Fimasartan, Anti-hypertension Drug, Suppressed Inducible Nitric Oxide Synthase Expressions via Nuclear Factor-Kappa B and Activator Protein-1 Inactivation

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Inflammatory processes are important participants in the pathophysiology of hypertension and cardiovascular disease. Hypertension patients have impaired functions of the endothelium, in which inflammation plays a key role.\textsuperscript{1} Angiotensin II is recognized as an important vascular pro-inflammatory factor in hypertension. Angiotensin II-driven vascular inflammation is considered the result of direct modulates on cytokine release and pro-inflammatory transcription factor including nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1), and angiotensin II receptor blockers (ARBs) reduce the peripheral and cerebrovascular inflammation associated with this disease.\textsuperscript{2,3}

Anti-inflammatory effects of ARBs, first established in the peripheral vasculature,\textsuperscript{4} were later demonstrated in the cerebral vasculature\textsuperscript{5} and in stress-induced gastric ulcerations.\textsuperscript{6} These observations suggest that ARBs may exert general anti-inflammatory effects beyond those associated with cardiovascular and metabolic disease.\textsuperscript{7} Thus, apart from being a major vasopressor effector of the renin-angiotensin system, ARBs are probably good candidate drugs for inflammation.\textsuperscript{8}

Since the important role of macrophages in inflammation, many investigations about ARBs (e.g., candesartan, losartan, telmisartan and valsartan) were performed in lipopolysaccharide (LPS)-induced macrophages.\textsuperscript{9–11} In fact, there are several physiological roles of angiotensin II type 1 (AT1) receptor in macrophages, such as positive regulation of peroxide production\textsuperscript{12} and shift M1/M2 polarization balance.\textsuperscript{13} However, in macrophages AT1a receptor expression level is reportedly low and AT1b receptor is not detected at all\textsuperscript{10} and the most important feature of macrophages is that macrophages are widely distributed in the body and provide an immediate defense against foreign agents, such as lipopolysaccharide (LPS).\textsuperscript{15} Nitric oxide (NO), a potent pro-inflammatory mediator, is mainly produced by macrophages and acts as a cytotoxic agent during immune and inflammatory responses, which is generated enzymatically by inducible nitric oxide synthase (iNOS) at inflammatory sites. iNOS is induced by pro-inflammatory transcription factors, such as NF-κB and AP-1 which are present in the cytoplasm under normal conditions and translocate to nucleus in response to the pro-inflammatory stimuli.\textsuperscript{16,17}

Fimasartan, 2-n-butyl-5-dimethylamino-thiocarboxyl-methyl-6-methyl-3-\{2-(1H-tetrazole-5-yl)biphenyl-4-yl)methyl\}-pyrimidin-4(3H)-one, formerly known as BR-A-657, is a novel angiotensin II receptor antagonist exhibiting potent and selective angiotensin II receptor type 1 (AT1) blocking activity\textsuperscript{18} (Fig. 1A). Fimasartan approved by Korean Food and Drug Administration (KFDA) for the treatment of essential hypertension in September 2010 exerts a direct antihypertensive effect on vascular smooth muscle cells and an indirect antihypertensive effect on sodium retention, fluid retention and vascular tone as mediated by blockade of angiotensin II.\textsuperscript{19} It seems that the identification of the mechanisms leading to inhibition of inflammation by ARBs including fimasartan would contribute to the development of specific therapeutic approaches to apply in hypertension and its complications. Therefore, it is necessary to investigate whether fimasartan has the anti-inflammatory potential, likewise other ARBs. In the present study, we investigated the anti-inflammatory effects of the fimasartan in LPS-stimulated RAW264.7 macrophages.

MATERIALS AND METHODS

Materials The fimasartan used for this study was synthesized at Boryung Pharm. Co. (Seoul, Republic of Korea)

The authors declare no conflict of interest.

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and its chemical structure was determined by spectral data as described previously.\(^{18}\) The identity of this compound was confirmed by LC-MS and was found to be $>98\%$ pure. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, U.S.A.). iNOS, p65, c-Jun, c-Fos, poly(ADP ribose)polymerase (PARP), β-actin monoclonal antibodies, and peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Random oligonucleotide primers and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase were purchased from Promega (Madison, WI, U.S.A.). SYBR green ex Taq was obtained from TaKaRa (Shiga, Japan). iNOS, β-actin oligonucleotide primers were purchased from Bioneer (Seoul, Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phenylmethylsulfonylfluoride (PMSF), dithiothreitol (DTT), NS-398, LPS (Escherichia coli, serotype 0111:B4), and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

**Cell Culture and Sample Treatment** The RAW264.7 macrophage cell line was obtained from the Korea Cell Line
Bank (Seoul, Korea). These cells were grown at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin sulfate (100 µg/mL) in a humidified atmosphere 5% CO₂. Cells were incubated with fimasartan at concentration of 62.5, 125 or 250 µM, and then stimulated with LPS 1 µg/mL for the indicated time.

**Nitrite Determination** RAW264.7 macrophages were plated at 5×10⁵ cells/well in 24 well-plates and then incubated with or without LPS (1 µg/mL) in the absence or presence of various concentrations (62.5, 125, or 250 µM) of fimasartan for 24h. The nitrite accumulated in culture medium was measured, based on the Griess reaction. Briefly, 100 µL of cell culture medium was mixed with 100 µL of Griess reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethlenediamine–HCl), incubated at room temperature for 10 min, and then the absorbance at 540 nm was measured in a microplate reader (Perkin Elmer Cetus, CA, U.S.A.). Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was measured with the sodium nitrite serial dilution standard curve and nitrite production was measured.

**Western Blot Analysis** RAW264.7 macrophages were collected by centrifugation and washed once with phosphate-buffered saline (PBS). The washed cell pellets were resuspended in PRO-PREP™ protein extraction solution (Intron Biotechnology, Seoul, Korea) and incubated with 20 min at 4°C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacturer’s instruction. Cellular protein from treated and untreated cell extracts was electroblotted onto a polyvinylidene difluoride (PVDF) membrane following separation on a 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The membrane was incubated overnight with blocking solution (5% skim milk) at 4°C, followed by incubation for 4 h with a primary antibody. Blots were washed four times with Tween 20/Tris-buffer and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Blots were again washed three times with Tween 20/Tris-buffer, and then developed by enhanced chemiluminescence (Amersham Life Science).

**Quantitative Real-Time Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR)** Total cellular RNA was isolated by Easy Blue® kits (Intron Biotechnology, Seoul, Korea). From each sample, 1 µg of RNA was reverse-transcribed (RT) using MuLV reverse transcriptase, 1 mm deoxyribonucleotide triphosphate (dNTP), and oligo (dT)₁₂₋₁₈ 0.5 µg/µL. PCR amplification was performed using the incorporation of SYBR green. The oligonucleotide primers for iNOS a designed for mouse (NM_010927) were 5’-CAT GCT ACT GGA GGT GGG TG3’ (forward) and 5’-CAT TTGA TCT CCG TGA CAG CC-3’ (reverse) and the suitable size of synthesized cDNA were 209 bp. The oligonucleotide primers for β-actin used as a house-keeping gene designed from mouse were (NM_007393) were 5’-ATC ACT ATT GGC AAC GAG CG-3’ (forward) and 5’-TCA GCA ATG CTC GGT TAC AT-3’ (reverse), and the suitable size of synthesized cDNA were 200 bp. Steady-state mRNA levels of iNOS, and β-actin were determined by quantitative real-time PCR (qPCR) using the Takara thermal cycler dice® (Takara Bio Inc., Japan).

**Nuclear Extraction and Electrophoretic Mobility Shift Assay (EMSA)** RAW264.7 macrophages were plated in 100-mm dishes (10⁶ cells/mL), and treated with fimasartan (62.5, 125, or 250 µM), stimulated with LPS for 1 h, washed once with PBS, scraped into 1 mL of cold PBS, and pelleted by centrifugation. Nuclear extracts were prepared as described previously.²⁰ Nuclear extracts (5 µg) were mixed with double-stranded NF-κB and AP-1 oligonucleotide, 5’-AGTTGAGG GAC TTT CCC AGG C-3’ and 5’- CGT TTG ATG ACT CAG CGC GAA-3’, respectively, and end-labeled by biotin. DNA-binding activities are measured by using LightShiftChemiluminescent EMSA kit (Thermo Scientific, U.S.A.)

**Transient Transfection and Luciferase Assay** The mouse iNOS promoter plasmid (pGL3-iNOS; −1592/+185) was prepared as described previously.²¹ RAW264.7 macrophages were co-transfected with pGL3-iNOS, NF-κB-Luc or AP-1-Luc reporter plasmid vector plus the phRL-TK plasmid (Promega, Madison, WI, U.S.A.) using Lipofectamine LTX™ (Invitrogen, CA, U.S.A.) as instructed by the manufacturer. After 4 h of transfection, cells were pretreated with fimasartan for 1 h and then stimulated with LPS (1 µg/mL) for 18 h. Each well was washed with cold-PBS and cells were lysed and the luciferase activity was determined using the Promega luciferase assay system (Promega).

**Statistical Analysis** Results are presented as the mean±S.D. of triplicate experiments. Statistically significant values were compared using ANOVA and Dunnett’s post-hoc test, and p-values of less than 0.05 were considered statistically significant.

**RESULTS**

**Fimasartan Inhibited LPS-Induced NO Production and iNOS Expression in RAW264.7 Macrophages** To determine the effects of fimasartan on LPS-induced pro-inflammatory mediators, such as NO, prostaglandin E₂ (PGE₂), tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 in RAW264.7 macrophages, cells were treated with/without fimasartan (62.5, 125, or 250 µM) for 1 h and then treated with LPS (1 µg/mL) for 24 h. As shown in Fig. 1B, fimasartan potently and concentration-dependently inhibited LPS-induced NO production. However, it did not show any inhibitory effects on the productions of PGE₂, TNF-α, IL-1β, and IL-6 (data not shown). 1-Nφ-(1-Iminoethyl)lysine (1-NIL) (20 µM) was used in this experiment as a positive NO production inhibitor. To determine whether inhibitory effect of fimasartan on NO production was related to the modulation of iNOS, we examined the protein and mRNA expressions of iNOS by Western blotting and qRT-PCR, respectively. The protein expression of iNOS was found to be up-regulated by LPS, but pretreatment with fimasartan (62.5, 125, or 250 µM) concentration-dependently inhibited these responses (Fig. 1C). qRT-PCR was used to confirm that fimasartan regulated LPS-induced iNOS expression at the transcriptional level, and as shown in Fig. 1D, pretreatment with fimasartan significantly reduced iNOS mRNA levels. Next, the transcriptional regulation of iNOS by fimasartan was further delineated using a promoter activity assay. As shown in Fig. 1E, LPS significantly enhanced iNOS promoter activity, and fimasartan concentration-dependently inhibited this increase. Furthermore, the possibility that cytotoxicity was responsible for this inhibitory effect was
eliminated by the finding that fimasartan did not affect cell viability (as determined by MTT assay) at concentrations of 62.5–250 µM, which suppressed the expressions of iNOS protein and mRNA (data not shown).

**Fimasartan Reduced LPS-Induced NF-κB and AP-1 Activation in RAW264.7 Macrophages** Since the activations of NF-κB and AP-1 are critically required for the LPS-induced activation of iNOS,22,23 we examined the effect of fimasartan on the LPS-induced NF-κB and AP-1-dependent reporter gene activity. A reporter gene assay for the NF-κB transcriptional activity was performed using a pNF-κB-luc plasmid, which was generated by inserting four spaced NF-κB binding sites into pLuc-promoter vector. Cells were transiently transfected with this plasmid and then stimulated with LPS, either in the presence or absence of fimasartan. It was found that fimasartan prevented LPS-induced increases in NF-κB-dependent luciferase activity (Fig. 2A). To further examine the DNA-binding activity of NF-κB, nuclear extracts isolated from cells stimulated with LPS for 30 or 60 min in the presence or absence of fimasartan were subjected to EMSA. Treatment with LPS at 30 or 60 min increased the DNA-binding activity of NF-κB to its consensus DNA sequence. However, pretreatment with fimasartan (250 µM) at both time significantly reduced this LPS-induced NF-κB-DNA binding (Fig. 2B). In addition, the binding activity of NF-κB to its consensus DNA sequence treated with LPS at 60 min was concentration-dependently reduced by fimasartan (Fig. 2C). The specificity of binding was examined by competition with the 100-fold
unlabeled oligonucleotide (lane 6) and competitive inhibition with p65 antibody (lane 7). In addition, the translocation of NF-κB to the nucleus is believed to be an essential requirement for the activation of inflammatory gene transcriptions, and thus, we investigated whether fimasartan prevents the nuclear translocations of the p65 and p50 subunits of NF-κB. It was found that LPS markedly induced the translocation of NF-κB to the nucleus, and pretreatment with fimasartan significantly suppressed this process (Fig. 2D). In addition, fimasartan also significantly decreased LPS-induced transcription activity of AP-1 (Fig. 3A). Moreover, DNA-binding activity of AP-1 was measured by EMSA. Treatment with LPS at 30 or 60 min increased the DNA-binding activity of AP-1 to its consensus DNA sequence, and pretreatment with fimasartan (250 µM) reduced this LPS-induced AP-1-DNA binding at both times (Fig. 3B). LPS stimulation at 30 min resulted in a significant increase in the DNA-binding activity of AP-1, as determined by EMSA, and fimasartan attenuated this LPS-induced AP-1 binding (Fig. 3C). The specificity of binding was also examined by competition with the 100-fold unlabeled AP-1 oligonucleotide (cp) and competitive inhibition with c-Jun monoclonal antibody. (D) Nuclear extracts were prepared for the Western blotting of c-Fos and c-Jun using a specific anti-c-Fos or anti-c-Jun monoclonal antibody. PARP was used as an internal control for nuclear fractions. *p<0.05 vs. control cells; **p<0.01, ***p<0.001 vs. LPS-stimulated cells. ANOVA and Dunnett’s post-hoc test were used to determine the significances of differences.

DISCUSSION

Since there is a potential link between inflammation and hypertension through sharing some pathophysiological mechanisms, this raises the question of whether the treatment of one of the two conditions could have some impact on the other.
It has been reported that the presence of a chronic low grade inflammatory status can anticipate the future development of hypertension, which suggests that the increase in plasma levels of inflammatory mediators observed among hypertensive patients cannot be solely attributed to the vascular damage induced by high blood pressure. Therefore, it is considered that anti-inflammatory therapy might be useful to treat severe forms of either resistant or malignant hypertension.

Angiotensin II promotes the innate immune response, inflammation and oxidative stress by mechanisms similar to those involved in the LPS effects and ARBs reverse the chronic peripheral and brain vascular inflammatory reaction associated with hypertension. Candesartan significantly decreased synthetic lipoprotein Pam3CSK4 or LPS-induced Toll-like receptor (TLR)2 or TLR4 expression along with decrease in the activity of NF-κB and the expression of IL-1β, IL-6, TNF-α, and monocyte chemotactic protein (MCP)-1 and it protects against Pam3CSK4 or LPS challenge in mice with down-regulation of TLR2 or TLR4. Moreover clinical studies have suggested that treatment with candesartan leads to decreased plasma levels of TNF-α, IL-6, MCP-1, soluble intercellular adhesion molecule (sICAM)-1, and soluble vascular cell adhesion molecule (sVCAM)-1 in patients with mild to moderate chronic heart failure and hypertension. Losartan also attenuated the LPS-induced expression of pro-inflammatory genes TNF-α, IL-8, and COX-2. In addition, telmisartan significantly reduced LPS-induced mitogen-activated protein kinase (MAPK) activation and reactive oxygen species (ROS) formation, intimately associated with NF-κB activation, resulting in inhibition of LPS-induced pro-inflammatory effects in human monocytes. Besides, valsartan inhibited LPS-induced cytokine productions in RAW264.7 macrophage cell line.

Fimasartan is a new ARB used as antihypertensive agent. The study of efficacy and tolerability of fimasartan compared with losartan suggested that fimasartan has superiority to losartan. However, no report has been issued on its anti-inflammatory effect or the molecular mechanisms involved. Therefore, we investigated for the first time the molecular mechanisms underlying the anti-inflammatory properties of fimasartan in activated macrophages.

The pharmacologically induced down-regulations of LPS-inducible inflammatory mediators (for example, NO, TNF-α, and ILs) are regarded as an essential requirement for the alleviation of a number of disorders attributed to macrophage activation. Thus, RAW264.7 macrophages provide us with an excellent model for anti-inflammatory drug screening and for the subsequent evaluations of the inhibitors of the pathways that lead to the inductions of pro-inflammatory enzymes and to the productions of pro-inflammatory cytokines. One of the key steps during inflammation is leukocyte infiltration, which is controlled chiefly by chemokines for neutrophils and monocytes. The production of these chemokines is regulated positively or negatively by iNOS-derived NO. Although the mechanisms underlying such dual effects of NO remain unknown, the level of NO and duration of NO exposure appear to be determining factors for pro-inflammatory cytokine expression during the induction and resolution of inflammation. After all, attenuation of NO production and iNOS expression is a good target for anti-inflammatory drugs developments. So it makes sense that losartan and candesartan which is well known to exert anti-inflammatory effects have the attenuating effect on iNOS expression.

In the present study, fimasartan also clearly suppressed induction of iNOS through down-regulation of its promoter activity and subsequent production of NO in LPS-stimulated macrophages. As mentioned above, Angiotensin II promotes the innate immune response, by mechanisms similar to those involved in the LPS effects, however, the possibility that AT1 receptor blockade was responsible for this inhibitory effect was eliminated by the finding macrophages treated with angiotensin II did not show significant effect on NO release compared with control cells, which imply that inhibitory effect of fimasartan on LPS-induced NO production is independent of AT1 receptor signaling (data not shown).

NF-κB and AP-1 are known to play a critical role in the regulation of gene expressions involved in cell survival and to coordinate the expression of the pro-inflammatory enzymes. In the promoter sequence of the iNOS gene, there are a number of sites that bind various transcription factors, including NF-κB and AP-1. Especially, NF-κB regulates the expressions of pro-inflammatory enzymes, cytokines, and chemokines, it has been described “a central mediator of immune response,” and thus, the NF-κB signaling cascade is an attractive therapeutic target for the development of treatments for inflammatory and autoimmune disorders. Additionally, AP-1 is able to regulate expression of a large number of genes. As a result, AP-1 has been implicated in a wide range of cellular processes, including proliferation, death, survival and differentiation, and participates in diverse biological and pathological processes, such as epidermal and neuronal development, immune and inflammatory responses, and tumorigenesis. Related to inflammation, AP-1 is an important modulator in inflammatory diseases because it regulates pro-inflammatory mediator which attract or activate immune cells. Based on these reports, we tested whether fimasartan inhibited NF-κB or AP-1 activity in RAW264.7 macrophages by using reporter gene assay and EMSA. We found that fimasartan inhibited LPS-induced transcriptional activity and DNA binding of NF-κB or AP-1 in a concentration-dependent manner in RAW264.7 macrophages, and these results were correspondence with inhibition of iNOS expression. To identify the mechanisms involved in the inhibition of NF-κB or AP-1 activity by fimasartan, we tested the effect of fimasartan on NF-κB or AP-1 activation signals. NF-κB or AP-1 is composed mainly of two proteins: p65 and p50 or c-jun and c-fos, respectively. In unstimulated cells, NF-κB or AP-1 exists in the cytosol in a quiescent form. Upon stimulation with LPS, NF-κB or AP-1 becomes activated and translocates to the nucleus where it activates its target genes, like that for iNOS, by binding to its consensus sequence in their promoter regions. In the present study, Western blotting revealed that fimasartan inhibited LPS-induced the nuclear translocations of p65 and p50 (NF-κB subunits) or c-fos and c-jun (AP-1 subunits), in a concentration-dependent manner. These data were consistent with the previous report that most of the ARBs, such as candesartan, telmisartan and valsartan, have the inhibitory effects on translocation of p65 subunit of NF-κB or promoter activity of NF-κB. Among these ARBs, valsartan inhibits AP-1 activation as well. Valsartan suppressed LPS-induced NF-κB activation with p65 phosphorylation and AP-1 activation with JNK phosphorylation in RAW264.7 macrophages. The
author described that valsartan inhibits LPS-induced NF-κB activation via p65 phosphorylation and JNK/AP-1 activity in RAW264.7 cells. In this regard, to clarify how fimasartan inhibits these two kinds of transcription factor, NF-κB and AP-1, we investigated whether fimasartan has the inhibitory effects on LPS-induced phosphorylation of p65 and JNK by Western blotting. However we could not notice any inhibitory effect of fimasartan on the phosphorylation of p65 and JNK (data not shown). Also, we examined the effect of fimasartan on LPS-induced phosphorylation of IκB-α, which functions to inhibit the NF-κB transcription factor by masking the nuclear localization signals of NF-κB proteins. Phosphorylation of IκB-α then leads to its degradation and allows NF-κB to enter the nucleus. It was found that fimasartan did not show any effect on LPS-induced IκB-α phosphorylation or degradation in macrophages (data not shown). Additionally, we evaluated its effects in a murine model of LPS-induced sepsis to confirm whether fimasartan also inhibits inflammatory responses in vivo. Pretreatment with fimasartan increased the survival rates of mice with established LPS-induced endotoxaemia (Suppl. Fig. 1). Although we could not ignore the possible involvement of endogenous pro-inflammatory angiotensin II and the activity of fimasartan as an ARB in vivo, because sepsis is a whole-body inflammatory state instead of limited macrophages, these result suggests that the suppressive effects of fimasartan on NF-κB and AP-1-regulated gene transcription in macrophages could partially result in an anti-inflammatory effect in an animal model of sepsis. However, further investigation is needed to clarify how fimasartan inhibits NF-κB and AP-1 activation.

In summary, our findings show that fimasartan inhibits the LPS-induced NO production through the suppression of iNOS expression at the transcription level via down-regulation of NF-κB and AP-1 activation in RAW264.7 macrophages. Although further investigation is needed to clarify the precise mechanisms by which fimasartan inhibits NF-κB and AP-1 activation, we conclude that fimasartan appears to have the potential to ameliorate the inflammatory disease.

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