5-Lipoxygenase plays a pivotal role in endothelial adhesion of monocytes via an increased expression of Mac-1

Seung Jin Lee¹,²†, Eun Kyoung Choi¹,²†, Kyo Won Seo¹,², Jin Ung Bae¹,², Yun Hak Kim¹,², So Youn Park¹,², Sae Ock Oh²,³, and Chi Dae Kim¹,²*

¹Department of Pharmacology, School of Medicine, Pusan National University, Yangsan, Gyeongnam 626-870, Korea; ²MRC for Ischemic Tissue Regeneration and Medical Research Institute, Pusan National University, Busan 602-739, Korea; and ³Department of Anatomy, School of Medicine, Pusan National University, Yangsan, Gyeongnam, Korea

Received 14 January 2013; revised 20 May 2013; accepted 22 May 2013; online publish-ahead-of-print 29 May 2013

Time for primary review: 24 days

Aims
5-Lipoxygenase (5-LO) is known to participate in the pathogenesis of atherosclerosis; however, the underlying mechanisms are unclear. Thus, this study investigated the molecular mechanisms responsible for 5-LO expression in monocytes as well as the role of 5-LO in monocyte adhesion to the vascular endothelium, which is a key early event in macrophage foam cell formation.

Methods and results
An en face immunohistochemistry of endothelial surfaces revealed a marked increase in monocyte adhesion to the aortic endothelium in wild-type (WT) mice treated with lipopolysaccharide (LPS), which was significantly attenuated in 5-LO(-/-) mice. Likewise, the adhesion capacity of primary monocytes isolated from LPS-treated WT mice was higher than those of monocytes from 5-LO(-/-) mice. In an in vitro study, LPS increased monocyte adhesion to endothelial cells with an enhanced Mac-1 expression. These were attenuated by a 5-LO inhibitor, MK886, as well as by molecular depletion of 5-LO in monocytes. Furthermore, LPS-induced Mac-1 expression on monocytes was significantly inhibited by pre-treatment with U-75302, a BLT1-receptor antagonist, suggesting a pivotal role of 5-LO-derived leukotrienes. In promoter activity analysis and chromatin immunoprecipitation assays to identify transcription factors involved in 5-LO expression, both NF-kB and Sp1 played central roles to increase 5-LO expression in LPS-treated monocytes.

Conclusion
5-LO expression in monocytes is modulated via NF-kB and Sp1 signalling pathways, and 5-LO plays a pivotal role in LPS-mediated monocyte adhesion to the vascular endothelium through an increased expression of Mac-1 on monocytes.

Keywords
5-Lipoxygenase • Mac-1 • Monocyte adhesion • Vascular inflammation • TLR4

1. Introduction
Macrophage foam cell formation is an important aspect of atherosclerosis,¹ ² 1 and monocyte adhesion to the endothelium in blood vessels is a key early event leading to macrophage foam formation.³–⁵ Recent evidence demonstrates that products of the 5-LO pathway, which metabolizes free-arachidonic acid and leads to the formation of pro-inflammatory leukotrienes (LT), are implicated in the development and progression of atherosclerosis.⁶–⁹ However, the precise mechanisms whereby 5-LO-mediated monocyte adhesion to the endothelium contributes to the development and progression of atherosclerosis remain unknown.

Monocytes play a central role during the progression of atherosclerosis from underlying inflammatory reactions,¹⁰–¹³ and the monocyte-endothelial cell interaction may play a crucial role in atherosclerotic plaque formation.¹⁴ Indeed, the adhesion of circulating monocytes to the intimal endothelial cell monolayer is considered one of the earliest events, and is mediated by complex interactions between multiple adhesion molecules. These adhesion molecules on monocyte surfaces include members of the β-2 integrin family, LFA-1 (CD11a/CD18), Mac-1(CD11b/CD18), CD11c/CD18, β-1 integrin, and VLA-4 (CD49d/29), which interact with endothelial counter-ligands, such as ICAM-1 and VCAM-1.¹⁵,¹⁶ Monocyte adhesion molecules required for...
adhesion to the endothelium play important roles during the development and progression of atherosclerosis.17–19

5-LO is a potent pro-inflammatory mediator in several inflammatory diseases, including atherosclerosis.20–22 In our previous studies, we found that 5-LO is involved in the development and progression of atherosclerosis.24–28 Several independent studies have indicated that toll-like receptor (TLR)-mediated responses are characterized by synthesis of the 5-LO-derived lipid mediator leukotriene B4 (LTB4),29 and 5-LO metabolites have been suggested to participate in in vivo and in vitro responses to lipopolysaccharide (LPS).30,31 Accordingly, the modulation of monocyte adhesion to the vascular endothelium suggests an important interaction between 5-LO-mediated inflammation and the development of atherosclerosis.

In the present in vitro study, we investigated the signalling pathways responsible for the regulation of 5-LO expression in monocytes as well as the potential role of 5-LO on TLR4-mediated monocyte adhesion to the vascular endothelium. In addition, we also determined the pivotal role of 5-LO on monocyte adhesion to the vascular endothelium in vivo study using 5-LO−/− mice.

2. Methods

2.1 Chemicals and antibodies

Lipopolysaccharide from Escherichia coli was purchased from Sigma-Aldrich (Saint Louis, MO, USA). MK-886 and various signal pathway inhibitors were purchased from EMD Seron (Rockland, MA, USA) and Sigma-Aldrich. Purified anti-human TLR4 antibody (Cat No. 14-9917), anti-human Mac-1 antibody (Cat No. 16-0113), and anti-mouse IgG isotype control antibody (Cat No. 16-4714) were purchased from eBioscience (San Diego, CA, USA). R-phycocerythrin (PE)-conjugated mouse anti-human Mac-1 (clone ICRF44; BD) with matched pairs of PE-conjugated mouse IgG isotype control (clone MOPC-21) antibody. Analysis was performed using FACSCalibur and CELLQUESTPRO software (BD), recording 10,000 cells in each individual sample. Live cells were gated based on size (FSC) and granularity (SSC), and then Mac-1 expression was analysed.

2.6 Adhesion assay

THP-1 cells were labelled with 0.2 mg/L calcein-AM for 30 min at 37°C, and labelled cells were seeded onto confluent HUVECs. After 2 h, co-cultured cells were washed with 1× PBS containing 1% bovine serum albumin, and images were obtained using an inverse optical microscope (Axiovert 25) and Axios Vision Release 4.7 software (Carl Zeiss Microlmaging GmbH, Oberkochen, Germany). Data were quantified using a Metamorph image analysis system (Molecular Devices, LLC, Downingtown, PA, USA).

2.7 En face immunohistochemistry

Fixation and tissue preparation were performed by systemic perfusion at a pressure of 180 cm H2O via the left ventricle with 500 mL of normal saline followed by 300 mL of 10% buffered formalin. After fixation, aortas were divided into 8–12 mm long segments and the proximal ends of segments were marked. Segment were then placed in 0.05% hydrogen peroxidase in methanol for 20 min at room temperature, rinsed three times with PBS, placed in boiling citrate buffer, and then cooled gradually under running water. After cooling, segments were incubated with anti-rat CD11b monoclonal antibody then with biotinylated goat anti-mouse IgG and reacted with horseradish peroxidase-conjugated streptavidin (Dako).

2.8 RNA isolation and RT–PCR

Total RNA was isolated with TRIzol reagent (Life Technologies), and reverse transcribed using the Improm II reverse transcription system according to the manufacturer’s instructions. Reverse transcribed cDNA was amplified by PCR using specific primers. PCR conditions and the information on the gene sequence for the primers are listed in Supplementary material online. Table S1. PCR products were separated on 1.2% agarose gels and stained with EtBr solution.

2.9 Measurement of 5-LO activity and expression

As an indicator for 5-LO activity, LTB4 production in the culture media was quantified using a commercially available ELISA assay kit (R&D Systems, Minneapolis, MN, USA). To determine 5-LO expression, THP-1 cells were washed with ice-cold PBS and lysed in lysis buffer. Cellular proteins were
resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After blocking membranes with 1 × TBST containing 5% skim milk, they were probed with an anti-5-LO (1:1000) or anti-β actin antibody (1:10 000). Immune complexes were then detected using ECL reagents after a second incubation with secondary antibodies according to the manufacturer’s instructions.

2.10 Preparation of 5-LO promoter constructs

The plasmids carrying different sizes of the 5′ flanking promoter region from the genomic DNA were amplified by PCR using the specific upstream primers (−1128; 5′-AGA AGA GAG CTC ACT TAG ACT GTGCCA-3′; −614; 5′-AGTCGCG CTC TCAAGTCGATGCGTACGTA-3′; −409; 5′-AC CGTG GAG CTA GCCGTA CTC TCCTTG T T-3′; −213; 5′-TGAGGG AAA ATA AAA GCTAGC TAG AGA TGG G-3′), and the downstream primer (5′-ATGAGGGCTGG TAG ATC TAG TCGTCG -3′). Underlined are restriction enzyme sites. The amplified fragments were cloned into luciferase vector pGL3 basic (Promega, WI, USA). Primers were designed based on the sequence retrieved from GenBank Accession No. NM009662 and AF 393814. The 5-LO promoter sequence was analysed for any transcription primers with the required nucleotide substitutions and pGL3-5LO plasmid including protease inhibitors (1 mM PMSF, 1 mM pepstatin A). Samples were sonicated with a Misonixsonicator 3000 (Misonix, Farmingdale, NY, USA), centrifuged and diluted 10-fold in ChIP dilution buffer. After removing an aliquot (whole-cell extract input), the chromatin samples were incubated at 4°C overnight with antibodies against NF-κB: WT, 5′-TGGGAGCTGGCCA-3′; Mutant, 5′-TATCGTCTGGATA-3′; Sp1: WT, 5′-CAGGGGCCGAGCC-3′; Mutant, 5′-CAGTGTTCATGCC-3′. PCR was performed using two antiparallel primers with the required nucleotide substitutions and pGL3-5LO plasmid as a template, and the PCR product was then treated with DpnI endonuclease. The sequences of the mutation constructs were confirmed by bidirectional DNA sequencing.

2.11 Site-directed mutagenesis

Mutagenesis of NF-κB and Sp1 in the 5-LO promoter region of the pGL3-SLO plasmid was generated using a Quick change site-directed mutagenesis system (Stratagene, CA, USA). The mutated primers for NF-κB and Sp1 were as follows: NF-κB: WT, 5′-TGGGAGCTGGCCA-3′; Mutant, 5′-TATCGTCTGGATA-3′; Sp1: WT, 5′-CAGGGGCCGAGCC-3′; Mutant, 5′-CAGTGTTCATGCC-3′. PCR was performed using two antiparallel primers with the required nucleotide substitutions and pGL3-SLO plasmid as a template, and the PCR product was then treated with DpnI endonuclease. The sequences of the mutation constructs were confirmed by bidirectional DNA sequencing.

2.12 Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChiP) analysis was performed using the Millipore ChiP kit (Millipore, Billerica, MA, USA) following the manufacturer’s instructions with minor modifications. For each assay, THP-1 cells were inoculated into a 10-cm dish (a total of 5 × 10⁶ cells) and fixed with 1% of formaldehyde. Cell pellets were resuspended in SDS lysis buffer including protease inhibitors (1 mM PMSF, 1 μg/mL aprotinin and 1 μg/mL pepstatin A). Samples were sonicated with a Misonixsonicator 3000 (Misonix, Farmingdale, NY, USA), centrifuged and diluted 10-fold in ChiP dilution buffer. After removing an aliquot (whole-cell extract input), the chromatin samples were incubated at 4°C overnight with antibodies against NF-κB p65 (ab79970 abcam) or Sp1 (ab13370). The samples were then precipitated by binding to protein A-Agarose/Salmon Sperm DNA beads (Millipore, Billerica, MA, USA). The immunoprecipitated chromatin was analysed by PCR using the primers of a 5-LO gene promoter. Cycling parameters were 50°C for 1 min, 95°C for 30 s, followed by 40–45 cycles.

2.13 Transient transfection and luciferase assay

THP-1 cells were grown to 90–95% confluence in 12-well plates. One microgram of plasmid DNA and 2 µL of Lipofectamine LTX reagent (Invitrogen, CA, USA) were separately diluted in 50 µL of Opti-MEM medium (GIBCO, NY), mixed together, and incubated at room temperature for 30 min. Cells were then washed with serum-free medium before adding 400 µL of Opti-MEM medium, and then the diluted mixed solution was added to the cells. Plates were incubated at 37°C for 6 h, subsequently, the conditioned medium was removed, and then the cells were grown in fresh medium containing 10% FBS for 24 h. Cells were untreated or treated with LPS. Cell lysates were prepared using passive lysis buffer from the Promega assay system (Promega, WI) and were used for the measurement of luciferase activity according to the manufacturer’s instructions for the dual luciferase reporter assay (Promega, WI, USA). All firefly luciferase values were normalized to Renilla luciferase in order to compare the transfection efficiencies.

2.14 Measurement of nuclear localization of NF-κB and Sp1 phosphorylation

Cytoplasmic and nuclear extracts were separated using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher: Rockford, IL, USA) according to manufacturer instructions. Briefly, cells were harvested and subsequently washed with chilled PBS. Cytosolic proteins were first extracted by disrupting cell membranes, followed by centrifugation. Intact nuclei were washed by cold PBS and then lysed with high salt NE-PER buffer. NF-κB in cytosolic and nuclear fractions was determined by immunoblotting. Controls for cross-contamination of individual fractions were confirmed by immunoblotting with antibodies against β-actin (cytosolic extract) and Histone H1 (nuclear extract). For Sp1 phosphorylation, cell lysates extracted from monocytes were electrophoretically resolved and transferred to nitrocellulose membranes. Membranes were blocked in 5% skim milk in TBST and then incubated overnight with total Sp1 and phosphospecific Sp1 antibody.

2.15 Statistical analysis

The results are expressed as means ± SEM. Statistical significance was determined using Student’s t-test for unpaired observations between two groups or by ANOVA with Bonferroni correction for multiple group comparisons. P-values of <0.05 were considered significant.

3. Results

3.1 Role of 5-LO on adhesion of monocytes to endothelial cells

This study examined the role of 5-LO on monocyte adhesion to the endothelial surface surrounding the aortic orifice in mice using an enface method for optimal observation of the endothelial surface in the in vivo system. The number of monocytes adhering to the aortic endothelium was significantly increased in LPS-injected mice relative to vehicle-treated mice, which was markedly attenuated in the 5-LO−/− mice (Figure 1A). When monocyte adhesion to the endothelium was evaluated using primary monocytes isolated from mice in the ex vivo system, endothelial adhesion of primary monocytes isolated from LPS-treated WT mice was markedly increased, which was attenuated in primary monocytes isolated from 5-LO−/− mice treated with LPS (Figure 1B). These results indicate that 5-LO plays a key role in monocyte adhesion to the endothelium in vivo and ex vivo studies.

3.2 Role of Mac-1 on monocyte adhesion to endothelial cells

The endothelial adhesion of mouse primary monocytes or THP-1 cells stimulated with LPS was significantly increased in a dose-dependent manner (Supplementary material online, Figure S1A and B). To determine the functional role of adhesion molecules expressed on monocytes, we examined the mRNA expression of various adhesion molecules on THP-1 cells stimulated with LPS. As shown in Figure 2A, LPS-stimulated THP-1 cells showed an increased expression of adhesion molecule, Mac-1 mRNA in a time-dependent manner; however, the mRNA expression of LFA-1, VLA-4, or PSGL-1 was not increased. Consistent with the up-regulation of Mac-1 mRNA by LPS, flow cytometric analysis also demonstrated a time-dependent increase in Mac-1 protein expression.
expression after LPS treatment (Figure 2B). In addition, to determine whether monocyte adhesion to endothelial cells is mediated by the expression of Mac-1, THP-1 cells were pre-treated with anti-human Mac-1 antibody or anti-mouse IgG isotype control antibody, and then LPS-stimulated monocytes were co-cultured with HUVECs. As shown in Figure 2C, the LPS-induced monocyte adhesion to HUVECs was significantly inhibited in a concentration-dependent manner by pre-treatment with anti-human Mac-1 antibody, demonstrating the involvement of Mac-1 in monocyte adhesion to endothelial cells.

3.3 Role of 5-LO on Mac-1 expression in monocytes

To assess the role of 5-LO on LPS-induced Mac-1 expression in monocytes, THP-1 cells were pre-treated with MK886, a 5-LO inhibitor, and then stimulated with LPS for 24 h. As shown in Figure 3A and B, LPS-induced expression of Mac-1 mRNA and protein was significantly inhibited by pre-treatment with MK886 in a concentration-dependent manner. Moreover, in an additional study to determine the potential involvement of PPAR-α, a specific antagonist for PPAR-α, GW6471, had no effect on LPS-induced Mac-1 expression (Supplementary material online, Figure S2), suggesting a role for 5-LO in LPS-induced Mac-1 expression. These results were also confirmed using primary monocytes isolated from WT and 5-LO(-/-) mice treated with LPS. As shown in Figure 3C, Mac-1 expression in primary monocytes of WT mice treated with LPS was significantly increased, which was markedly attenuated in primary monocytes of LPS-treated 5-LO(-/-) mice, demonstrating an inhibitory effects of 5-LO in LPS-induced Mac-1 expression in vivo condition.

3.4 Involvement of BLT₁-receptor in 5-LO-mediated Mac-1 expression

To assess the 5-LO signalling pathway involved in LPS-induced Mac-1 expression, we examined the effects of BLT₁- and cysLT₁-receptor antagonists. LPS-induced Mac-1 expression in THP-1 cells and primary monocytes of mice (Figure 4A and B and Supplementary material online, Figure S3A) was inhibited by pre-treatment with U-75302, a BLT₁-receptor antagonist, but not by REV-5901, a cysLT₁-receptor antagonist, which suggested that the BLT₁-receptor on monocytes plays a pivotal role in LPS-induced Mac-1 expression. To confirm the role of BLT₁-receptor, we determined the effects of LTB4 on Mac-1 expression. As shown in Figure 4C and D, LTB4 increased Mac-1 mRNA and protein expression dose-dependently in THP-1 cells as well as in primary monocytes of mice (Supplementary material online, Figure S2B). These results suggest that 5-LO metabolites induce Mac-1 expression via BLT₁-receptor on monocytes.
3.5 Involvement of TLR4 in LPS-induced 5-LO expression

To examine the effect of LPS on the regulation of 5-LO activity, monocytes were stimulated with LPS for the indicated time, and then 5-LO product formation was determined as a marker for 5-LO activity. As shown in Figure 5A, LTB₄ production was markedly increased at 1 h of LPS treatment and remained high up to 24 h. In parallel, 5-LO mRNA and protein expression were increased at 1 h of LPS treatment, and then further increased with maximum induction of the mRNA and protein expression of 5-LO at 4 and 12 h, respectively (Figure 5B and C). The LPS-induced 5-LO expression was significantly attenuated by pre-treatment an anti-TLR4 antibody in a concentration-dependent manner (Figure 5D), suggesting a pivotal role of TLR4 on LPS-induced 5-LO expression in monocytes.

3.6 Involvement of NF-κB and Sp1 in LPS-induced 5-LO transcription

As shown in Figure 6A, promoters containing progressive 5’ deletions from nt-1128 to nt-213 remained highly inducible in response to LPS. The luciferase activities of LPS-stimulated p5LO-213 were ≈2.3 (± 0.7)-fold higher than those of controls. A sequence motif search of GenomeNet further identified transcription factor binding sites,
including NF-κB and Sp1 sites, within the nt -213 region of the 5-LO gene\textsuperscript{28} (Figure 6B). To confirm the effects of NF-κB and Sp1 on LPS-induced 5-LO transcription, the luciferase activities of mutant constructs of NF-κB and Sp1-binding sites in p5LO-213 promoter were measured. Mutation of the NF-κB or Sp1-binding sites in the p5LO-213 promoter reporter constructs greatly reduced promoter activity by 70–80% (Figure 6C), indicating the importance of these cis-acting elements and their involvements in 5-LO promoter activation by LPS in THP-1 cells.

To directly assess whether LPS increases binding of NF-κB and Sp1 to the corresponding binding sites of the 5-LO promoter, ChIP assays were carried out. Nuclear extracts from THP-1 cells stimulated with LPS were used for immunoprecipitation with anti-NF-κB p65 or anti-Sp1 antibodies. PCR amplification of the 5-LO promoter showed an increased NF-κB or Sp1 binding to the 5-LO promoter (Figure 6D). Moreover, the nuclear translocation of NF-κB and Sp1 phosphorylation by LPS was significantly increased in a time-dependent manner (Figure 6E and F), demonstrating a role for NF-κB and Sp1 in LPS-induced 5-LO expression.

4. Discussion

In this study, we found that monocyte adhesion to the aortic endothelium was significantly higher in LPS-treated WT mice than in controls, and this was significantly attenuated in 5-LO\textsuperscript{−/−} mice. The results of our in vitro study showed that LPS, a TLR4 ligand, enhanced monocyte adhesion to endothelial cells, which was mediated by Mac-1 on monocytes. Furthermore, the increased expression of Mac-1, along with monocyte adhesion to endothelial cells by LPS, was significantly attenuated by inhibiting 5-LO with MK886, a 5-LO inhibitor, and in primary monocytes isolated from 5-LO\textsuperscript{−/−} mice, thus confirming a role of 5-LO in LPS-mediated Mac-1 expression. In addition, we found that LPS-enhanced 5-LO expression in monocytes was regulated by the transcription factors, NF-κB and Sp1. These results support the hypothesis that 5-LO contributes to monocyte adhesion to the endothelium via Mac-1 expression on monocytes, which is mediated by increased 5-LO expression through NF-κB and Sp1 signaling pathways.
S-LO in atherosclerotic lesions co-localizes to macrophage-derived foam cells, and LTβ4 receptor blockade inhibited atherosclerotic progression by inhibiting monocyte recruitment. Thus, the modulation of monocyte adhesion to the endothelium, which is one of the earliest detectable cellular responses and processes leading to macrophage foam cell formation, could be an important target for preventing the development and progression of atherosclerosis. Our NEMO in vivo studies demonstrated that S-LO deficiency markedly attenuated LPS-induced monocyte adhesion to aortic endothelium relative to controls, and these results were also confirmed using primary monocytes isolated from LPS-injected S-LO−/− mice. These results suggest a pivotal role of S-LO in monocyte adhesion to the vascular endothelium.

In vitro study, we evaluated the effects of LPS on monocyte adhesion to endothelial cells, and found that the endothelial adhesion of LPS-stimulated monocytes was increased in a concentration-dependent manner (Supplementary material online, Figure S1). The adhesion of circulating monocytes to the endothelium involves a tightly regulated multistep process that is mediated by a combination of cell surface adhesion
Our present study suggested a potential role of adhesion molecules on monocytes for adhesion to endothelial cells in response to LPS because LPS was found to induce the expression of Mac-1 in THP-1 monocytes. Based on the facts that the interaction between Mac-1 and ICAM-1 is known to be required for cell adhesion, LPS-induced cell adhesion appears to be resulted from the up-regulated expression of Mac-1 on monocytes. In the present study, a functional role of Mac-1 on endothelial adhesion of monocytes was confirmed by blocking monocyte adhesion to endothelial cells with a Mac-1 antibody. Thus, the participation of Mac-1 in LPS-mediated monocyte adhesion to endothelial cells suggests a pivotal role of Mac-1 in the pathogenesis of atherosclerosis.

Our present study indicated that the LPS-induced expression of Mac-1 was attenuated by inhibiting 5-LO pathways with MK886, a 5-LO inhibitor, and also inhibited in the primary monocytes isolated from 5-LO−/− mice, which suggest that 5-LO is involved in Mac-1 expression. Furthermore, when THP-1 cells were pre-treated with antagonists of BLT1-receptor or cysteine XT-receptor followed by stimulation with LPS, U-75302 (a BLT1-receptor antagonist), but not REV-5901 (a cysteine XT-receptor antagonist), attenuated LPS-induced expression of Mac-1 mRNA and protein. Likewise, LT4, a ligand for BLT1-receptor, was capable of stimulating the mRNA and protein expression of Mac-1 in THP-1 monocytes, suggesting that LT4/BLT1-receptors are major contributors of Mac-1 expression in monocytes.

To evaluate the role of LPS on the regulation of 5-LO activity, we stimulated monocytes with LPS, and then 5-LO product formation was determined as a marker for 5-LO activity. As shown in Figure 5A, LPS induced LTB4 production in association with an increased expression of 5-LO mRNA and protein. The LPS-induced 5-LO expression was...
significantly attenuated by pre-treatment an anti-TLR4 antibody. In the previous study, LPS induced expression of 5-LO activating protein gene in monocytes via a transcriptional mechanism. Thus, our present study provides an important insight into the mechanism of TLR4 signalling in LPS-induced modulation of 5-LO expression in monocytes via transcriptional mechanisms. In the present study, the promoter activity of LPS-stimulated p5LO-213 was \( \approx 3.2 \) \( \pm 0.5 \) times higher than in untreated control THP-1 cells. In our previous study, sequence analysis of the region between nt -213 and +1 in the 5-LO promoter demonstrated the presence of consensus binding sites for NF-kB and Sp1. This result was confirmed by observations made during a site-directed mutagenesis study, in which mutations of the NF-kB and Sp1 binding sites in the 5-LO promoter completely abolished the responsiveness of THP-1 cells to LPS, indicating the importance of these cis-acting elements and their involvement in 5-LO promoter activation by LPS in THP-1 cells. ChIP assays of the present study, LPS enhanced binding of NF-kB and Sp1 to the corresponding binding sites of the 5-LO promoter. Moreover, the nuclear translocation of NF-kB and Sp1 phosphorylation by LPS was significantly increased, demonstrating that NF-kB and Sp1 are essential transcription factors of LPS-induced 5-LO transcription. In another series of experiments, LTB4-induced Mac-1 expression was not inhibited by pre-treatment with an NF-kB inhibitor or a Sp1 inhibitor (Supplementary material online, Figure S4). Thus, it was suggested that NF-kB and Sp1 do not directly interact with Mac-1, but are specific for Mac-1 expression via an activation of the 5-LO signalling pathway.

In summary, this study provides important evidences that 5-LO deficiency markedly inhibits monocyte adhesion to endothelial cells and suppresses the expression of Mac-1 on monocytes. Furthermore, LPS-induced 5-LO expression was found to be regulated at the

Figure 6  Determination of transcription factors involved in LPS-induced 5-LO expression in monocytes. (A) THP-1 cells were transiently co-transfected with various promoter constructs and an empty luciferase vector, pRL CMV for 24 h, and then stimulated with LPS for 4 h. Relative luciferase activities were quantified, and presented as the mean \pm SEM of representative experiments performed in triplicate. **P < 0.01, vs. value in corresponding control. (B) The 213-bp sequence of the S′-flanking region of the 5-LO promoter region. The transcription start site is indicated by +1. The arrow indicates the translation start site. The underlined sequences are the possible transcription factor binding sites, as predicted by GenomeNet. (C) THP-1 cells were transiently co-transfected with the empty luciferase vector pRL CMV and promoter constructs or mutant constructs of the NF-kB and Sp1 binding sites; WT5LO (wt5LO), NF-kB mutant (mtNF-kB), Sp1 mutant (mtSp1). The cells were stimulated with LPS for 4 h, and then assayed for relative luciferase activity. **P < 0.01 vs. value in a control. (D) Binding of NF-kB and Sp1 to the 5-LO promoter was detected with the ChIP assay. Immunocomplexes of NF-kB and Sp1 associated with DNA were obtained from LPS-stimulated THP-1 cells for the indicated time. Specific DNA fragments were quantified by PCR, as detailed in Methods. DNA purified from lysates incubated without antibody was used as input control (Input). (E) The levels of cytosolic and nuclear NF-kB p65 were determined by immunoblotting in THP-1 cells stimulated with LPS for the indicated time. Histone H1 and \( \beta \)-actin were used as an internal control for nuclear and cytosolic protein, respectively. (F) The lysates of LPS-stimulated THP-1 cells were analysed for total and phosphorylated levels of Sp1. Each bar in right panel represents the means \pm SEM from three to five independent experiments. *P < 0.05, **P < 0.01 vs. value at time 0.
transcriptional level in monocytes by activation of NF-kB and Sp1 pathways. Collectively, these results suggest that 5-LO expression in monocytes is modulated via NF-kB and Sp1 pathways, and that 5-LO plays a pivotal role in LPS-mediated monocyte adhesion to the endothelium via an increased expression of Mac-1, which may participate in the pathogenesis of atherosclerosis.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

**Conflict of interest:** none declared.

**Funding**

This study was supported by the MRC program of MEST/KOSEF (2005-0049477), and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0005969).

**References**

10. 5-LO mediates monocyte adhesion to ED via Mac-1.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

**Conflict of interest:** none declared.

**Funding**

This study was supported by the MRC program of MEST/KOSEF (2005-0049477), and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0005969).

**References**

10. 5-LO mediates monocyte adhesion to ED via Mac-1.