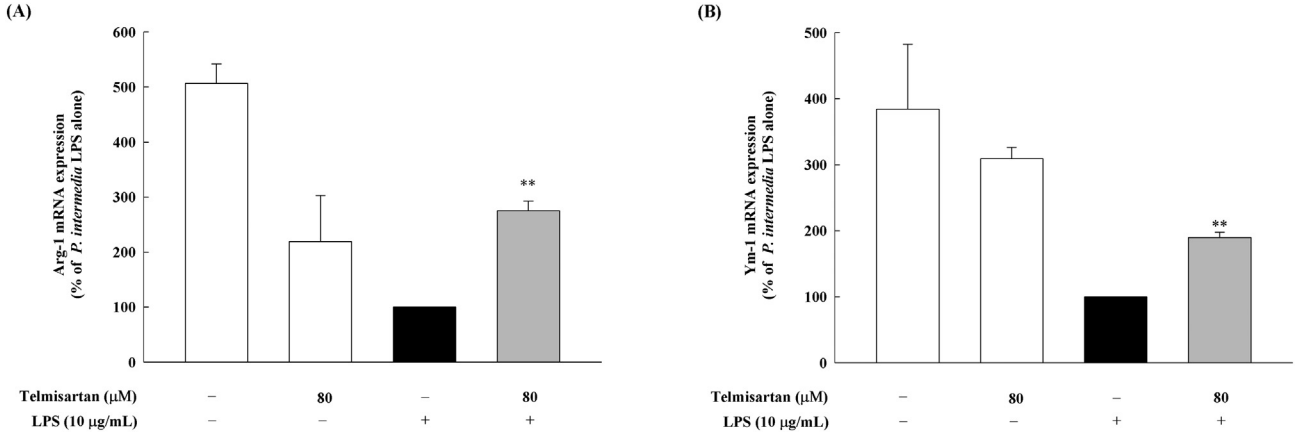

Fig. 2. Effect of telmisartan on the expression of macrophage M2 biomarkers in RAW264.7 cells activated by *Prevotella intermedia* LPS. Cells were treated with telmisartan (80 μM) in the absence or presence of *P. intermedia* LPS (10 μg/ml) for 24 h, after which real-time PCR was carried out for the measurement of Arg-1 (A) and Ym-1 (B) mRNA expression. The results are means ± S.D. of three independent experiments. $^{**}P < 0.01$ versus *P. intermedia* LPS alone.

inhibitory activity of telmisartan on NO and IL-1β was not due to its direct toxicity.

Telmisartan suppressed the protein level of iNOS induced by *P. intermedia* LPS in the similar fashion as it inhibited NO production (Figs. 1A, S2). We also analyzed whether the suppressive influence of telmisartan upon NO and IL-1β was due to the reduction of mRNA encoding iNOS and IL-1β. As was expected, telmisartan also distinctly decreased the gene expression of iNOS and IL-1β which were notably elevated by *P. intermedia* LPS (Fig. 1C, D).

3.2. Telmisartan increases the expression of M2 macrophage polarization markers in P. intermedia LPS-stimulated cells

We examined the effects of telmisartan on the properties that define M2 (alternative) macrophage polarization in *P. intermedia* LPS-stimulated RAW264.7 cells. The expression of M2 markers was determined by real-time PCR assay. Telmisartan treatment of LPS-activated cells significantly up-regulated arginase 1 (Arg-1) and chitinase-like 3 (Ym-1), which are specific markers of M2 macrophages (Fig. 2).

3.3. Telmisartan-induced expression of HO-1 mediates the inhibition of P. intermedia LPS-evoked generation of NO

Next, we investigated the possible function of HO-1 in the suppression by telmisartan of *P. intermedia* LPS-induced generation of NO and IL-1β. Here we show that administration of telmisartan caused a significant increase in HO-1 expression at both protein and mRNA levels in murine macrophages stimulated with LPS (Fig. 3A, B).

Because telmisartan triggered HO-1 expression in cells stimulated with *P. intermedia* LPS, we next tested whether HO-1 expression induced by telmisartan is related to the modulation of NO and IL-1β production. Cells were exposed to LPS (10 μg/ml) and telmisartan (80 μM) together with increasing dosages of SnPP, a potent HO-1 inhibitor, and the generation of NO and IL-1β was analyzed. Although the inhibitory action of telmisartan against NO production was notably attenuated in a dosage-dependent manner by treatment with SnPP (Fig. 3C), similar result was not observed for IL-1β synthesis (data not shown).

3.4. PPAR-γ is not involved in the inhibitory activity of telmisartan on P. intermedia LPS-evoked generation of NO and IL-1β

Telmisartan, in addition to being an AT_1R antagonist, activates peroxisome proliferator-activated receptor (PPAR)-γ, thereby acting as a partial agonist of PPAR-γ [44–46]. In the light of the partial PPAR-γ agonist activity of telmisartan, we tested whether telmisartan mediates its inhibitory influence on *P. intermedia* LPS-induced synthesis of NO and IL-1β via PPAR-γ activation. It was found that treatment of cells with 80 μM of telmisartan increased the expression of PPAR-γ mRNA by about 95% as compared to that of vehicle-treated cells (Fig. 4A). However, we observed significant decrease of PPAR-γ mRNA following treatment with *P. intermedia* LPS, and telmisartan did not reversed the LPS-induced decrease of PPAR-γ (Fig. 4A). As shown in Fig. 4B, whereas treatment of cells with telmisartan resulted in a notable inhibition of *P. intermedia* LPS-induced generation of NO and IL-1β, neither ciglitazone nor pioglitazone, two PPAR-γ agonists, was active in this respect. In addition, PPAR-γ antagonism by GW9662 and T0070907 did not reverse the suppressive effect of telmisartan against LPS-induced production of NO and IL-1β (Fig. 4C).

3.5. Telmisartan does not suppress phosphorylation of JNK and p38 evoked by P. intermedia LPS

Our previous works have shown that the JNK and p38 as well as NF-κB and JAK2/STAT1 mediate the synthesis of NO in RAW264.7 cells stimulated with *P. intermedia* LPS [16]. These signaling molecules, except JNK, are known to mediate LPS-induced generation of IL-1β as well [47]. Additionally, the STAT3 signaling is involved in the production of NO and IL-1β induced by this LPS [47]. First, we investigated the possibility that the inhibitory influence of telmisartan on *P. intermedia* LPS-induced generation of NO and IL-1β might be due to inhibition of JNK and p38 signaling. As the results showed in Fig. 5A, the phosphorylation levels of JNK and p38 markedly enhanced in response to LPS were not affected by telmisartan treatment.

3.6. Telmisartan does not suppress transcriptional activity of NF-κB evoked by P. intermedia LPS

In subsequent experiments, we determined the possible influences of telmisartan upon NF-κB signaling, which plays a role in the generation of NO and IL-1β from *P. intermedia* LPS-stimulated murine macrophages. To ascertain whether telmisartan is able to suppress transcriptional activity of NF-κB, we conducted SEAP assay using RAW-Blue cells. As shown in Fig. 5B, NF-κB-dependent SEAP release was markedly enhanced after 24 h exposure of RAW-Blue cells to *P. intermedia* LPS compared with vehicle-treated cells, whereas treatment with telmisartan did not reduced SEAP release evoked by LPS.

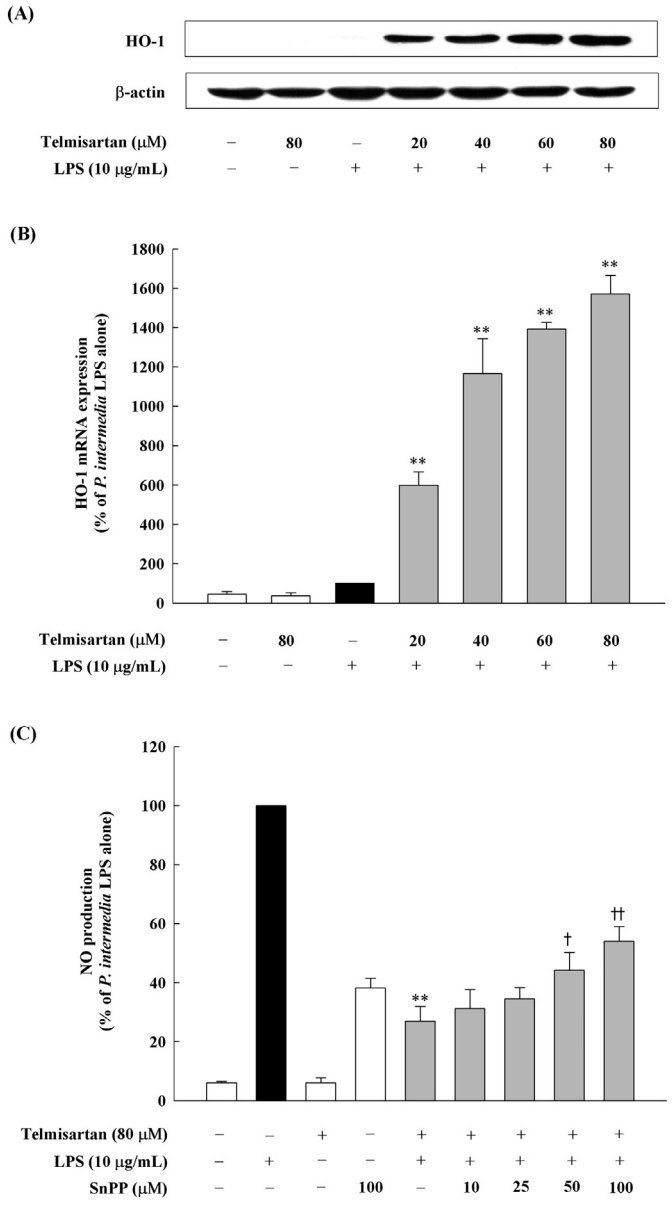

Fig. 3. Effect of telmisartan-mediated HO-1 induction upon suppression of *Prevotella intermedia* LPS-induced generation of NO in RAW264.7 cells. (A, B) Cells were treated with different concentrations of telmisartan in the absence or presence of *P. intermedia* LPS (10 μg/ml) for 24 h. (A) HO-1 protein synthesis was measured by immunoblot analysis of cell lysates using HO-1-specific antibody. A representative immunoblot from three separate experiments with similar results is shown. (B) Real-time PCR was performed with EvaGreen Supermix. Data are presented as percentage of *P. intermedia* LPS alone. The results are means ± S.D. of three independent experiments. $^{**}P < 0.01$ versus *P. intermedia* LPS alone. (C) Cells were treated with telmisartan (80 μM) and *P. intermedia* LPS (10 μg/ml) for 24 h in the presence of different doses of SnPP, after which, supernatants were removed and assayed for NO. Data are presented as percentage of *P. intermedia* LPS alone. The results are means ± S.D. of three independent experiments. $^{**}P < 0.01$ versus *P. intermedia* LPS alone; $^{\dagger}P < 0.05$ versus *P. intermedia* LPS plus telmisartan; $^{\dagger\dagger}P < 0.01$ versus *P. intermedia* LPS plus telmisartan.

3.7. Telmisartan attenuates phosphorylation of STAT1 and STAT3 evoked by P. intermedia LPS

To further elucidate the mechanism by which telmisartan inhibits *P. intermedia* LPS-induced increase of NO and IL-1β, we investigated the possibility that telmisartan inhibits the phosphorylation of STAT1 and STAT3. As shown in Fig. 5C, activation of STAT1 and STAT3 induced by LPS were dosage-dependently reduced by telmisartan.

4. Discussion

In this work, we assessed the influences of telmisartan, an ARB, on the production of proinflammatory mediators in murine macrophages stimulated with *P. intermedia* LPS. The structure and function of *P. intermedia* LPS are shown to be distinct from those of classical enterobacterial LPS [48]. In addition, unlike *Salmonella* LPS, LPS isolated from *P. intermedia* activated macrophages isolated from C3H/HeJ mice, which are LPS-resistant [49]. Further, in contrast to LPS from *Enterobacteriaceae*, the activities of *P. intermedia* LPS were not suppressed by polymyxin B, a cationic polypeptide antibiotic primarily used for resistant gram-negative infections [49].

In the present study, we found that production of NO and IL-1β as well as their gene expressions was notably down-regulated when murine macrophages activated with *P. intermedia* LPS were challenged with telmisartan (Fig. 1). The results obtained here indicate that the suppression of these inflammatory mediators by telmisartan occurred mainly at the transcription level.

It is well-known that NO has dual behavior depending on its concentration, which is dictated by NOS isoforms. Very low concentrations of NO induced by constitutive eNOS and nNOS have been shown to be protective [50,51]. In contrast, massive amounts of NO produced by iNOS play an important role in the development of pathological conditions like inflammation, sepsis and atherosclerosis [52,53].

In the present study, we found that telmisartan treatment of *P. intermedia* LPS-activated cells significantly up-regulated Arg-1 and Ym-1, which are specific markers of M2 macrophages (Fig. 2). These results indicate that telmisartan prevents the induction of the M1 macrophage phenotype by LPS and promotes the switch to the M2 phenotype.

HO-1 is the inducible isoform of heme oxygenase involved in heme catabolism and has been reported to convert proinflammatory free heme to carbon monoxide (CO), biliverdin and ferrous iron [54]. This cytoprotective enzyme exerts beneficial effects like anti-inflammatory and antioxidant properties [54–56]. Studies have shown that mice lacking in HO-1 demonstrated serious inflammation, while overexpression of this cytoprotective enzyme contributed to the reduction of inflammatory response [55,57]. In this study, our data revealed that HO-1 induction is achieved by telmisartan in cells activated with LPS from *P. intermedia*, and the suppressive influence of telmisartan upon LPS-induced NO generation was apparently reversed by SnPP treatment (Fig. 3), indicating that NO inhibition by telmisartan is partially ascribed to the induction of HO-1. However, similar effect was not noticed for IL-1β synthesis. Evidence from studies suggests that the biological effects of HO-1 are attributable to CO and bilirubin, the products of heme catabolism [58–60]. Thus, we suppose that the inhibitory effect of telmisartan on *P. intermedia* LPS-elicited elaboration of NO is ascribed to CO and bilirubin generated as a consequence of HO-1 induction. We believe that the present study is the first report about the function of telmisartan in HO-1 modulation.

PPARs are the nuclear receptor family of ligand-activated transcription factors and have 3 isotypes encoded by different genes, PPAR-α, PPAR-β/δ and PPAR-γ [61,62]. It was recognized that activation of PPAR signaling can ameliorate the inflammatory responses [63]. In particular, PPAR-γ exerts anti-inflammatory activity by blocking the transcription of a diverse group of genes involved in inflammation and is a negative regulator of macrophage activation [64–67]. Therefore, targeted induction of PPAR-γ may be beneficial for treatment of inflammatory disease. Reports have shown that telmisartan, an ARB, also functions as a partial agonist of the PPAR-γ [44–46]. In the present study, whereas treatment of cells with telmisartan significantly increased the PPAR-γ expression as compared to vehicle-treated cells, diminished expression of PPAR-γ induced by *P. intermedia* LPS was not reversed in the presence of telmisartan (Fig. 4A). In addition, inhibition

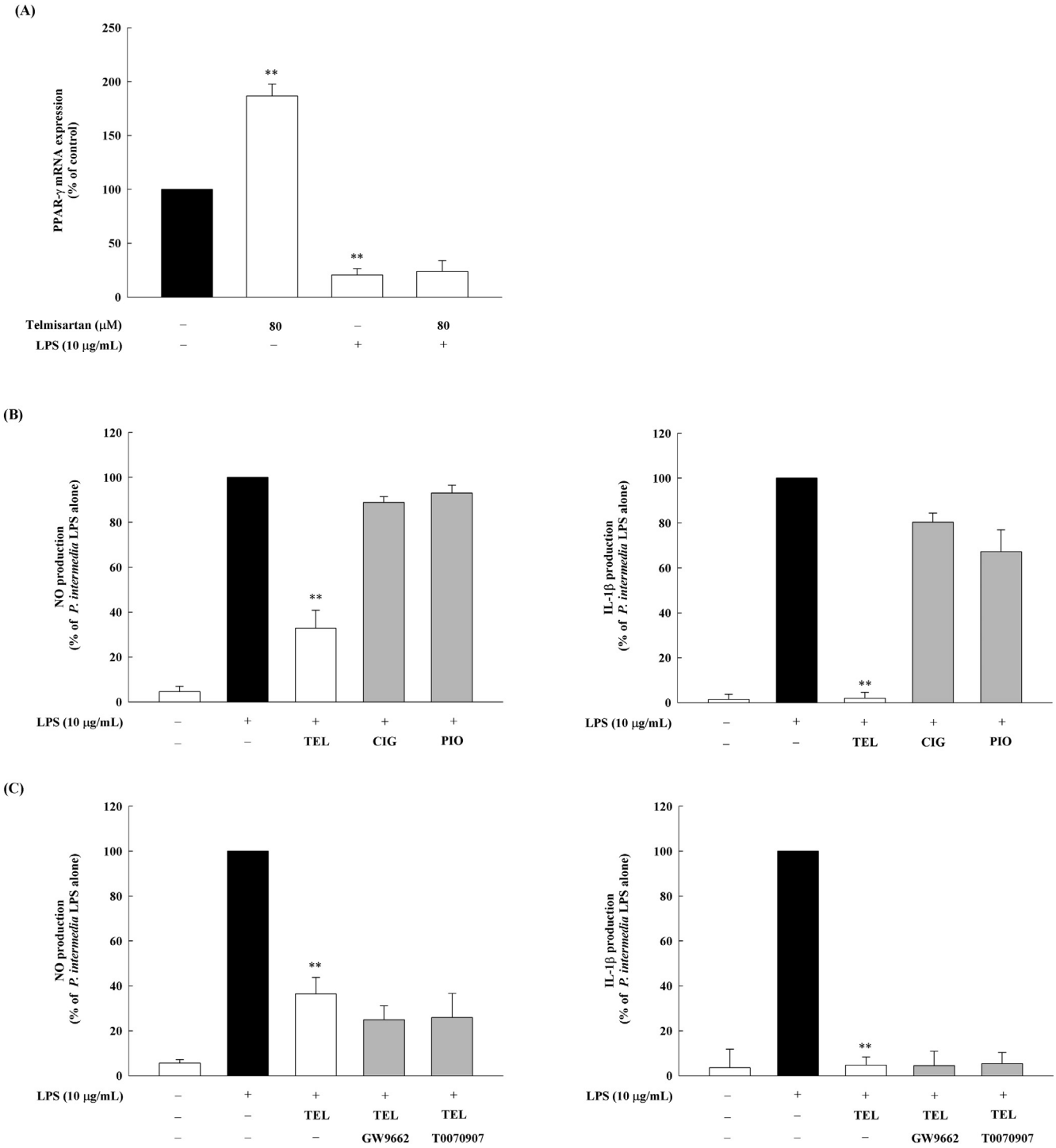

Fig. 4. Role of PPAR-γ in telmisartan-mediated inhibition of NO and IL-1β production in *Prevotella intermedia* LPS-activated RAW264.7 cells. (A) Cells were treated with telmisartan (80 μM) in the absence or presence of *P. intermedia* LPS (10 μg/ml) for 3 h, after which real-time PCR was carried out for the measurement of PPAR-γ mRNA expression. The results are means ± S.D. of three independent experiments. $^{**}P < 0.01$ versus control. (B) Cells were exposed to *P. intermedia* LPS (10 μg/ml) together with telmisartan (TEL; 80 μM) and PPAR-γ agonists (each at a concentration of 10 μM), ciglitazone (CIG) and pioglitazone (PIO), for 24 h (for NO) or 48 h (for IL-1β), after which the supernatants were removed and assayed. Data are presented as percentage of *P. intermedia* LPS alone. The results are means ± S.D. of three independent experiments. $^{**}P < 0.01$ versus *P. intermedia* LPS alone. (C) Cells were exposed to telmisartan (TEL; 80 μM) and *P. intermedia* LPS (10 μg/ml) together with PPAR-γ antagonists (each at a concentration of 10 μM), GW9662 and T0070907, for 24 h (for NO) or 48 h (for IL-1β), after which the supernatants were removed and assayed. Data are presented as percentage of *P. intermedia* LPS alone. The results are means ± S.D. of three independent experiments. $^{**}P < 0.01$ versus *P. intermedia* LPS alone.

of LPS-induced generation of NO and IL-1β by telmisartan was not mimicked by PPAR-γ agonists (Fig. 4B). Further, the telmisartan-mediated suppression of NO and IL-1β was not abrogated by PPAR-γ antagonists (Fig. 4C). Hence, it can be concluded from these observations that telmisartan inhibits *P. intermedia* LPS-induced generation of NO and IL-1β independently of PPAR-γ activation in RAW264.7 cells.

We also investigated whether the inhibitory activity of telmisartan

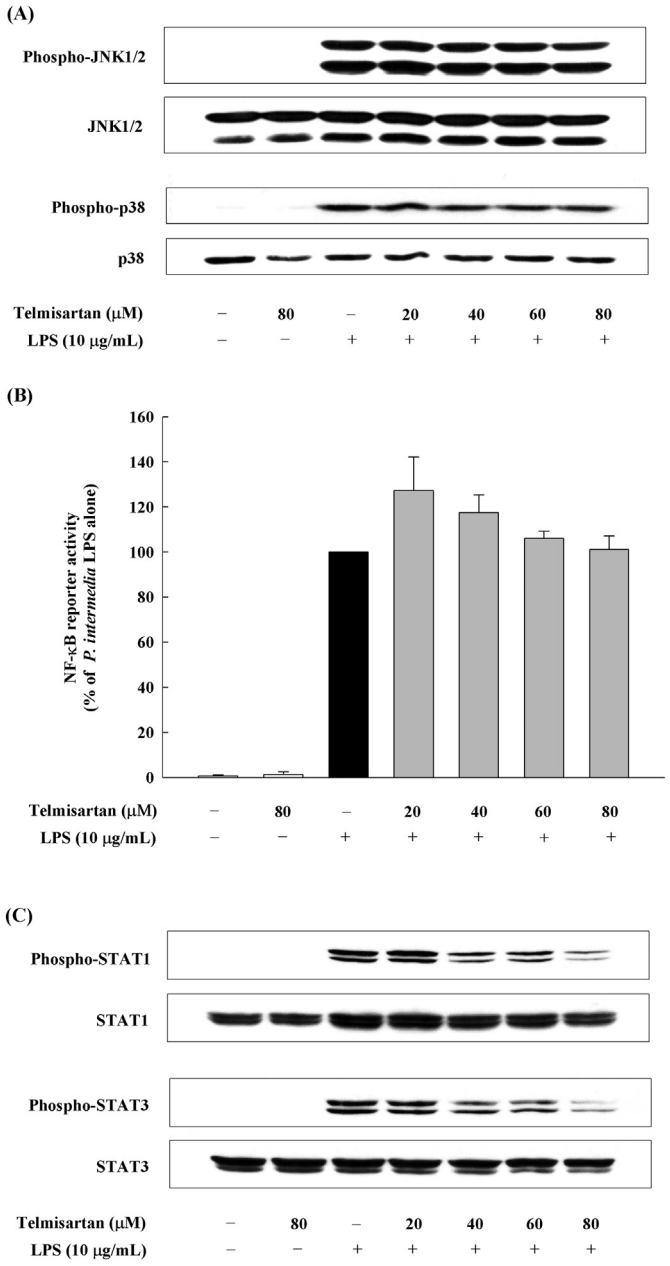

Fig. 5. Effects of telmisartan on JNK and p38 phosphorylation (A), NF-κB transcriptional activity (B) and STAT1/3 phosphorylation (C) induced by *Prevotella intermedia* LPS. (A, C) RAW264.7 cells were incubated with different doses of telmisartan in the absence or presence of *P. intermedia* LPS (10 μg/ml) for 15 min (for p38), 30 min (for JNK), or 4 h (for STAT1/3). Cells lysates were subjected to immunoblot analysis using specific antibodies. A representative immunoblot from three separate experiments with similar results is shown. (B) RAW-Blue cells, stably expressing the gene for SEAP inducible by NF-κB transcription factor, were treated with different concentrations of telmisartan in the absence or presence of *P. intermedia* LPS (10 μg/ml) for 24 h, after which the supernatants were collected for SEAP secretion assay. The results are means ± S.D. of three independent experiments.

on *P. intermedia* LPS-elicited generation of NO and IL-1β might be ascribed to blocking of JNK and p38 signaling. Our findings have shown that phosphorylation of JNK and p38 caused by LPS was not modulated by telmisartan (Fig. 5A), which indicates that these signaling molecules do not play a role in the suppression of NO and IL-1β by telmisartan in RAW264.7 cells stimulated by *P. intermedia* LPS.

The NF-κB is an important transcriptional factor involved in the induction of various genes associated with the inflammatory responses [68–70]. In this work, it was observed that NF-κB activation evoked by *P. intermedia* LPS was not influenced by telmisartan treatment (Fig. 5B). Therefore, the modulating influence of telmisartan on LPS-induced generation of NO and IL-1β was unlikely to be mediated at the NF-κB level.

The signal transducer and activator of transcription (STAT) plays a vital role in LPS-evoked inflammatory reactions [71]. STAT1 and STAT3, in particular, are key modulators actively involved in regulation of LPS-elicited expression of genes encoding a variety of inflammatory mediators in macrophages [72,73]. Telmisartan diminished the phosphorylation of STAT1 and STAT3 in cells exposed to *P. intermedia* LPS (Fig. 5C), indicating that the suppression of NO and IL-1β production by telmisartan seems to involve these signaling molecules.

In summary, we can infer from the results reported in this study that telmisartan is a potent inhibitor of *P. intermedia* LPS-induced generation of NO and IL-1β in RAW264.7 cells used here and promotes macrophage phenotype switching toward the M2 phenotype. Telmisartan acts by inducing anti-inflammatory HO-1 expression and blocking STAT1/3 activation independently of both PPAR-γ and NF-κB as well as JNK and p38 signaling.

It is clear that the pathogenesis of periodontal disease involves the host immune-inflammatory responses to periodontopathic bacteria leading to tissue breakdown. Thus, various therapeutic strategies have been evolved to modulate or block the host component of periodontal disease [26,27]. Telmisartan may have potential to be developed into host modulatory agent for plaque-induced periodontal disease, although additional studies are needed to confirm the therapeutic effect.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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References

[1] R.C. Williams, Periodontal disease, N. Engl. J. Med. 322 (1990) 373–382.
[2] G.J. Seymour, P.J. Ford, M.P. Cullinan, S. Leishman, K. Yamazaki, Relationship between periodontal infections and systemic disease, Clin. Microbiol. Infect. 13 (2007) 3–10.
[3] R.R. Braga, M.A. Carvalho, O. Bruña-Romero, R.E. Teixeira, J.E. Costa, E.N. Mendes, L.M. Farias, P.P. Magalhães, Quantification of five putative periodontal pathogens in female patients with and without chronic periodontitis by real-time polymerase chain reaction, Anaerobe 16 (2010) 234–239.
[4] C.P. Chung, R.J. Nisengard, J. Slots, R.J. Genco, Bacterial IgG and IgM antibody titers in acute necrotizing ulcerative gingivitis, J. Periodontol. 54 (1983) 557–562.
[5] K.S. Kornman, W.J. Loesche, The subgingival microbial flora during pregnancy, J. Periodontal Res. 15 (1980) 111–122.
[6] R.C. Jacinto, B.P. Gomes, C.C. Ferraz, A.A. Zaia, F.J. Filho, Microbiological analysis of infected root canals from symptomatic and asymptomatic teeth with periapical periodontitis and the antimicrobial susceptibility of some isolated anaerobic bacteria, Oral Microbiol. Immunol. 18 (2003) 285–292.
[7] M. Ulrich, I. Beer, P. Braitmaier, M. Dierkes, F. Kummer, B. Krismer, U. Schumacher, U. Gräpler-Mainka, J. Riethmüller, P.Ø. Jensen, T. Bjarnsholt, N. Høiby, G. Bellon, G. Döring, Relative contribution of *Prevotella intermedia* and *Pseudomonas aeruginosa* to lung pathology in airways of patients with cystic fibrosis, Thorax 65 (2010) 978–984.
[8] I. Brook, E.H. Frazier, Immune response to *Fusobacterium nucleatum* and *Prevotella intermedia* in the sputum of patients with acute exacerbation of chronic bronchitis, Chest 124 (2003) 832–833.
[9] A. Pucar, J. Milasin, V. Lekovic, M. Vukadinovic, M. Ristic, S. Putnik, E.B. Kenney, Correlation between atherosclerosis and periodontal putative pathogenic bacterial infections in coronary and internal mammary arteries, J. Periodontol. 78 (2007) 677–682.
[10] B.J. Shenker, L. Vitale, J. Slots, Immunosuppressive effects of *Prevotella intermedia* on in vitro human lymphocyte activation, Infect. Immun. 59 (1991) 4583–4589.
[11] J.E. Beem, W.E. Nesbitt, K.P. Leung, Identification of hemolytic activity in *Prevotella*

intermedia, Oral Microbiol. Immunol. 13 (1998) 97–105.
[12] S.M. Guan, L. Shu, S.M. Fu, B. Liu, X.L. Xu, J.Z. Wu, *Prevotella intermedia* upregulates MMP-1 and MMP-8 expression in human periodontal ligament cells, FEMS Microbiol. Lett. 299 (2009) 214–222.
[13] D.C. Morrison, J.L. Ryan, Endotoxins and disease mechanisms, Annu. Rev. Med. 38 (1987) 417–432.
[14] S.J. Kim, M.S. Ha, E.Y. Choi, J.I. Choi, I.S. Choi, *Prevotella intermedia* lipopolysaccharide stimulates release of nitric oxide by inducing expression of inducible nitric oxide synthase, J. Periodontal Res. 39 (2004) 424–431.
[15] S.J. Kim, E.Y. Choi, E.G. Kim, S.H. Shin, J.Y. Lee, J.I. Choi, I.S. Choi, *Prevotella intermedia* lipopolysaccharide stimulates release of tumor necrosis factor-alpha through mitogen-activated protein kinase signaling pathways in monocyte-derived macrophages, FEMS Immunol. Med. Microbiol. 51 (2007) 407–413.
[16] E.Y. Choi, J.Y. Jin, J.Y. Lee, J.I. Choi, I.S. Choi, S.J. Kim, Melatonin inhibits *Prevotella intermedia* lipopolysaccharide-induced production of nitric oxide and interleukin-6 in murine macrophages by suppressing NF-κB and STAT1 activity, J. Pineal Res. 50 (2011) 197–206.
[17] E.Y. Choi, J.Y. Jin, J.I. Choi, I.S. Choi, S.J. Kim, DHA suppresses *Prevotella intermedia* lipopolysaccharide-induced production of proinflammatory mediators in murine macrophages, Br. J. Nutr. 111 (2014) 1221–1230.
[18] P. Pelt, B. Zimmermann, N. Ulbrich, J.P. Bernimoulin, Effects of lipopolysaccharide extracted from *Prevotella intermedia* on bone formation and on the release of osteolytic mediators by fetal mouse osteoblasts in vitro, Arch. Oral Biol. 47 (2002) 859–866.
[19] E.F. Rossomando, J.E. Kennedy, J. Hadjimichael, Tumour necrosis factor alpha in gingival crevicular fluid as a possible indicator of periodontal disease in humans, Arch. Oral Biol. 35 (1990) 431–434.
[20] M. Matejka, L. Partyka, C. Ulm, P. Solar, H. Sinzinger, Nitric oxide synthesis is increased in periodontal disease, J. Periodontal Res. 33 (1998) 517–518.
[21] M. Mogi, J. Otogoto, N. Ota, H. Inagaki, M. Minami, K. Kojima, Interleukin 1β, interleukin 6, β_2-microglobulin, and transforming growth factor-α in gingival crevicular fluid from human periodontal disease, Arch. Oral Biol. 44 (1999) 535–539.
[22] D.N. Tatakis, Interleukin-1 and bone metabolism: a review, J. Periodontol. 64 (1993) 416–431.
[23] C.R. Irwin, T.T. Myrillas, The role of IL-6 in the pathogenesis of periodontal disease, Oral Dis. 4 (1998) 43–47.
[24] K. Kobayashi, N. Takahashi, E. Jimi, N. Udagawa, M. Takami, S. Kotake, N. Nakagawa, M. Kinosaki, K. Yamaguchi, N. Shima, H. Yasuda, T. Morinaga, K. Higashio, T.J. Martin, T. Suda, Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction, J. Exp. Med. 191 (2000) 275–286.
[25] X.H. Liu, A. Kirschenbaum, S. Yao, A.C. Levine, Cross-talk between the interleukin-6 and prostaglandin E_2 signaling systems results in enhancement of osteoclastogenesis through effects on the osteoprotegerin/receptor activator of NF-κB (RANK) ligand/RANK system, Endocrinology 146 (2005) 1991–1998.
[26] M.S. Reddy, N.C. Geurs, J.C. Gunsolley, Periodontal host modulation with anti-proteinase, anti-inflammatory, and bone-sparing agents. A systematic review, Ann. Periodontol. 8 (2003) 12–37.
[27] P.M. Preshaw, Host response modulation in periodontics, Periodontol. 2000 48 (2008) 92–110.
[28] B.E. Karlberg, L.E. Lins, K. Hermansson, Efficacy and safety of telmisartan, a selective AT1 receptor antagonist, compared with enalapril in elderly patients with primary hypertension. TEES Study Group, J. Hypertens. 17 (1999) 293–302.
[29] N. Nagai, Y. Oike, K. Noda, T. Urano, Y. Kubota, Y. Ozawa, H. Shinoda, T. Koto, K. Shinoda, M. Inoue, K. Tsubota, K. Yamashiro, T. Suda, S. Ishida, Suppression of ocular inflammation in endotoxin-induced uveitis by blocking the angiotensin II type 1 receptor, Invest. Ophthalmol. Vis. Sci. 46 (2005) 2925–2931.
[30] K. Fujita, M. Yoneda, K. Wada, H. Mawatari, H. Takahashi, H. Kirikoshi, M. Inamori, Y. Nozaki, S. Maeyama, S. Saito, T. Iwasaki, Y. Terauchi, A. Nakajima, Telmisartan, an angiotensin II type 1 receptor blocker, controls progress of non-alcoholic steatohepatitis in rats, Dig. Dis. Sci. 52 (2007) 3455–3464.
[31] S. Cianchetti, A. Del Fiorentino, R. Colognato, R. Di Stefano, F. Franzoni, R. Pedrinelli, Anti-inflammatory and anti-oxidant properties of telmisartan in cultured human umbilical vein endothelial cells, Atherosclerosis 198 (2008) 22–28.
[32] A. Nakano, Y. Hattori, C. Aoki, T. Jojima, K. Kasai, Telmisartan inhibits cytokine-induced nuclear factor-κB activation independently of the peroxisome proliferator-activated receptor-γ, Hypertens. Res. 32 (2009) 765–769.
[33] Q. Tian, R. Miyazaki, T. Ichiki, I. Imayama, K. Inanaga, H. Ohtsubo, K. Yano, K. Takeda, K. Sunagawa, Inhibition of tumor necrosis factor-α-induced interleukin-6 expression by telmisartan through cross-talk of peroxisome proliferator-activated receptor-γ with nuclear factor κB and CCAAT/enhancer-binding protein-β, Hypertension 53 (2009) 798–804.
[34] Araújo, A.A., Souza, T.O., Moura, L.M., Brito, G.A., Aragão, K.S., Araújo, L.S., Medeiros, C.A., Alves, M.S., Araújo, R.F. Jr., 2013. Effect of telmisartan on levels of IL-1, TNF-α, down-regulated COX-2, MMP-2, MMP-9 and RANKL/RANK in an experimental periodontitis model. J. Clin. Periodontol. 40, 1104–1111.
[35] N. Suda, K. Moriyama, G. Ganburged, Effect of angiotensin II receptor blocker on experimental periodontitis in a mouse model of Marfan syndrome, Infect. Immun. 81 (2013) 182–188.
[36] R.D. Stout, C. Jiang, B. Matta, I. Tietzel, S.K. Watkins, J. Suttles, Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences, J. Immunol. 175 (2005) 342–349.
[37] D.M. Mosser, J.P. Edwards, Exploring the full spectrum of macrophage activation, Nat. Rev. Immunol. 8 (2008) 958.
[38] S.K. Biswas, A. Mantovani, Orchestration of metabolism by macrophages, Cell Metab. 15 (2012) 432–437.
[39] L.B. Ivashkiv, Epigenetic regulation of macrophage polarization and function, Trends Immunol. 34 (2013) 216–223.
[40] R.P. de Campos, J.M. Siegel, C.G. Fresta, G. Caruso, J.A. da Silva, S.M. Lunte, Indirect detection of superoxide in RAW 264.7 macrophage cells using microchip electrophoresis coupled to laser-induced fluorescence, Anal. Bioanal. Chem. 407 (2015) 7003–7012.
[41] G. Caruso, C.G. Fresta, F. Martinez-Becerra, L. Antonio, R.T. Johnson, R.P.S. de Campos, J.M. Siegel, M.B. Wijesinghe, G. Lazzarino, S.M. Lunte, Carnosine modulates nitric oxide in stimulated murine RAW 264.7 macrophages, Mol. Cell. Biochem. 431 (2017) 197–210.
[42] G. Caruso, C.G. Fresta, J.M. Siegel, M.B. Wijesinghe, S.M. Lunte, Microchip electrophoresis with laser-induced fluorescence detection for the determination of the ratio of nitric oxide to superoxide production in macrophages during inflammation, Anal. Bioanal. Chem. 409 (2017) 4529–4538.
[43] L.C. Green, D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok, S.R. Tannenbaum, Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids, Anal. Biochem. 126 (1982) 131–138.
[44] S.C. Benson, H.A. Pershadsingh, C.I. Ho, A. Chittiboyina, P. Desai, M. Pravenec, N. Qi, J. Wang, M.A. Avery, T.W. Kurtz, Identification of telmisartan as a unique angiotensin II receptor antagonist with selective PPARgamma-modulating activity, Hypertension 43 (2004) 993–1002.
[45] M. Schupp, J. Janke, R. Clasen, T. Unger, U. Kintscher, Angiotensin type 1 receptor blockers induce peroxisome proliferator-activated receptor-gamma activity, Circulation 109 (2004) 2054–2057.
[46] T.W. Kurtz, M. Pravenec, Antidiabetic mechanisms of angiotensin-converting enzyme inhibitors and angiotensin II receptor antagonists: beyond the renin-angiotensin system, J. Hypertens. 22 (2004) 2253–2261.
[47] E.Y. Choi, S.H. Choe, J.Y. Hyeon, J.I. Choi, I.S. Choi, S.J. Kim, Effect of caffeic acid phenethyl ester on *Prevotella intermedia* lipopolysaccharide-induced production of proinflammatory mediators in murine macrophages, J. Periodontal Res. 50 (2015) 737–747.
[48] S. Hamada, H. Takada, T. Ogawa, T. Fujiwara, J. Mihara, Lipopolysaccharides of oral anaerobes associated with chronic inflammation: chemical and immunomodulating properties, Int. Rev. Immunol. 6 (1990) 247–261.
[49] T. Kirikae, T. Nitta, F. Kirikae, Y. Suda, S. Kusumoto, N. Qureshi, M. Nakano, Lipopolysaccharides (LPS) of oral black-pigmented bacteria induce tumor necrosis factor production by LPS-refractory C3H/HeJ macrophages in a way different from that of Salmonella LPS, Infect. Immun. 67 (1999) 1736–1742.
[50] L.J. Ignarro, G.M. Buga, K.S. Wood, R.E. Byrns, G. Chaudhuri, Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide, Proc. Natl. Acad. Sci. U. S. A. 84 (1987) 9265–9269.
[51] J. Garthwaite, Neural nitric oxide signaling, Trends Neurosci. 18 (1995) 51–52.
[52] J.M. Wong, T.R. Billiar, Regulation and function of inducible nitric oxide synthase during sepsis and acute inflammation, Adv. Pharmacol. 34 (1995) 155–170.
[53] M. Takahashi, K. Fukuda, T. Ohata, T. Sugimura, K. Wakabayashi, Increased expression of inducible and endothelial constitutive nitric oxide synthases in rat colon tumors induced by azoxymethane, Cancer Res. 57 (1997) 1233–1237.
[54] D. Morse, A.M. Choi, Heme oxygenase-1: the "emerging molecule" has arrived, Am. J. Respir. Cell Mol. Biol. 27 (2002) 8–16.
[55] L.E. Otterbein, M.P. Soares, K. Yamashita, F.H. Bach, Heme oxygenase-1: unleashing the protective properties of heme, Trends Immunol. 24 (2003) 449–455.
[56] S.W. Ryter, J. Alam, A.M. Choi, Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications, Physiol. Rev. 86 (2006) 583–650.
[57] K.D. Poss, S. Tonegawa, Reduced stress defense in heme oxygenase 1-deficient cells, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 10925–10930.
[58] L.E. Otterbein, F.H. Bach, J. Alam, M. Soares, H. Tao Lu, M. Wysk, R.J. Davis, R.A. Flavell, A.M. Choi, Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway, Nat. Med. 6 (2000) 422–428.
[59] D. Morse, S.E. Pischke, Z. Zhou, R.J. Davis, R.A. Flavell, T. Loop, S.L. Otterbein, L.E. Otterbein, A.M. Choi, Suppression of inflammatory cytokine production by carbon monoxide involves the JNK pathway and AP-1, J. Biol. Chem. 278 (2003) 36993–36998.
[60] J.K. Sarady-Andrews, F. Liu, D. Gallo, A. Nakao, M. Overhaus, R. Ollinger, A.M. Choi, L.E. Otterbein, Biliverdin administration protects against endotoxin-induced acute lung injury in rats, Am. J. Physiol. Lung Cell. Mol. Physiol. 289 (2005) L1131–L1137.
[61] V. Laudet, C. Hanni, J. Coll, F. Catzeflis, D. Stehelin, Evolution of the nuclear receptor gene superfamily, EMBO J. 11 (1992) 1003–1013.
[62] C.K. Glass, S. Ogawa, Combinatorial roles of nuclear receptors in inflammation and immunity, Nat. Rev. Immunol. 6 (2006) 44–55.
[63] J.H. Chung, A.Y. Seo, S.W. Chung, M.K. Kim, C. Leeuwenburgh, B.P. Yu, H.Y. Chung, Molecular mechanism of PPAR in the regulation of age-related inflammation, Ageing Res. Rev. 7 (2008) 126–136.
[64] M. Ricote, C.K. Glass, PPARs and molecular mechanisms of transrepression, Biochim. Biophys. Acta 1771 (2007) 926–935.
[65] M.A. Bouhlel, B. Derudas, E. Rigamonti, R. Dièvart, J. Brozek, S. Haulon, C. Zawadzki, B. Jude, G. Torpier, N. Marx, B. Staels, G. Chinetti-Gbaguidi, PPARγ activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties, Cell Metab. 6 (2007) 137–143.
[66] K. Saijo, A. Crotti, C.K. Glass, Regulation of microglia activation and deactivation by nuclear receptors, Glia 61 (2013) 104–111.
[67] V.R. Narala, P.A. Subramani, V.R. Narasimha, F.B. Shaik, K. Panati, The role of nitrated fatty acids and peroxisome proliferator-activated receptor gamma in modulating inflammation, Int. Immunopharmacol. 23l (2014) 283–287.
[68] P.A. Baeuerle, T. Henkel, Function and activation of NF-κB in the immune system, Annu. Rev. Immunol. 12 (1994) 141–179.

[69] D.M. Rothwarf, M. Karin, The NF-κB activation pathway: a paradigm in information transfer from membrane to nucleus, Sci. STKE 1999 (1999) RE1.
[70] M. Karin, Y. Ben-Neriah, Phosphorylation meets ubiquitination: the control of NF-κB activity, Annu. Rev. Immunol. 18 (2000) 621–663.
[71] E. Pfitzner, S. Kliem, D. Baus, C.M. Litterst, The role of STATs in inflammation and inflammatory diseases, Curr. Pharm. Des. 10 (2004) 2839–2850.
[72] J.J. Gao, M.B. Filla, M.J. Fultz, S.N. Vogel, S.W. Russell, W.J. Murphy, Autocrine/paracrine IFN-αβ mediates the lipopolysaccharide-induced activation of transcription factor Stat1α in mouse macrophages: pivotal role of Stat1α in induction of the inducible nitric oxide synthase gene, J. Immunol. 161 (1998) 4803–4810.
[73] L. Samavati, R. Rastogi, W. Du, M. Hüttemann, A. Fite, L. Franchi, STAT3 tyrosine phosphorylation is critical for interleukin 1 beta and interleukin-6 production in response to lipopolysaccharide and live bacteria, Mol. Immunol. 46 (2009) 1867–1877.