Lipopolysaccharide augments the uptake of oxidized LDL by up-regulating lectin-like oxidized LDL receptor-1 in macrophages

Ekhtear Hossain · Akinobu Ota · Sivasundaram Karnan · Miyuki Takahashi · Shahnewaj B. Mannan · Hiroyuki Konishi · Yoshitaka Hosokawa

Abstract There is a growing body of evidence supporting an intimate association of immune activation with the pathogenesis of cardiovascular diseases, including atherosclerosis. Uptake of oxidized low-density lipoprotein (oxLDL) through scavenging receptors promotes the formation of mature lipid-laden macrophages, which subsequently leads to exacerbation of regional inflammation and atherosclerotic plaque formation. In this study, we first examined changes in the mRNA level of the lectin-like oxLDL receptor-1 (LOX-1) in the mouse macrophage cell line RAW264.7 and the human PMA-induced macrophage cell line THP-1 after LPS stimulation. LPS significantly up-regulated *LOX-1* mRNA in RAW264.7 cells; LOX-1 cell-surface protein expression was also increased. Flow cytometry and fluorescence microscopy analyses showed that cellular uptake of fluorescence (Dil)-labeled oxLDL was significantly augmented with LPS stimulation. The augmented uptake of Dil-oxLDL was almost completely abrogated by treatment with an anti-LOX-1 antibody. Of note, knockdown of Erk1/2 resulted in a significant reduction of LPS-induced LOX-1 up-regulation. Treatment with U0126, a specific inhibitor of MEK, significantly suppressed LPS-induced expression of LOX-1 at both the mRNA and protein levels. Furthermore, *LOX-1* promoter activity was significantly augmented by LPS stimulation; this augmentation was prevented by U0126 treatment. Similar results were also observed in human PMA-induced THP-1 macrophages. Taken together, our results indicate that LPS up-regulates LOX-1, at least in part through activation of the Erk1/2 signaling pathway, followed by augmented cellular oxLDL uptake, thus highlighting a critical role of TLR4-mediated aberrant LOX-1 signaling in the pathogenesis of atherosclerosis.

Keywords LPS · LOX-1 · Atherosclerosis · Erk1/2 · Molecular biology

E. Hossain · A. Ota · S. Karnan · M. Takahashi · S. B. Mannan · H. Konishi · Y. Hosokawa
Department of Biochemistry, Aichi Medical University School of Medicine, Nagakute, Aichi, Japan

A. Ota (✉)
Department of Biochemistry, Aichi Medical University School of Medicine, 1-1 Yazakokarimata, Building #2, Room 362, Nagakute, Aichi 480-1195, Japan
e-mail: aota@aichi-med-u.ac.jp

M. Takahashi
Division of Hematology, Department of Internal Medicine, Aichi Medical University School of Medicine, Nagakute, Aichi, Japan

Abbreviations

ABCA1	ATP-binding cassette, sub-family A, member 1
AP-1	Activator protein 1
CAPE	Caffeic acid phenethylester
DAPI	4′,6-Diamidine-2′-phenylindole dihydrochloride
DCFH-DA	2,7-Dichlorofluorescin diacetate
Dil	1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate
Erk1/2	Extracellular signal-regulated protein kinases 1 and 2
FACS	Fluorescence-activated cell sorting
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
JNK	C-Jun N-terminal kinases

LOX-1	Lectin-like oxidized low-density lipoprotein receptor-1
MAPK	Mitogen-activated protein kinase
MEK	MAPK/Erk kinase
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B cells
oxLDL	Oxidized low-density lipoprotein
SR-A	Scavenger receptor A

Introduction

Atherosclerosis, which is globally recognized as a major cause of cardiovascular disease, results from a passive accumulation of cholesterol in the artery wall. There is a growing body of evidence supporting an intimate association of immune activation and atherogenesis [1–4]. In the initial step of atherogenesis, monocytes adhere to the endothelial monolayer, migrate into the intima during endothelial dysfunction, and mature to macrophages, which actively uptake low-density lipoprotein (LDL) and/or modified LDL, such as oxidized LDL (oxLDL). Uptake of oxLDL through scavenging receptors (SRs) leads to the formation of mature lipid-laden macrophages, which are called foam cells. Consequently, these cells are further activated and can supply tissue factor pro-coagulants and pro-inflammatory cytokines, thereby promoting further regional inflammation and atherosclerotic plaque formation [4–6].

Lectin-like oxidized LDL receptor-1 (LOX-1) was initially identified as a major SR for oxLDL [7, 8]. Although basal LOX-1 expression level in monocytes/macrophages is thought to be lower than that in the endothelium, over-expression of LOX-1 has been observed in endothelial cells, intimal smooth muscle cells, and macrophages in human atherosclerotic lesions [9]. In addition, ApoE$^{-/-}$ mice overexpressing LOX-1 have been reported to show increases in atheroma-like lesions [10], whereas macrophages from LOX-1 knockout mice exhibited a marked reduction in migration compared to those from wild-type mice both in vitro and in vivo [11]. Thus, accumulating evidences implicate the involvement of LOX–1 in the pathogenesis of atherosclerosis.

Recently, TLR4, which recognizes bacterial LPS, has been reported to mediate LOX-1 up-regulation in human monocyte-derived dendritic cells [12]. Another report showed that LPS increases lipid accumulation, which results in enhancement of foam cell formation [13]. However, to date, the role of LOX-1 in cellular uptake of oxLDL and the molecular mechanisms underlying LPS-induced up-regulation of LOX-1 after LPS stimulation are poorly understood. Given the association between the expression of LOX-1 and the pathophysiology of atherosclerosis, it would be of particular interest to determine whether up-regulation of LOX-1 indeed contributes to cellular uptake of oxLDL and also to determine how LOX-1 is up-regulated in activated macrophages.

In the present study, we examined LOX-1 expression and cellular uptake of oxLDL after LPS stimulation in the mouse macrophage cell line RAW264.7 and the human PMA-induced macrophage cell line THP-1. To our knowledge, the present study is the first to report the involvement of LOX-1 in the cellular uptake of oxLDL through LPS-mediated ERK signaling. We also discuss a possible molecular mechanism underlying LPS-induced LOX-1 up-regulation.

Materials and methods

Reagents

RPMI-1640, penicillin–streptomycin solution, SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), and caffeic acid phenethylester (CAPE, NF-κB inhibitor) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). LPS was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Protease-free cell dissociation buffer was obtained from Life Technologies Japan (Tokyo, Japan). Goat anti-mouse and anti-human LOX-1/OLR1 antibodies were obtained from R & D Systems (Minneapolis, MN, USA). PE-labeled anti-mouse LOX-1, anti-mouse SR-A, anti-mouse CD36, and isotype-matched control antibody were obtained from Biolegend (San Diego, CA, USA). All other primary and secondary antibodies for Western blot analysis and U0126 (MEK inhibitor) were obtained from Cell Signaling Technology Japan, K.K. (Tokyo, Japan). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate(Dil)-labeled human oxLDL (Dil-oxLDL) was obtained from KALEN BioMedical, LLC (Montgomery Village, MD, USA). Human unlabeled oxLDL was obtained from Biomedical Technologies, Inc. (Stoughton, MA, USA).

Cell culture

RAW264.7 cells (RAW cells, a mouse macrophage cell line), kindly provided by Takashi Yokochi (Aichi Medical University School of Medicine, Aichi, Japan), were maintained in RPMI1640 medium supplemented with 5 % FBS and penicillin–streptomycin at 37 °C in 5 % CO$_2$ humidified air. THP-1 cells (human acute monocytic leukemia cell line), kindly provided by Hiroshi Miwa (Aichi Medical University School of Medicine, Aichi, Japan), were

Table 1 Primers applied for qRT-PCR

Gene symbol		Sequences (5'-)	Product size (bp)
Mouse			
LOX-1	Sense	5'- ACA AGA TGA AGC CTG CGA AT	213
	Antisense	5'- GCT GAG TAA GGT TCG CTT GG	
SR-A	Sense	5'- TCA CTG GAT GCA ATC TCC AA	194
	Antisense	5'- ACG TGC GCT TGT TCT TCT TT	
CD36	Sense	5'- TGC TGG AGC TGT TAT TGG TG	190
	Antisense	5'- TGG GTT TTG CAC ATC AAA GA	
GAPDH	Sense	5'- GAT GAC ATC AAG AAG GTG GTG A	199
	Antisense	5'- TGC TGT AGC CGT ATT CAT TGT C	
Human			
LOX-1	Sense	5'- AAG GAC CAG CCT GAT GAG AA	227
	Antisense	5'- GCT GAG ATC TGT CCC TCC AG	
GAPDH	Sense	5'- GAG TCA ACG GAT TTG GTC GT	209
	Antisense	5'- GAC AAG CTT CCC GTT CTC AG	

maintained in RPMI1640 medium supplemented with 10 % FBS and penicillin–streptomycin at 37 °C in 5 % CO_2. For differentiation of THP-1 monocytes into macrophages, cells were incubated in a medium containing 5 ng/ml PMA as described elsewhere with slight modifications [14, 15]. After incubation for 48 h, the cells were washed with PBS, and further incubated in a fresh culture medium for 24 h. Thereafter, the cells were treated with LPS (100 ng/ml) for the indicated durations. In order to achieve a sufficient augmentative effect of LPS on the uptake of ox-LDL and expression of LOX-1, RAW cells were treated with 100 ng/ml of LPS (unless otherwise stated).

Quantitative (q) RT-PCR analysis

RAW cells (2×10^5 cells/well) were seeded in 6-well plates and incubated for 24 h. The cells were incubated in a medium containing LPS (100 ng/ml) for the indicated times. qRT-PCR analysis using SYBR Green I was performed as described previously [16]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers used in this study are detailed in Table 1.

Flow cytometry analysis

RAW cells were stimulated with LPS as described above. Cells were scraped using enzyme-free cell dissociation buffer, washed with culture medium, and re-suspended in ice-cold PBS containing 2 % FBS, 1 mM EDTA, and 0.05 % NaN_3 (FACS buffer). The cells were then incubated with PE-labeled anti-mouse LOX-1 antibody, anti-mouse SR-A, anti-mouse CD36, or isotype-matched control antibody on ice for 1 h. After incubation, the cells were washed twice, re-suspended in FACS buffer, and examined using a FACSCantoII

system (BD Bioscience; Tokyo, Japan), in which 10,000 events (determined by forward and side scatter) were analyzed. The data provided represent the mean fluorescence intensity of triplicate determinations ± SE.

Western blot analysis

RAW cells (2×10^5 cells/well) were incubated with LPS as described above. The cells were washed with ice-cold PBS and lysed in loading buffer containing 125 mmol/l Tris (pH 6.8), 4 % SDS, 10 % β-mercaptoethanol, 20 % glycerol, and 0.02 % bromophenol blue. For analysis of the supernatant, the cells were incubated in a serum-free medium containing LPS (100 ng/ml) for the indicated times (0, 3, 6, 12, 24, and 36 h). After stimulation, cell culture supernatants were collected, centrifuged at $10,000 \times g$ for 5 min at 4 °C to remove cell debris, and then concentrated by ultrafiltration. Western blot analysis was performed as described previously [17]. Immune complexes were detected with ImmunoStar LD (Wako) using an LAS-4000 image analyzer (GE Healthcare; Tokyo, Japan). The band intensity was measured using ImageQuant TL software (GE Healthcare). Relative protein levels were calculated after normalization against an internal control, β-actin.

LOX-1 promoter constructs, transient transfection, and dual luciferase assay

The 3058-bp (−3,000 to +58) murine *LOX-1* promoter was amplified from genomic DNA using the forward primer 5'-GCTCCCCTTCCATTCTGTAT-3' and the reverse primer 5'-CTCCAAATTCCTGCTAAGAG-3', and cloned into the pGL3 basic vector (Promega). The LOX-1 promoter sequence was confirmed by automatic sequencing. RAW

cells (1×10^4 cells/well) were plated in a 96-well plate. The following day, the cells were transiently transfected with 0.15 µg of the *LOX-1* promoter construct and 0.03 µg of the phRL-TK vector (Promega) as an internal control using *Trans*IT-2020 (Mirus Bio LLC; Science Drive Madison, WI, USA). After 24 h, the cells were incubated with 1 µg/ml LPS for the indicated durations. The cells were lysed and assayed for both firefly and *Renilla* luciferase activity using the dual luciferase assay system (Promega) and a SpectraMAX M5 spectrophotometer (Molecular Devices; Sunnyvale, CA, USA).

Cellular uptake of Dil-OxLDL

Cellular uptake of oxLDL was measured using both confocal microscopy and FACS as described previously [18]. For confocal microscopy, RAW cells or THP-1 cells were seeded on a poly-L-lysine-coated culture cover glass (Matsunami Glass Ind., Ltd.; Osaka, Japan). After the cells were incubated overnight, they were incubated in a medium containing 5 ng/ml PMA as described in above. The cells were next incubated in a medium containing the indicated concentration (RAW cells, 100 ng/ml; THP-1 cells, 1,000 ng/ml) of LPS for 6 h, and then treated with 1 µg/ml Dil-oxLDL for another 3 h in the presence of LPS. For a neutralizing assay, cells were incubated with 10 µg/ml anti-LOX-1 antibody or normal goat IgG for 1 h prior to LPS (100 ng/ml) stimulation. Then, the cells were washed 3 times with PBS and fixed with 1 % ice-cold paraformaldehyde in PBS. For counter-staining, cell nuclei were stained with DAPI (l µg/ml) for 10 min at room temperature. The cells were then analyzed using an LSM710 confocal microscope (Carl Zeiss; Oberkochen, Germany). Cellular uptake of Dil-oxLDL was confirmed by competition with excess (50 µg/ml) unlabeled human ox-LDL (Biomedical Technologies). For FACS analysis, cells (2×10^5 cells/well) were seeded in a 6-well plate. The cells were treated with LPS (1, 10, 25, 50, 100, and 1,000 ng/ml) as described above for microscopy analysis. Following LPS stimulation for the indicated times (6, 9, and 15 h), the cells were detached, washed, re-suspended in PBS, and then examined as described above.

RNA interference

RAW cells (7.5×10^4 cells/well) or THP-1 cells (2.5×10^4 cells/well) were plated in a 6-well culture plate. The following day, the THP-1 cells were incubated in a medium containing 5 ng/ml PMA, as described above. The cells were then transfected using Lipofectamine RNAi/ MAX (Life Technologies Japan) to deliver 100 nM Erk1 or Erk2 small interference RNA (siRNA; Cell Signaling Technology Japan) according to the manufacturer's

protocol. siGENOME RISC-Free siRNA (Dharmacon-Thermo FisherScientific; Tokyo, Japan) was used as a negative control.

Statistical analysis

At least three independent experiments and three replication per experiment were performed. The results are expressed as the mean ± SE. Statistical significance between groups was determined using one-way ANOVA and Dunnett's comparison. Statistical analyses were performed using SPSS 15.0 (SPSS Inc; Chicago, IL, USA).

Results

LPS up-regulates LOX-1 in RAW cells

We first performed qRT-PCR to examine whether LPS affects the mRNA expression of three scavenger receptors, *LOX-1*, *SR-A*, and *CD36*, in RAW cells. Notably, *LOX-1* mRNA expression was found to significantly increase at 2 h after LPS stimulation ($p < 0.005$, Fig. 1a), whereas *SR-A* and *CD36* mRNA expressions were found to slightly increase at 8 h and 24 h after LPS stimulation, respectively (Fig. 1b, c). We next performed FACS analyses to examine whether LPS affects the cell-surface expression of LOX-1, SR-A, and CD36 proteins. As shown in Fig. 1d, cell-surface expression of LOX-1 increased 6 h after LPS stimulation, whereas no changes were observed in the expression of SR-A and CD36 (Supplementary Fig. S1). The cell-surface expression of SR-A, CD36, and LOX-1 increased at both 12 and 24 h after LPS stimulation (Fig. 1d and Supplementary Fig. S1). We also found that the LOX-1 expression at both the mRNA and protein levels significantly increased after LPS stimulation in a concentration-dependent manner (Supplementary Fig. S2a, b). Moreover, Western blot analysis revealed that the LOX-1 protein level increased over time after LPS stimulation (Fig. 1e). This increase was accompanied by an increase in the soluble LOX-1 protein level in the cell culture supernatant (Fig. 1e). These results suggest that LPS stimulation preferentially up-regulates LOX-1 rather than CD36 or SR-A.

LPS augments the cellular uptake of Dil-oxLDL through up-regulation of LOX-1

Several studies have reported an association between inflammation and atherosclerosis, in which macrophages uptaking oxLDL secrete pro-coagulants and pro-inflammatory cytokines/chemokines, thereby promoting further regional inflammation and atherosclerotic plaque formation [3–6, 19]. The results of these studies prompted us to
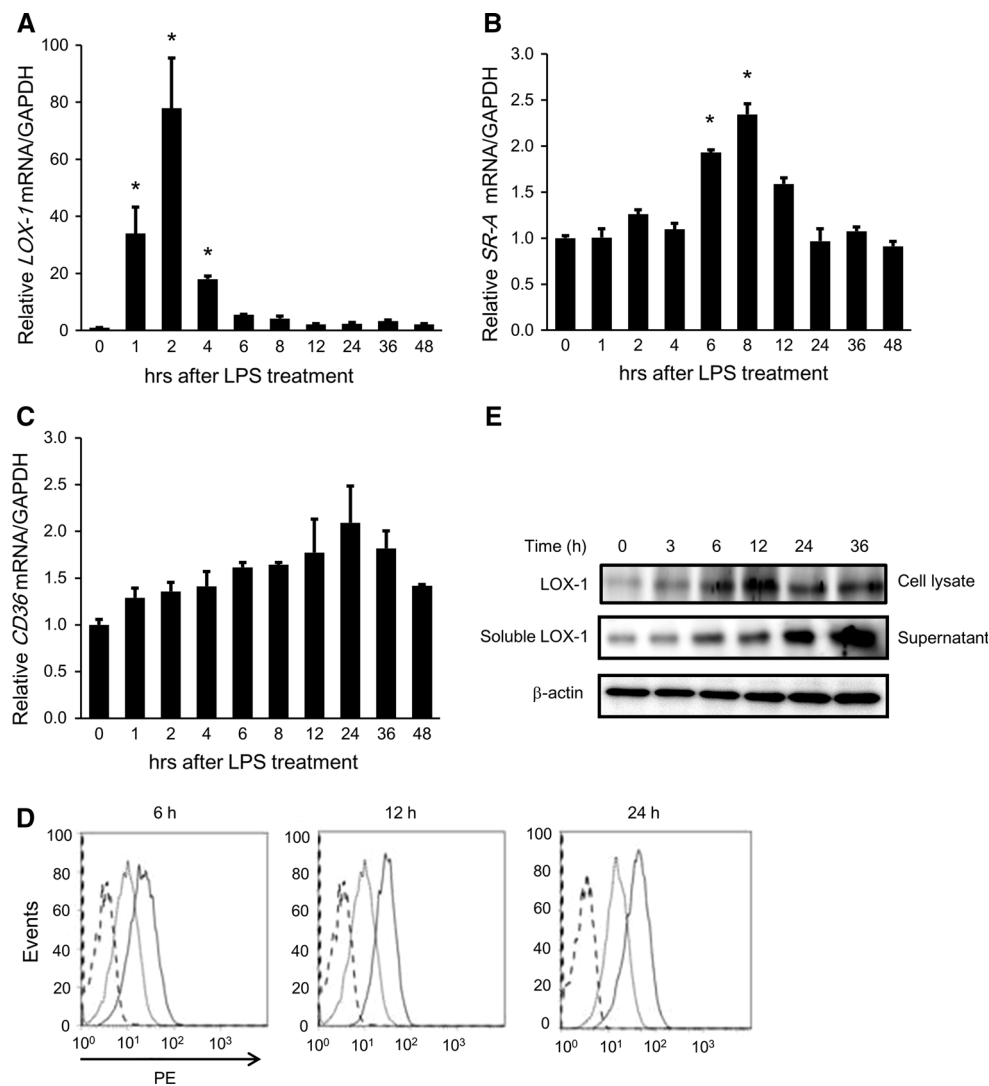
Fig. 1 Expression of LOX-1, SR-A, and CD36 in LPS-stimulated RAW cells. **a–c** mRNA expression of *LOX-1* (**a**), *SR-A* (**b**), and *CD36* (**c**) was examined using qRT-PCR analysis. RAW cells were stimulated with LPS (100 ng/ml) for the indicated durations. The relative mRNA expression levels are shown after normalization against *GAPDH* mRNA expression. The data are expressed relative to the mRNA levels found in the control (at 0 h after LPS), which was arbitrarily defined as 1. The values shown represent the mean ± SE ($n = 3$). *Asterisks* (******) or (*****) indicate a statistically significant difference at $p < 0.005$ or $p < 0.05$, respectively ($n = 3$). **d** The cell-surface expression of LOX-1 was examined using FACS analysis. RAW cells were stimulated with LPS (100 ng/ml) for 6 h (left panel), 12 h (middle panel), and 24 h (right panel). The cells were subsequently stained with PE-labeled anti-LOX-1 antibody. *Dashed line*, stained with control antibody; *dotted line*, stained with a specific antibody in the absence of LPS; *solid line*, stained with a specific antibody in the presence of LPS. **e** The protein expression level of LOX-1 was examined using Western blot analysis. RAW cells were stimulated with LPS (100 ng/mL) for the indicated durations, and 1 µg of cell lysate was subjected to Western blot analysis to detect both LOX-1 and β-actin proteins. In addition, 5 µl of 20-fold-concentrated cell culture supernatant was applied to detect soluble LOX-1 protein. The values shown represent the mean ± SE of three separate experiments

further investigate whether LPS affects the cellular uptake of oxLDL. As evidenced by the results of the FACS analysis (Fig. 2a), the uptake of Dil-oxLDL was significantly augmented 6 h after LPS stimulation in a concentration-dependent manner, relative to untreated cells. The cellular uptake of Dil-oxLDL was confirmed by competition with an excess amount of unlabeled oxLDL (data not shown). We next examined the effect of LPS on the formation of foam cells using Oil Red O staining-based microscopy.

Foam cell formation was enhanced after LPS treatment in a concentration-dependent manner (Supplementary Fig. S3). Therefore, we further investigated the involvement of LOX-1 in the cellular uptake of oxLDL. FACS analysis revealed that the LPS-augmented uptake of Dil-oxLDL was significantly prevented by treatment with an anti-LOX-1 antibody following 6 h ($p < 0.005$), 9 h ($p < 0.05$), and 15 h ($p < 0.05$) of LPS treatment, but not by treatment with a control antibody (Fig. 2b). The preventive effect
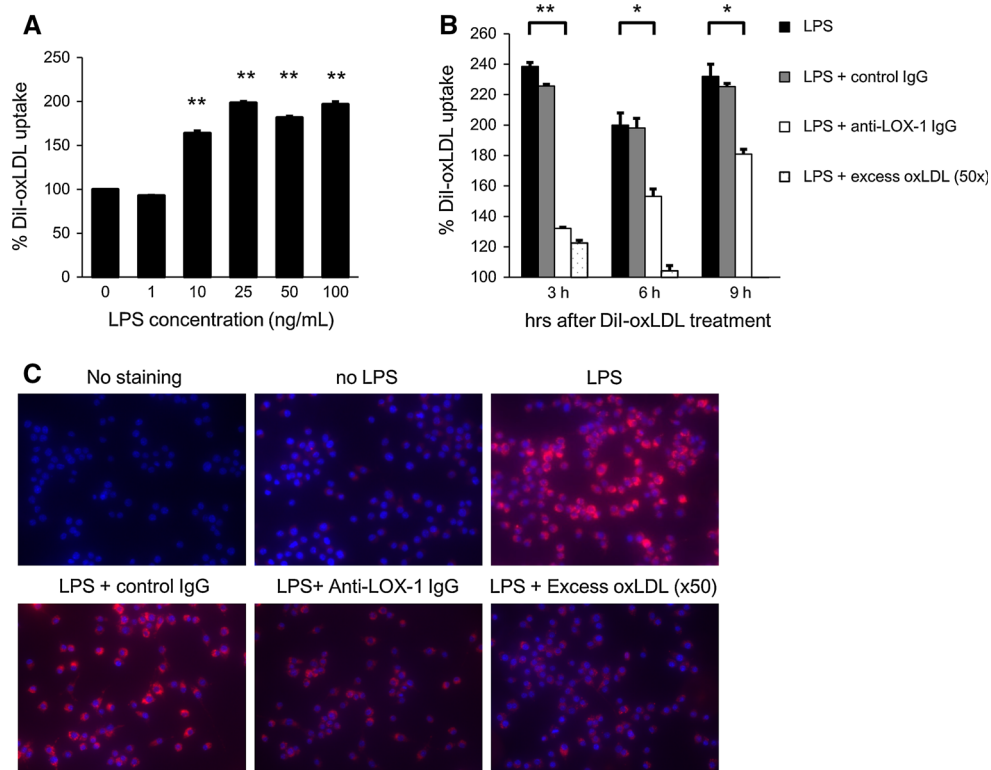
Fig. 2 Augmented effect of LPS on uptake of DiI-labeled oxLDL in RAW cells. **a–b** Cellular uptake of DiI-oxLDL was examined using flow cytometry. **a** RAW cells were stimulated with the indicated concentrations (0, 1, 10, 25, 50, and 100 ng/ml) of LPS for 6 h, and then further incubated with DiI-oxLDL (1 μg/ml) for 3 h. **b** RAW cells were incubated with a control goat antibody or anti-LOX-1 antibody at 5 μg/ml, or an excess amount of unlabeled oxLDL (50-fold excess amount) for 1 h prior to LPS stimulation. The cells were stimulated with LPS (100 ng/ml) for 6 h and then further incubated with DiI-oxLDL (1 μg/ml) for 3 h, 6 h, and 9 h in the presence of the corresponding antibody. The data are expressed relative to the mean

fluorescence intensity found in untreated cells, which was arbitrarily defined as 100 % ($n = 3$). *Asterisks* (**) or *Asterisk* (*) indicate a statistically significant difference at $p < 0.005$ or $p < 0.05$, respectively ($n = 3$). **c** RAW cells were treated as described in the legend for Fig. 2b. After the treatments, the cells were fixed with 1 % formaldehyde, stained with DAPI (1 μg/ml), and visualized using fluorescence microscopy. The DiI-oxLDL signals are shown in *red*. DAPI (*blue*) was used to counter-stain the cell nuclei. No staining, cells incubated without LPS and DiI-oxLDL; no LPS, cells incubated with DiI-oxLDL in the absence of LPS. Magnification: ×200

was found to decrease over time (Fig. 2b), suggesting that other SRs, such as SR-A or CD36, may contribute to the augmentation of DiI-oxLDL uptake. To further obtain experiment evidences for the involvement of LOX-1, we visualized the uptake of DiI-oxLDL using confocal fluorescence microscopy. The intracellular fluorescence signals of DiI-oxLDL clearly increased after LPS treatment; this increase was again blocked by anti-LOX-1 antibody treatment (Fig. 2c). Taken together, these results indicate that LPS augments the cellular uptake of oxLDL, at least in part through up-regulation of LOX-1.

Possible molecular mechanism underlying LPS-induced LOX-1 expression

It has been reported that NF-κB is involved in up-regulation of *LOX-1* mRNA after angiotensin II or pro-inflammatory stimulation in human endothelial cells [20], and that AP-1 plays an important role in up-regulation of *LOX-*

1 mRNA after PMA treatment [21]. To clarify the molecular basis underlying LPS-induced *LOX-1* expression, we examined the effect of CAPE (an NF-κB inhibitor) or MAPK inhibitors, including SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), and U0126 (MEK inhibitor), on mRNA expression of *LOX-1*. qRT-PCR analysis revealed that LPS-induced *LOX-1* mRNA expression was significantly attenuated by treatment of SP600125 ($p < 0.05$), U0126 ($p < 0.005$), and CAPE ($p < 0.005$) (Fig. 3a). Similarly, FACS analysis revealed that LPS-induced cell-surface expression of LOX-1 was significantly attenuated by treatment with SP600125 ($p < 0.05$), U0126 ($p < 0.005$), and CAPE ($p < 0.05$) (Fig. 3b). We unexpectedly found that the p38 inhibitor SB203580 augmented the cell-surface expression of LOX-1 without any changes in the *LOX-1* mRNA level (Fig. 3a and b). To further confirm the involvement of Erk on LPS-induced LOX-1 expression, we examined the cell-surface expression of LOX-1 after LPS under knockdown of Erk1/
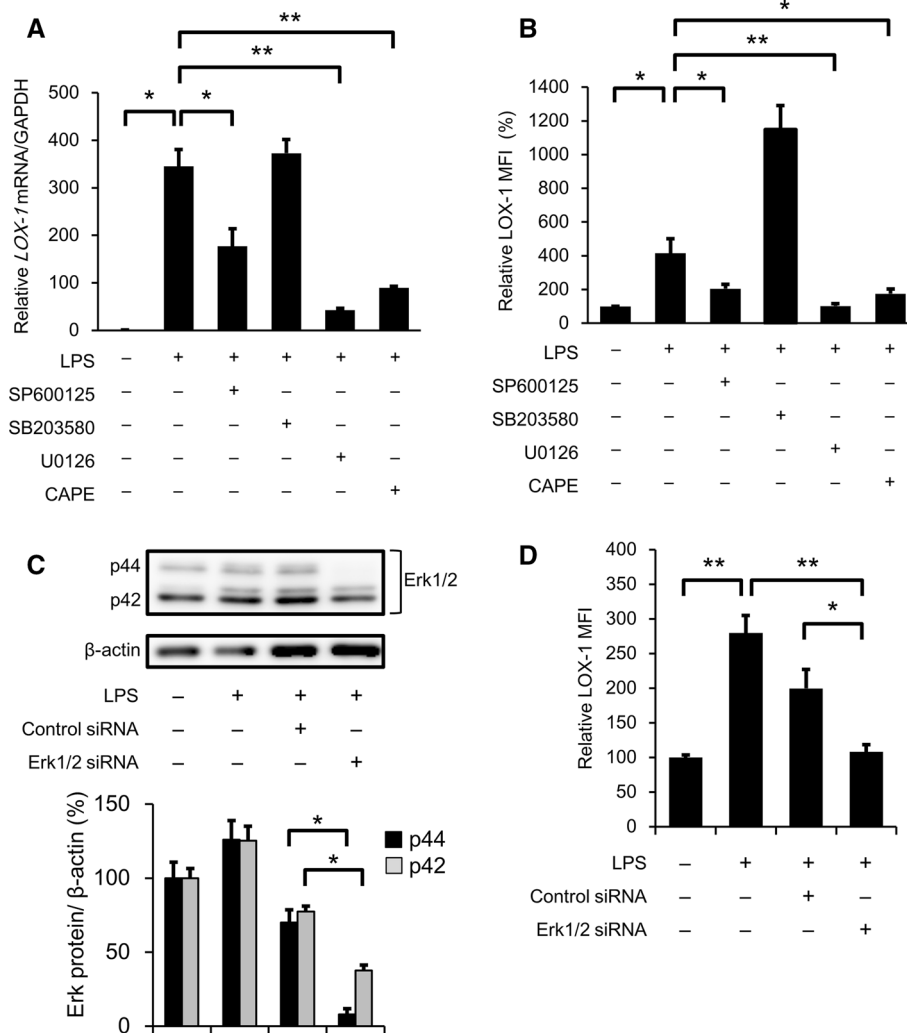
Fig. 3 Possible molecular mechanism underlying LPS-induced LOX-1 expression. **a** The effect of CAPE and MAPK inhibitors on LPS-induced *LOX-1* mRNA expression was examined using qRT-PCR analysis. RAW cells were treated with CAPE (NF-κB inhibitor, 10 μmol/l), SP600125 (JNK inhibitor, 5 μmol/l), SB203580 (p38 inhibitor, 10 μmol/l), or U0126 (MEK inhibitor, 10 μmol/l) for 1 h and then stimulated with LPS (100 ng/ml) for 2 h in the presence of CAPE (10 μmol/l), SP600125 (5 μmol/l), SB203580 (10 μmol/l), or U0126 (10 μmol/l), respectively. The data are expressed as described in the legend for Fig. 1a. **b** The effect of CAPE and MAPK inhibitors on LPS-induced cell-surface protein expression of LOX-1 was examined using FACS analysis. RAW cells were treated as described in **a**. After LPS stimulation (100 ng/ml) for 6 h, the cells were stained with PE-labeled anti-LOX-1 antibody. The data are expressed relative to the mean fluorescence intensity found in the untreated cells, which was arbitrarily defined as 100 % (*n* = 3). Asterisks (**) or (*) indicate a statistically significant difference at *p* < 0.005 or *p* < 0.05,

respectively. **c** Effect of Erk1/2 siRNA on protein expression of Erk1/2. RAW cells were transfected with 100 nmol/l siRNA specific to Erk1/2 or non-specific control siRNA. After 48 h of transfection, the cells were stimulated with LPS (100 ng/ml) for 2 h, and 5 μg of the protein was used to detect the total Erk1/2. β-actin was used as an internal control. After normalization of the β-actin protein, the values in the bar graph are calculated relative to the protein levels found in the untreated cells, which were arbitrarily defined as 100 %. An *asterisk* (*) indicates statistically significant differences of *p* < 0.05 (*n* = 3). *Black bar*, p44; *gray bar*, p42. **d** RAW cells were transfected as described in **c**. After 48 h of transfection, the cells were detached and stimulated with LPS (100 ng/ml) for 6 h. The cells were then stained with a PE-labeled anti-LOX-1 antibody. The data are expressed relative to the mean fluorescence intensity found in the untreated cells, which was arbitrarily defined as 100 % (*n* = 3). *Asterisks* (**) or (*) indicate a statistically significant difference at *p* < 0.005 or *p* < 0.05, respectively

2 using RNA interference. As shown in Fig. 3c and d, knockdown of Erk1/2 resulted in a significant reduction of the cell-surface LOX-1 levels, providing experimental evidence that LPS up-regulates LOX-1 through ERK signaling. We also found that LPS-induced SR-A mRNA and protein expression were prevented by either U0126 or

CAPE after 8 h of LPS treatment (Supplementary Fig. 4a and B), whereas CD36 mRNA and protein expression were prevented by SP600125 and CAPE after 24 h of LPS stimulation (Supplementary Fig. 4c and d).

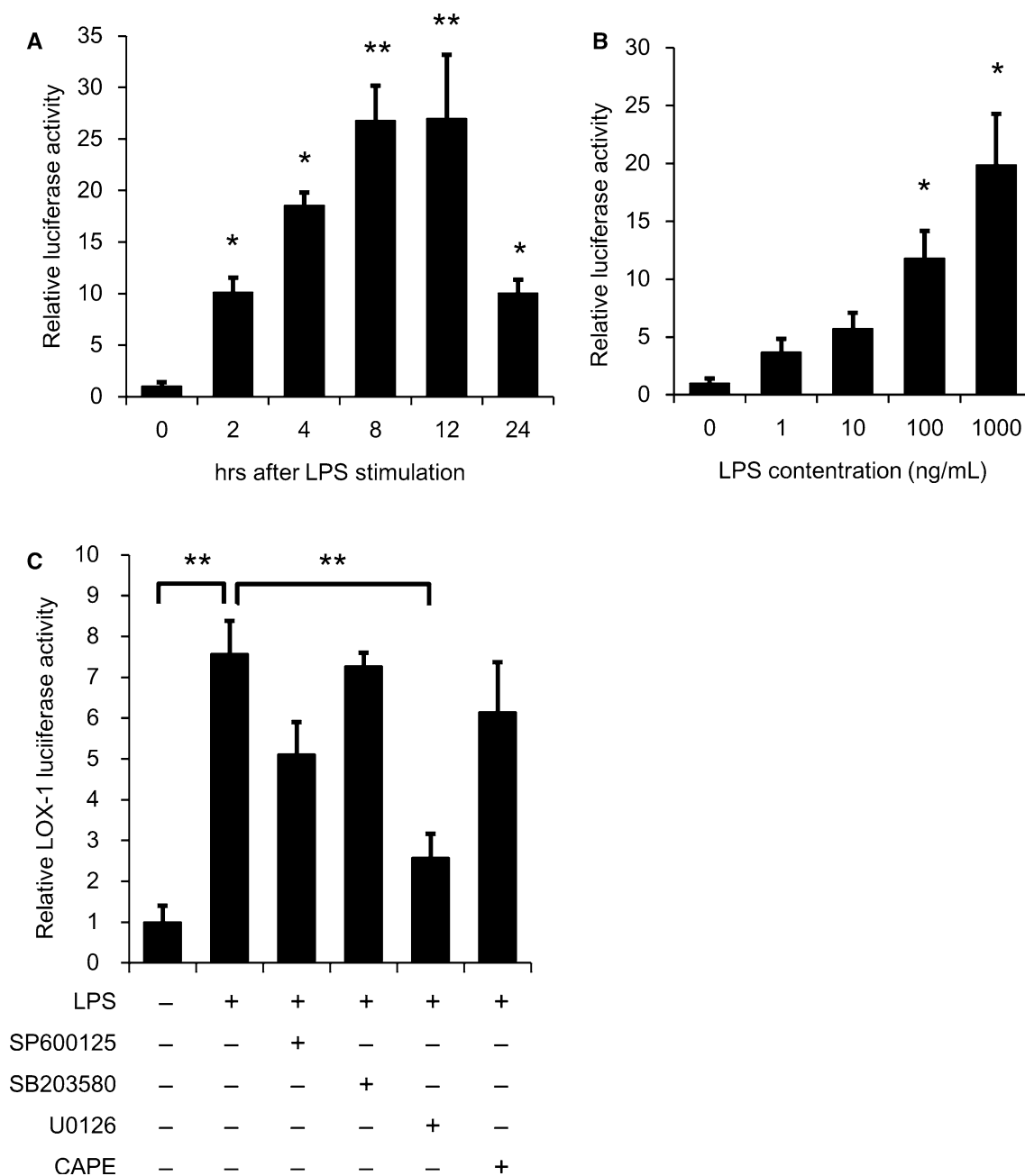To further investigate the LPS-induced LOX-1 expression at the transcriptional level, we examined *LOX-1*

Fig. 4 Analysis of *LOX-1* promoter activity after LPS stimulation. **a–b** RAW cells were co-transfected with 0.15 μg of the mouse LOX-1/ pGL3 vector for LOX-1 firefly luciferase activity and 0.03 μg of the phRL-TK vector for internal control *Renilla* luciferase activity. After 24 h, the cells were stimulated with LPS (100 ng/ml) for the indicated times (0, 2, 4, 8, 12, and 24 h) (**a**) and were stimulated with the indicated concentrations (0, 1, 10, 100, and 1,000 ng/ml) of LPS for 8 h (**b**). After normalization against *Renilla* luciferase activity, the *LOX-1* promoter activity was expressed relative to that found in the control group (at 0 h after LPS), which was arbitrarily defined as 1

(n = 6). **c** Effects of CAPE and MAPK inhibitors on *LOX-1* promoter activity. RAW cells were co-transfected as described above. After 24 h, the cells were treated with CAPE (10 μmol/L) or MAPK inhibitors (SP600125, 5 μmol/l; SB203580, 10 μmol/l; U0126, 10 μmol/l) for 1 h and then stimulated with LPS (100 ng/ml) as described in the legend for Fig. 3a. After stimulation with LPS (100 ng/ml) for 8 h, the relative *LOX-1* promoter activity was measured as described above. *Asterisks* (**) or (*) indicate a statistically significant difference at $p < 0.005$ or $p < 0.05$, respectively (n = 6)

promoter activity using the mouse *LOX-1* promoter region (−3,000 to +58). Luciferase reporter assays revealed that *LOX-1* promoter activity was increased at 2 h ($p < 0.05$), 4 h ($p < 0.05$), 8 h ($p < 0.005$), 12 h ($p < 0.005$), and

24 h ($p < 0.05$) after LPS stimulation (Fig. 4a). In addition, LPS increased *LOX-1* promoter activity 4 h after LPS stimulation in a concentration-dependent manner (Fig. 4b). We also examined the effect of SP600125, U0126, and
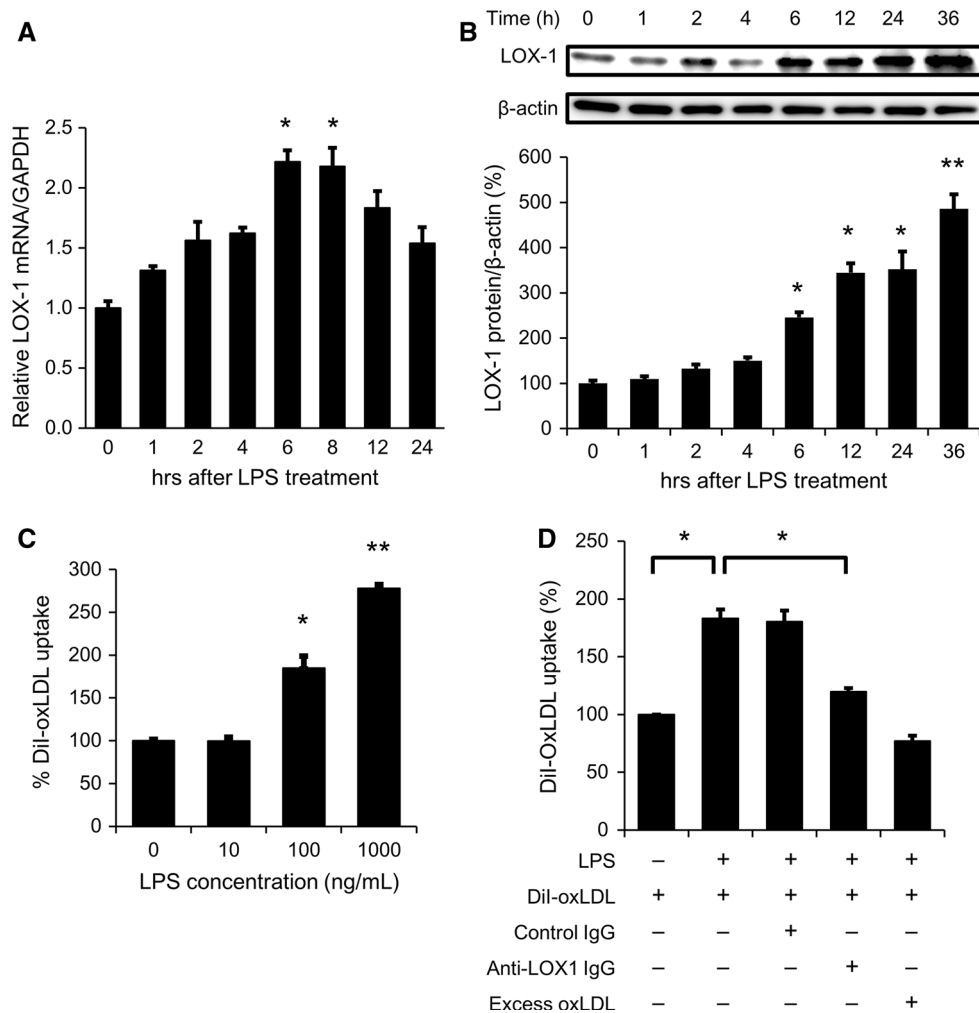
Fig. 5 Analyses of LOX-1 expression and uptake of DiI-labeled oxLDL after LPS treatment in PMA-stimulated human THP-1 macrophages. **a–g** THP-1 cells were incubated with PMA (100 ng/ml) for 48 h. After incubation, the cells were washed, cultured without PMA for a further 24 h, and used for further analyses. **a** *LOX-1* mRNA expression was examined using qRT-PCR. PMA-stimulated THP-1 cells were stimulated with the LPS (1 µg/ml) for the indicated durations (0, 1, 2, 4, 6, 8, 12, and 24 h). The relative mRNA expression levels are shown as described in the legend for Fig. 1a. The values shown represent the mean ± SE ($n = 3$). An *asterisk* (*) indicates a statistically significant difference at $p < 0.05$. **b** The protein expression level of LOX-1 was examined using Western blot analysis. PMA-stimulated THP-1 cells were stimulated with the LPS (1 µg/ml) for the indicated durations (0, 1, 2, 4, 6, 12, 24, and 36 h). In total, 10 µg of protein was subjected to Western blot analysis using a goat anti-human LOX-1 polyclonal antibody (0.1 µg/ml). β-actin protein was used as an internal control. After normalization against the β-actin protein level, the bar graph values were calculated relative to the protein levels found in the untreated cells (at 0 h after LPS), which were arbitrarily defined as 100 %. The values shown represent the mean ± SE of 3 separate experiments. *Asterisks* (**) or (*) indicate a statistically significant difference at $p < 0.005$ or $p < 0.05$, respectively ($n = 3$). **c** Cellular uptake of DiI-oxLDL after LPS stimulation was examined using flow cytometry. PMA-stimulated THP-1 cells were stimulated with the indicated concentration (0, 10, 100, and 1,000 ng/ml) of LPS for 9 h and then incubated with DiI-

oxLDL (1 µg/ml) for 3 h. The data are expressed as described in the legend for Fig. 2a. **d** The effect of an anti-LOX-1 antibody on uptake of DiI-oxLDL after LPS treatment was examined using flow cytometry. PMA-stimulated THP-1 cells were incubated with a control antibody or an anti-human LOX-1 antibody at 10 µg/ml, or an excess amount of oxLDL (50-fold excess amount) for 1 h prior to LPS treatment. The cells were stimulated with LPS (1 µg/mL) for 9 h and then incubated with DiI-oxLDL (1 µg/ml) for 3 h. The data are expressed as described in the legend of Fig. 2a. An *asterisk* (*) indicates a statistically significant difference at $p < 0.05$ ($n = 3$), compared to the control (at 0 h after LPS). **e** PMA-stimulated THP-1 cells were stimulated as described in **d**. The cells were analyzed and visualized using fluorescence microscopy as described in the legend for Fig. 2c. Magnification: ×400. **f–g** Effects of the MEK inhibitor U0126 (**f**) and Erk1/2 siRNA (**g**) on LPS-induced LOX-1 protein expression were examined using Western blot analysis. **f** PMA-stimulated THP-1 cells were treated with U0126 (10 µmol/l) for 1 h prior to LPS, and then stimulated with LPS (100 ng/ml) for 12 h in the presence of U0126 (10 µmol/l). **g** PMA-stimulated THP-1 cells were transfected with 100 nmol/l siRNA specific to Erk1/2 or non-specific control siRNA. After 48 h of transfection, the cells were stimulated with LPS (100 ng/ml) for 12 h, and 10 µg of protein was subjected to Western blot analysis to detect LOX-1 and total Erk1/2 protein, whereas 1 µg of the protein was used to detect the β-actin protein

Fig. 5 continued
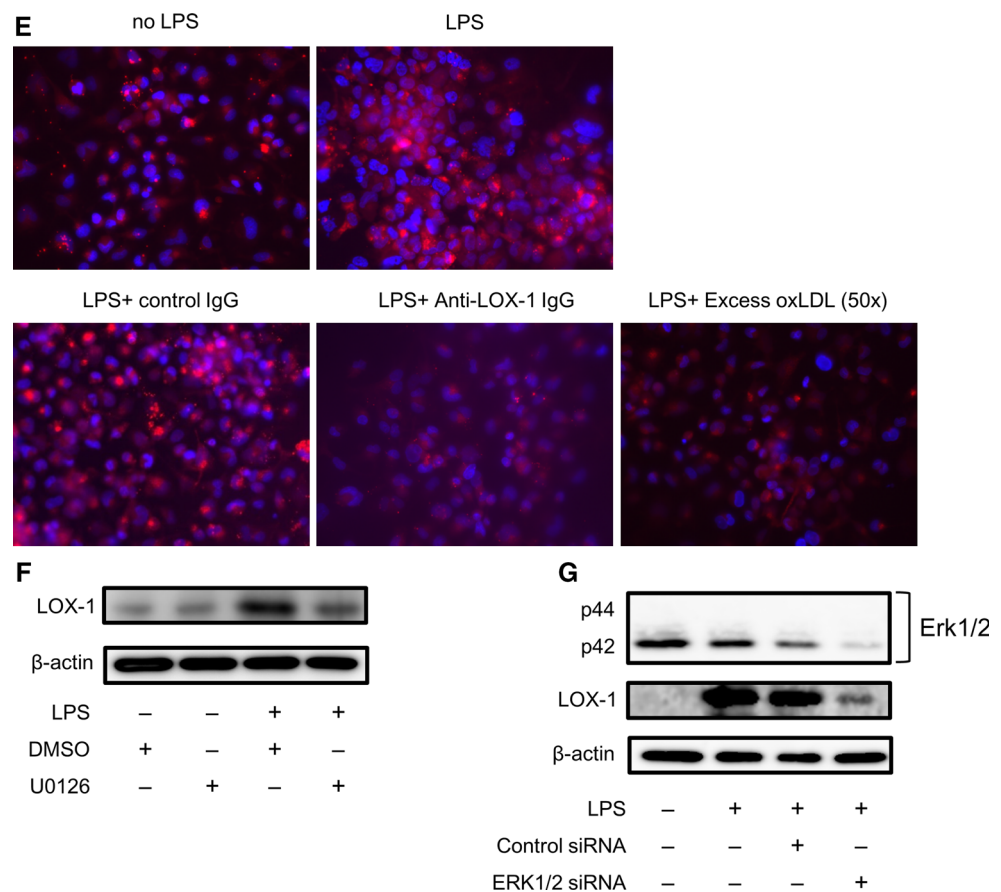
CAPE on *LOX-1* promoter activity. As shown in Fig. 4c, LPS-activated *LOX-1* promoter activity was significantly inhibited by treatment with the MEK inhibitor U0126, but not with the JNK inhibitor SP600125 or the NF-κB inhibitor CAPE. Taken together, these results strongly indicate that LPS-induced LOX-1 expression is activated by ERK signaling in RAW cells.

LPS augments DiI-oxLDL uptake through up-regulating LOX-1 in human THP-1 macrophages

Given our experimental results in mouse macrophage RAW cells, we next sought to examine whether LPS affects LOX-1 expression in the human monocyte/macrophage cell line, THP-1. As shown in Fig. 5a, b, LOX-1 expression was found to significantly increase at both mRNA and protein levels after LPS treatment of PMA-activated THP-1 macrophages. In addition, FACS analysis revealed that uptake of DiI-oxLDL was augmented 6 h after LPS treatment in a concentration-dependent manner (Fig. 5c). Accordingly, we further investigated the involvement of LOX-1 in the cellular uptake of oxLDL. The LPS-augmented uptake of DiI-oxLDL was almost completely abrogated by the treatment with an anti-LOX-1

antibody, but not with the control antibody (Figs. 5d). Confocal fluorescence microscopy revealed similar results in which the intracellular fluorescence signals of DiI-ox-LDL increased after LPS stimulation, and this increase was again abrogated by an anti-LOX-1 antibody (Fig. 5e), strongly suggesting that LOX-1 plays an important role in the uptake of oxLDL in human macrophages. Finally, we examined the effect of *ERK1/2* gene knockdown and treatment with the MEK inhibitor U0126 on LPS-induced LOX-1 expression using Western blot analysis. As shown in Fig. 5f, g, LPS-induced LOX-1 protein expression was prevented by both U0126 and Erk1/2 siRNA treatment. Taken together, these results strongly indicate that LPS up-regulates LOX-1 through activation of ERK signaling, thereby enhancing the cellular uptake of oxLDL in human and mouse macrophages.

Discussion

Immune activation and inflammation contribute to the development of atherosclerosis, in which activated macrophages are thought to play a pivotal role [1–4]. Activated macrophages express SRs, which mediate macrophage

uptake of oxLDL particles. Subsequently, uptake of oxLDL leads to intracellular cholesterol accumulation and the formation of foam cells, thereby promoting atherogenesis. However, the molecular basis underlying LPS-augmented uptake of oxLDL remains obscure. In the present study, we demonstrated for the first time that LOX-1 expression was significantly up-regulated after LPS stimulation in both mouse RAW264.7 cells and PMA-stimulated THP-1 human macrophages. We also demonstrated that LOX-1 is involved in uptake of oxLDL by activated macrophages.

LOX-1, initially identified as a receptor for oxLDL, has been shown to mediate oxLDL-induced vascular dysfunction, induce foam cell accumulations, and promote atheroma formation [10, 22, 23]. To date, *LOX-1* has been reported to be up-regulated by angiotensin II [20], pro-inflammatory molecules [24, 25], phorbol ester [24], oxLDL [26], fluid shear stress [27], and a ligand for a lymphocyte-expressed G protein-coupled receptor, lysophosphatidylcholine [28]. Our quantitative analyses revealed that *LOX-1* mRNA expression was significantly increased after 1, 2, and 4 h of LPS stimulation, whereas *SR-A* and *CD36* mRNA expressions were slightly increased after 6 and 8 or 24 h, respectively. Furthermore, our FACS analysis revealed that cell-surface expression of LOX-1 increased after 6, 12, and 24 h of LPS stimulation, whereas that of SR-A and CD36 increased after 12 and 24 h. These results suggest that the molecular basis underlying LPS-induced LOX-1 expression is distinct from that underlying LPS-induced SR-A and CD36 expression. It has been reported that LOX-1 accounts for 5–10 % of ox-LDL uptake in unstimulated macrophages, whereas internalization of oxLDL increases by more than 40 % when LOX-1 is up-regulated after lysophosphatidylcholine stimulation [28]. Another study reported that LPS stimulation induces lipid accumulation and foam cell formation in RAW267.4 cells [13]. These results prompted us to examine whether LPS enhances the cellular uptake of oxLDL by up-regulating LOX-1 in macrophages. Indeed, we found that cellular uptake of Dil-oxLDL was augmented after LPS in both human and mouse macrophages. The uptake of Dil-oxLDL augmented by LPS was almost completely abrogated by treatment with an anti-LOX-1 antibody, strongly suggesting that LOX-1 plays an important role in oxLDL uptake in LPS-stimulated macrophages. Furthermore, the preventive effects against oxLDL uptake were increasingly impaired over time, suggesting that other SRs and LOX-1 may cooperatively enhance the uptake of oxLDL in the activated macrophages.

It is known that several stimulants modulate *LOX-1* transcription [28]. OxLDL is known to activate *LOX-1* transcription through an Oct-1 motif located at nt −1,556 in the human *LOX-1* gene locus [29]. PMA and angiotensin II stimulation also activate *LOX-1* transcription through AP-1 and NF-κB, respectively [19, 20]. Therefore, we

further sought to clarify the molecular mechanism underlying LPS-induced LOX-1 expression. In this study, we found that LPS-induced LOX-1 expression was significantly prevented by treatment with an MEK inhibitor (U0126) and an NF-κB inhibitor (CAPE) but was only moderately prevented by a JNK inhibitor (SP600125) at both the mRNA and protein levels. In addition, the luciferase reporter gene assay revealed that LPS-induced activation of *LOX-1* promoter activity was significantly prevented by treatment with U0126. Finally, the prevention of the LPS-induced LOX-1 expression in PMA-activated human THP-1 macrophages by treatment with Erk siRNA and U0126 provided the experimental evidence that ERK signaling plays a pivotal role in LPS-induced LOX-1 expression. CAPE, an NF-κB inhibitor, significantly suppressed LPS-induced LOX-1 expression at both the mRNA and protein levels, but did not suppress *LOX-1* promoter activity, suggesting that NF-κB may be involved in the *LOX-1* mRNA stability at the post-transcriptional level. Recently, Zhao et al. reported that knockdown of TLR4 resulted in a significantly decreased LOX-1 expression in LPS-stimulated human umbilical vein endothelial cells [30]. Thus, it may be possible that TLR4 mediates cellular uptake of oxLDL through up-regulation of LOX-1 in LPS-stimulated macrophages.

It has been reported that LPS induces SR-A expression in mouse macrophages, whereas LPS reduces the receptor expression in human macrophages [31]. LPS has been shown to reduce CD36 expression in primary human macrophages [32]. LOX-1 is expressed in macrophages of human carotid atherosclerotic plaques [33]. Although we used cell lines in this study, it would be of particular interest to use primary human monocyte-derived macrophages to examine whether LPS up-regulates LOX-1 expression and/or enhances the cellular uptake of oxLDL.

In conclusion, this study is the first to demonstrate that LPS up-regulates LOX-1 through activation of the ERK signaling pathway, thereby enhancing the cellular uptake of oxLDL in macrophages. Although CD36 and/or SR-A have been shown to mediate oxLDL uptake in macrophages, our novel findings raise the possibility that LOX-1 may promote the pathogenic actions of activated macrophages by evoking uptake of oxLDL, thus playing a role in the pathophysiology of cardiovascular diseases. Further studies, including in vivo experiments, may contribute to a better understanding of the molecular basis underlying the LOX-1-mediated pathogenesis of atherosclerosis associated with immune activation and inflammation.

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