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Oxidized low density lipoprotein suppresses lipopolysaccharide-induced inflammatory responses in microglia: Oxidative stress acts through control of inflammation

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Abstract

Low density lipoprotein (LDL) is readily oxidized under certain conditions, resulting in the formation of oxidized LDL (oxLDL). Despite numerous in vitro reports that reveal the pathogenic role of oxidative stress, anti-oxidative strategies have underperformed in the clinic. In this study, we examine the role of oxLDL in brain inflammatory responses using cultured rat brain microglia. We demonstrate that oxLDL inhibits lipopolysaccharide (LPS)-induced inflammatory responses in these cells. It also decreases LPS-induced expression of inducible nitric oxide synthase and production of nitric oxide, and reduces LPS-induced secretion of tumor necrosis factor-α and monocyte chemoattractant protein-1. Oxysterols, known components of oxLDL and endogenous agonists of liver X receptor, can simulate the inhibitory effects of oxLDL in LPS-activated microglia. In addition, their inhibitory effects were mimicked by liver X receptor (LXR) agonists and potentiated by a retinoid X receptor agonist, suggesting these molecules heterodimerize to function as oxysterol receptors. Taken together, our results demonstrate that oxLDL inhibits LPS-induced inflammatory responses in brain microglia and that these inhibitory effects are mediated by oxysterols and, at least in part, by the nuclear receptor LXR. Our results suggest an additional mechanism of action for oxidative stress that acts indirectly via modulation of inflammatory responses. Although further studies are needed, these results answer in part the question of why anti-oxidative strategies have not been successful in clinical situations. Moreover, as brain inflammation participates in the initiation and progression of several neurodegenerative disorders, the present data provide information that should prove a useful guide for designing therapeutic strategies to combat oxidative brain diseases.

Keywords: Neuroinflammation; Oxidative stress; LDL; LXR

High levels of cholesterol and low density lipoprotein (LDL) are well-known risk factors for several conditions, including atherosclerosis and stroke. Despite reports that LDL itself could be harmful to cells, numerous studies are currently focusing on the role of oxidative stress in the pathogenesis elicited by LDL and cholesterol. LDL may be readily oxidized under certain conditions, resulting in the formation of oxidized LDL (oxLDL). Although the oxidants responsible for in vivo oxidation of LDL are unknown at present, several candidates have been suggested, including 15-lipoxygenase [1], myeloperoxidase [2], endothelial nitric oxide synthase [3], and transition metals [4–6]. Within the brain, LDL is highly vulnerable to oxidative modification. The central nervous system (CNS) is particularly sensitive to oxygen radical damage due to poor antioxidant defense and an abundant supply of transition metals [7]. Several reports have suggested that oxidative stress is associated with many neurodegenerative disorders, including Parkinson's disease (PD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS). Markers for lipid peroxidation, such as 4-hydroxynonal (4-HNE) and malondialdehyde (MDA), have been identified in the cortex and hippocampus of

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patients with AD [8], the substantia nigra of patients with PD [9], and the spinal fluid of patients with ALS [10]. In addition, brain injury is often associated with disruption of the blood–brain barrier (BBB), which increases the possibility of exposing the CNS to plasma LDL or oxLDL [11]. Indeed, Uno et al. [12] have demonstrated elevated plasma oxLDL levels in acute cerebral infarction cases. Together, these findings suggest that oxLDL may contribute to various brain pathologies.

Despite abundant data linking oxidative stress to the underlying pathogenesis of neurodegenerative disease, including stroke, anti-oxidative strategies have thus far not performed well in clinical situations. In this study, we suggest that oxidative modification of LDL and cholesterol suppress release of nitric oxide (NO), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-α (TNF-α) in LPS-activated brain microglia. Thus, oxidative stress acts indirectly, via modulation of brain inflammatory responses. Since brain inflammation participates in the initiation or progression of several neurodegenerative disorders [13–15], the present data, which reveal a new mechanism for oxidative stress modulating cellular function via control of inflammatory responses in the brain, provide information that should prove a useful guide for designing therapeutic strategies to combat oxidative brain disease.

Materials and methods

Reagents. Human LDL was purchased from Calbiochem (La Jolla, CA) and Cu-oxLDL was obtained from Biomedical Technologies (Stoughton, MA). *Salmonella typhimurium* LPS and 7-ketocholesterol (7-KC) were purchased from Sigma–Aldrich (St. Louis, MO). Bovine brain ganglioside mixture (Gmix) was purchased from Matreya (Pleasant Gap, PA). 22(R)-hydroxycholesterol (22(R)HC) and TO901317 were purchased from Cayman (Ann Arbor, MI). Methoprene acid (MA) was purchased from BIOMOL (Plymouth Meeting, PA). GW3965 was kindly provided by GlaxoSmithKline (Stevenage, UK).

Cell Culture. Primary microglia were cultured from the cerebral cortices of 1- to 3-day-old Sprague–Dawley rats as previously described [16]. Briefly, the cortices were triturated into single cells in minimal essential medium (MEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and plated in 75-cm^2 T-flasks (0.5 hemispheres/flask) for 2–3 weeks. Microglia were then detached from the flasks by mild shaking and filtered through a nylon mesh to remove astrocytes. Cells were plated in six-well plates (5×10^4 cells/well), 60-mm dishes (8×10^5 cells/dish), or 100-mm dishes (1.5×10^6 cells/well). One hour later, the cells were washed to remove unattached cells before being used in experiments.

Oxidation of LDL by AAPH. We prepared oxLDL using a standard method of 2,2′-azobis(2-amidimopropane) dihydrochloride (AAPH; Sigma–Aldrich)-mediated oxidation [17,18]. Oxidation of LDL was performed at 37 °C under the following conditions to obtain different degrees of oxidation. First, LDL was oxidized by 10 mM AAPH for 2, 6, or 18 h. Second, oxidation of LDL was performed for 18 h at AAPH concentrations of 1, 5, or 10 mM. Produced oxLDLs were represented as "oxLDL-concentration of AAPH (mM): oxidation period (h)".

Relative electrophoretic mobility. Electrophoretic mobility relative to LDL was measured by agarose gel (0.8% agarose in 0.08 mol/L Tris–HCl buffer [pH 8.3]) electrophoresis and Coomassie brilliant blue R250 staining. This allows detection of changes in electric charge induced by oxidation [19,20].

Thiobarbituric acid reacting substances assay. 100 μg of LDL/oxLDL was added to a test tube containing 200 μl SDS (8%, w/v), 400 μl acetic acid (20%, w/v), and 400 μl of thiobarbituric acid (0.8%, w/v). The mixture was vortexed well and boiled for 1 h. After cooling, the specimens were centrifuged (13,000 rpm, 10 min) and the absorbance of the supernatant was determined at 540 nm using a spectrophotometer. The amount of thiobarbituric acid reacting substances (TBARS) was determined by comparison to a standard of malondialdehyde (MDA) equivalents prepared using 1,1,3,3-tetraethoxypropane (Sigma–Aldrich).

Lipid hydroperoxide assay. The lipid hydroperoxide (LPO) level in oxLDL was determined by a LPO assay kit as provided by the manufacturer (Lipid Hydroperoxide Assay Kit, Cayman). 100 μg of LDL/oxLDL was used for LPO measurements. The absorbance at 500 nm was measured using a spectrophotometer (Amersham Pharmacia Biotech, San Francisco, CA).

Western blot analysis. Cells were washed twice with cold PBS and then lysed in ice-cold modified RIPA buffer (50 mM Tris–HCl [pH 7.4], 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM Na_3VO_4, and 1 mM NaF) containing protease inhibitors (2 mM PMSF, 100 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μg/ml aprotinin, and 2 mM EDTA). The lysates were centrifuged for 20 min at 12,000*g* at 4 °C and the supernatants were collected. Proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies (Abs) and peroxidase-conjugated secondary Abs (Vector Laboratories, Burlingame, CA), and then visualized using an enhanced chemiluminescence (ECL) system (Sigma–Aldrich). Primary Abs used in this study were as follows: STAT1, phospho-STAT1 (Tyr701), IκB, and phospho-IκB (Cell Signaling Technology, Beverley, MA); phospho-ERK, phospho-p38, and phospho-JNK (Calbiochem); inducible nitric oxide synthase (iNOS, Upstate Biotechnology, Lake Placid, NY); COX-2 and actin (Santa Cruz Biotechnology, Santa Cruz, CA).

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted using RNAzol B (Tel-Test, Friendswood, TX) and cDNA was prepared using reverse transcriptase that originated from avian myeloblastosis virus (Takara, Shiga, Japan), according to the manufacturer's instructions. PCR was performed with 30 cycles as follows: 94 °C for 60 s, 55 °C for 30 s, and 72 °C for 90 s. Oligonucleotide primers were purchased from Bioneer (Seoul, Korea). The sequences of the PCR primers are shown in Table 1. PCR products were separated by electrophoresis on a 1.5% agarose gel and detected under UV light.

Determination of nitric oxide release. Media nitrite concentration was measured as an indication of nitric oxide (NO) release. Following the

Table 1
Primer sequences for PCR

Genes	Primer sequences
ABCA1	F: 5′-GGG CGG GGG CCT GAA GAT C-3′
	R: 5′-AGA GCC ATT TGG GGA CTG AAC ATC-3′
Actin	F: 5′-GCC ATC TCC TGC TCG AAG TCT AG-3′
	R: 5′-CAT GTT TGA GAC CTT CAA CAC CCC-3′
CD36	F: 5′-TCA AGG TGT GCT CAA CAG CC-3′
	R: 5′-AGG ATA AAA CAC ACC AAC TGT-3′
CD68	F: 5′-TCC CTC TTG CTG CCT CTC ATC-3′
	R: 5′-GGT GGC TTA CAC AGT GGA CTG G-3′
IFNβ	F: 5′-GCC TTCG CTT ATC GCC ATC AAC-3′
	R: 5′-AAT GCA AAG ACC TGC TCC GAG-3′
LOX-1	F: 5′-GAC TGG ATC TGG CAT AAA GA-3′
	R: 5′-CCT TCT TCT GAC ATA TGC TG-3′
LXR-α	F: 5′-GCG AGG GCT GCA AGG GAT TCT-3′
	R: 5′-ATG GGC CAA GGC GTG ACG CG-3′
LXR-β	F: 5′-GCC TTG CTT ATC GCC ATC AAC-3′
	R: 5′-AAT GCA AAG ACC TGC TCC GAG-3′
RXR-α	F: 5′-AGA GGA CAG TAC GCA AAG AC-3′
	R: 5′-GTG AAG AGC TGC TTG TCT G-3′
SRA	F: 5′-TTG GCT TCC CTG GAG GTC GAG-3′
	R: 5′-ACA CAG GAA CCA ATG TCA TTT G-3′

indicated incubation periods, 50 μl culture medium was removed and mixed with an equal volume of Griess reagent (0.1% naphthylethylene diamine, 1% sulfanilamide, and 2.5% H_3PO_4), and the absorbance of the mixture at 540 nm was measured.

Enzyme-linked immunosorbent assay. TNF-α and MCP-1 levels in cell culture media were determined by enzyme-linked immunosorbent assay (ELISA) as described by the manufacturer (OptEIA Sets, Pharmingen, San Diego, CA). TNF-α and MCP-1 concentrations in the media were determined by spectrophotometer and calibrated from standards containing known concentrations of the cytokines.

Data analysis. Data were expressed as means ± SEM. Analysis of variance followed by Dunnett's multiple comparisons test were used for statistical comparisons.

Results

oxLDL potently inhibits LPS-induced expression of iNOS and COX-2 in primary microglia cultured from rat brain

To examine the effects of oxLDL on inflammatory responses, we first stimulated cultured microglia with 10 ng/ml LPS for 24 h in the absence or presence of LDL or modified LDLs, and measured the protein expression of iNOS and COX-2, two key inflammatory mediators in activated microglia. We used AAPH-mediated oxLDL (AAPH-oxLDL) synthesized using the standard method, as described in Experimental procedures. oxLDL 50 μg/ml markedly suppressed the protein expression of iNOS and COX-2, while LDL or AcLDL did not appear to influence expression of either molecule (Fig. 1A). These inhibitory effects of oxLDL were dose-dependent from 25 up to 100 μg/ml (Fig. 1B). Inhibitory effects of oxLDL were also observed when microglia were activated by ganglioside mixture (Gmix), another microglial activator (Fig. 1C). Although cytotoxic effects of oxLDL have been previously reported [21,22], no impact on cell viability has ever been noted at doses ⩽50 μg/ml using our experimental conditions (data not shown). Thus, we used 50 μg/ml oxLDL for all subsequent experiments.

oxLDL suppresses the expression and release of inflammatory mediators in an oxidation-dependent manner

Since LDL is a complex of lipid and protein molecules, oxidation of LDL results in diverse products, depending on the degree of and methods used to achieve oxidation. Accordingly, some groups have reported that minimally modified LDL appears to be more chemoattractive than fully oxidized LDL [23,24]. We measured the oxidation levels of LDL and modified LDLs using several methods. First, the oxidative modification of LDL was determined by relative electrophoretic mobility (REM) assay. Alteration of agarose gel electrophoretic mobility reflects the increase in negative charge of LDL particles that occurs during oxidation. oxLDL migrated faster than native LDL and appeared less visible using Coomassie blue staining, owing to increases in negative charge and partial degradation, respectively. AcLDL was loaded as a negatively modified LDL for comparison. As revealed by the REM data, electrophoretic mobility increased depending on the length of the oxidation period as well as oxidant concentration in AAPH-oxLDL (Fig. 2A, a and b). Lipid peroxidation is quantified either by measuring malondialdehyde (MDA), degradation by-products [25,26], or by directly measuring lipid hydroperoxide (LPO) [27]. As oxidation progressed, the MDA content of other oxLDL preparations increased depending on the length of the oxidation period and AAPH concentration as measured by TBARS assay (Table 2). The LPO content in the oxLDL preparations, measured by direct LPO assay, also increased

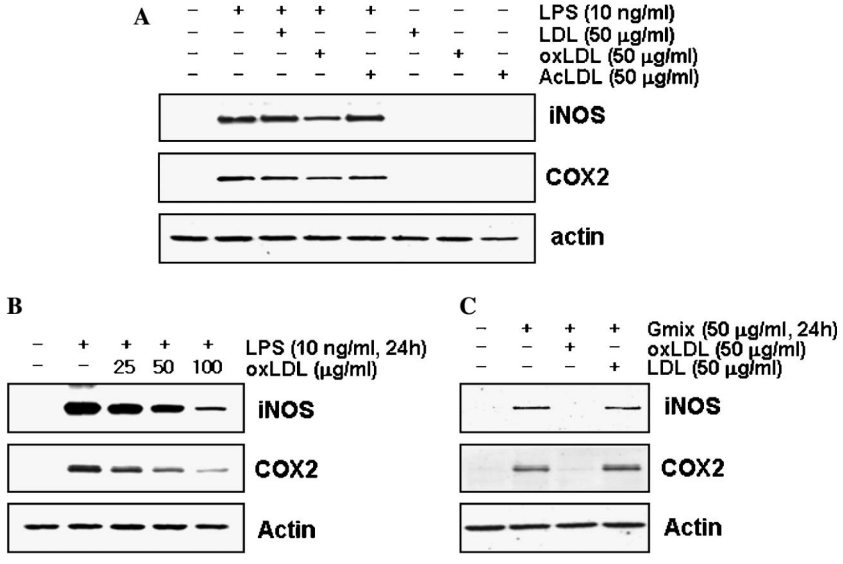

Fig. 1. oxLDL suppresses iNOS and COX2 expression in LPS-activated microglia. Rat primary microglia cultured from rat brain were incubated with LDL, AcLDL, or oxLDL for 1 h and then stimulated with LPS (10 ng/ml) (A,B) or Gmix (50 μg/ml) (C) for 24 h. Cell lysates were then separated using 10% SDS–PAGE, and Western blot analysis was performed using antibodies against iNOS and COX-2. Actin was used as a loading control. Data shown are representative of three independent experiments.

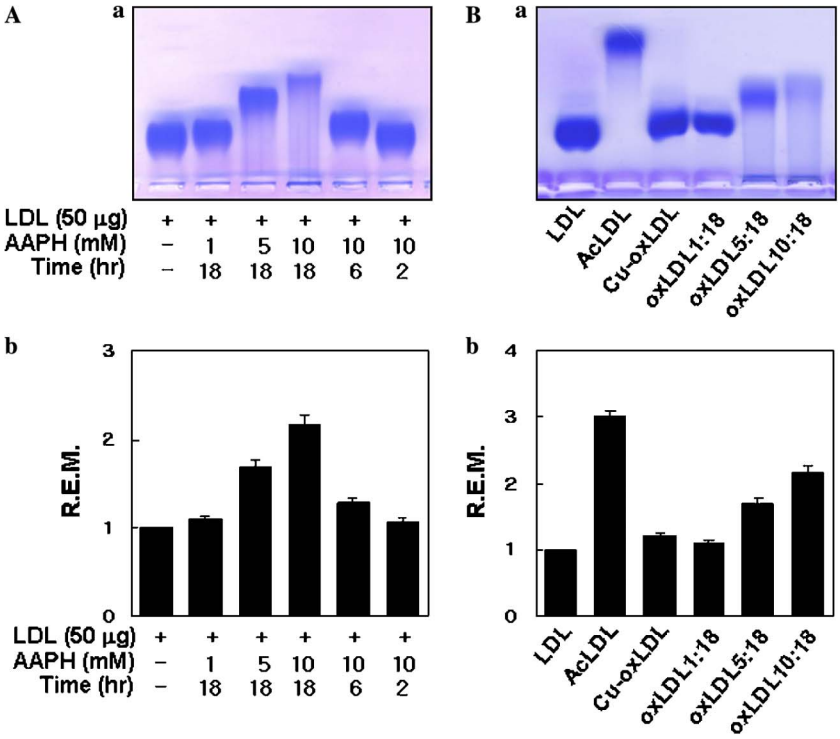

Fig. 2. Relative agarose gel electromobility of modified LDLs. AAPH-mediated oxLDLs at different oxidation levels yielded as described in Materials and methods (A) or several modified LDLs (B) were loaded onto 0.8% agarose gels for electrophoresis. The gels were then stained with Coomassie brilliant blue R-250 (a), and the migration distance was measured (b). The stained gel is representative of three independent experiments.

depending on the length of the oxidation period and AAPH concentration. Therefore, oxLDL yielded from LDL incubated with 10 mM AAPH for 18 h (oxLDL10:18) were used for all remaining experiments. Moreover, LDL oxidized by copper sulfate (Cu-oxLDL), which produced sufficient MDA in the TBARS assay but no apparent shift in REM or increase in LPO production (Fig. 2B, Table 2), did not display any significant inhibitory effects (data not shown).

We next determined the inhibitory effects of AAPH-oxLDL on inflammatory mediator release in LPS-stimulated microglia. Cells were pretreated for 1 h with several oxLDLs, as described in Fig. 2 and Table 2. oxLDL suppressed protein expression of iNOS and COX-2, depending on the degree of oxidation (Fig. 3A, a and b). Release of NO, TNF-α, and MCP-1 into the culture media was also suppressed in an oxidation-dependent manner (Figs. 3B–D, respectively).

Inhibitory effects of oxLDL are mediated by interference with LPS-induced NF-κB activation, IFN-β release, and STAT1 activation in brain microglia

We next addressed how oxLDL inhibits LPS-induced inflammatory responses. The first possibility is that oxLDL simply blocks LPS binding to its receptor. This was tested by adding oxLDL at several time points before and after LPS treatment. We stimulated cells with LPS in the absence and presence of oxLDL as depicted in Fig. 4A, a. The addition of oxLDL, regardless of the time added, suppressed the expression of iNOS and COX-2 in LPS-stimulated primary microglia (Fig. 4A, b). These results indicate that the inhibitory effects of oxLDL are not due to the displacement of LPS from binding to its extracellular receptors. Next, an RT-PCR-based study using primers for representative scavenger receptors that are known to bind to oxLDL demonstrated the presence of CD36 [28], CD68 [29], SRA [30], and LOX-1 [31] in control cells, and no changes were observed following the addition of LDL or oxLDL (Fig. 4B). Therefore, it is likely that oxLDL is internalized into microglia via scavenger receptors and can therefore affect intracellular signaling.

Since we and others have reported that LPS induces inflammatory responses via the NF-κB-IFN-β-STAT pathway [32,33], we tested the inhibitory effects of oxLDL on NF-κB activation. oxLDL suppressed the phosphorylation of IκB and reversed IκB degradation, thereby inhibiting NF-κB activation (Fig. 4C). Although various MAP kinases (MAPKs; ERK, JNK, and p38) are known to be involved in LPS-activated signaling [34], no MAPKs showed any apparent changes in phosphorylation following a 45-min or 3-h pretreatment of oxLDL in primary microglia (Fig. 4C and data not shown). We next determined the effects of oxLDL on LPS-induced IFN-β mRNA expression. IFN-β is a critical and necessary component of the signaling processes involved in inflammation in LPS-induced delayed STAT1 activation [35]. IFN-β mRNA expression and tyrosine phosphorylation of STAT1 by LPS were markedly suppressed in oxLDL-treated primary microglia (Fig. 4D).

Table 2
Thiobarbituric acid-reacting substances and lipid hydroperoxide assay

	LDL	oxLDL 1:18[a]	oxLDL 5:18	oxLDL 10:18	oxLDL 10:6	oxLDL 10:2	AcLDL	Cu-oxLDL
TBARS (mM MDA/mg LDL)	0.511 ± 1.016	7.938 ± 2.103	25.031 ± 2.266	32.394 ± 2.368	27.784 ± 2.464	21.382 ± 3.275	3.071 ± 1.040	17.540 ± 0.768
LPO (nM LPO/mg LDL)	5.310 ± 1.033	8.946 ± 0.735	17.159 ± 0.834	21.405 ± 0.627	18.709 ± 0.773	8.409 ± 2.054	5.733 ± 0.634	5.289 ± 0.783

[a] AAPH concentration (mM) : oxidized period (h).

Oxysterol/LXR agonists mimic anti-inflammatory effects of oxLDL on LPS-activated microglia

To identify which components of oxLDL contribute to the suppressive effects of oxLDL, we investigated lysophosphatidylcholine (LPC) and two oxysterols (7-KC and 22(R)HC), which are representative constituents of oxLDL. Pretreatment of LPC did not suppress LPS-induced inflammatory responses (data not shown). In contrast, 22(R)HC and 7-KC efficiently suppressed protein expression of both iNOS and COX-2 in LPS-stimulated primary microglia (Fig. 5A). As oxysterols are reported to act as endogenous agonists for liver X receptor (LXR), we tested whether this occurs through the activation of LXR. First, we examined the expression of LXR-α, LXR-β, and retinoid X receptor (RXR) by RT-PCR-based assays; all three are detected in control as well as agonist-stimulated cells. However, the transcript levels of these molecules were not changed despite the addition of agonists, while ATP binding cassette transporter-1 (ABCA1), a representative LXR-regulated gene, transcript was increased by all of the agonists, revealing that LXR is active under these experimental conditions (Fig. 5B). To confirm that oxLDL/oxysterols act via LXR activation, we compared their effects using synthetic LXR agonists (TO901317 and GW3965) [36]. Both of these compounds efficiently suppressed iNOS and COX-2 expression in LPS-activated microglia, thus mimicking the inhibitory effects of oxLDL/oxysterols (Fig. 5C). Furthermore, they also suppressed the release of NO and MCP-1 in a dose-dependent manner in LPS-activated cells (Figs. 5D and F). With respect to TNF-α release, oxysterol dose-dependently suppressed release, but GW3695 did not (Fig. 5E).

RXR agonist potentiates the inhibitory effects of oxLDL

To further explore the involvement of LXRs in the inhibitory effects of oxLDL, we tested whether they are potentiated following the addition of a synthetic RXR agonist (methoprene acid, MA), since LXRs/RXRs act by forming heterodimers. Pretreatment with MA potentiated the inhibitory effects of oxLDL on LPS-induced iNOS expression (Fig. 6A), NO production (Fig. 6B), and MCP-1 secretion (Fig. 6D). However, the inhibitory effects of oxLDL on LPS-induced TNF-α secretion were not affected by MA pretreatment (Fig. 6C). These observations suggest that the inhibition of inflammatory responses by oxLDL/oxysterols is mediated by both LXR-dependent (NO production and MCP-1 secretion) and independent (TNF-α secretion) pathways.

Discussion

Based on the controversial roles of LDL and oxLDL during cellular functioning, we explored their activity in the brain. Here, we demonstrate that oxLDL inhibits inflammatory responses in LPS-activated rat primary

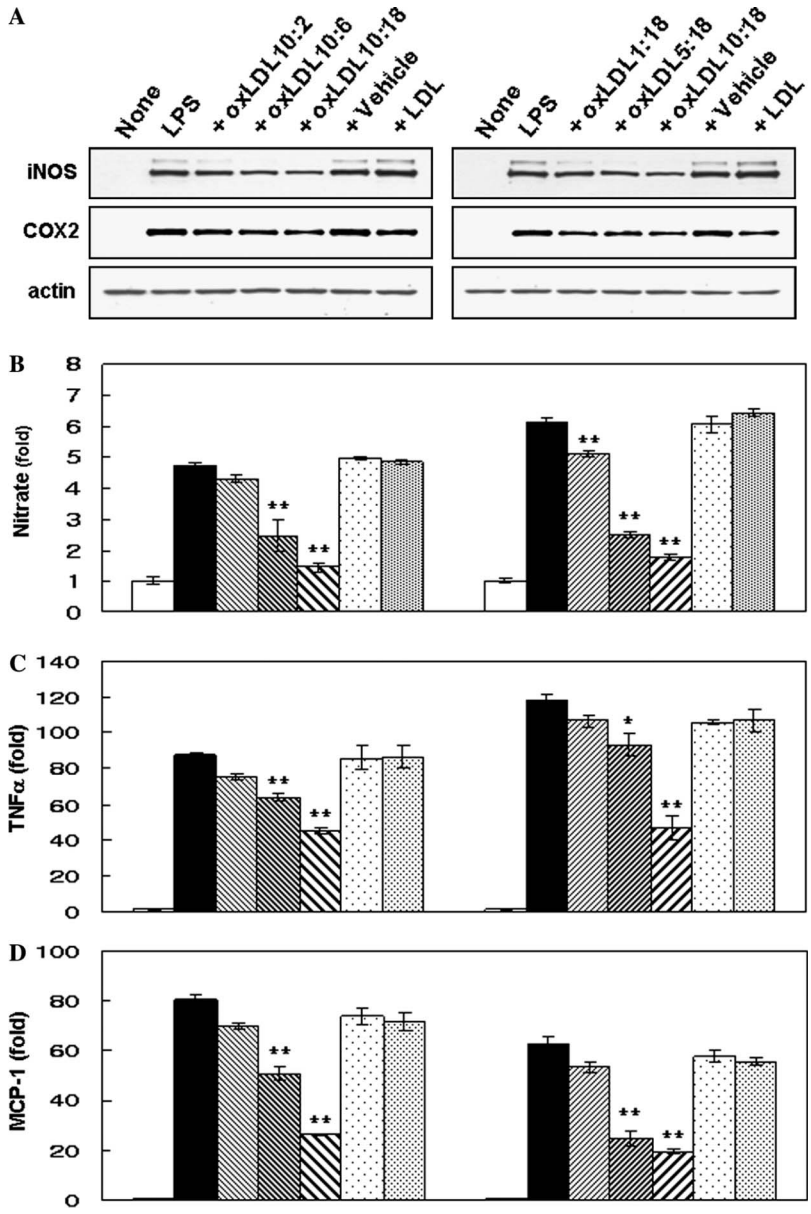

Fig. 3. oxLDL suppresses the expression and release of inflammatory mediators in LPS-stimulated microglia in an oxidation-dependent manner. Primary microglia were pretreated with different oxidation levels of oxLDLs as described in Fig. 2 for 1 h and then stimulated with LPS for 24 h. Cell lysates were then subjected to Western blot analysis (A) and media were used for measurement of NO (B), TNF-α (C), and MCP-1 (D), using Griess reagent (NO) and ELISA (TNF-α and MCP-1). Data represent means ± SEM of three independent experiments. Significant differences from the LPS group by Dunnett's multiple range test, $*P < 0.05$; $**P < 0.01$.

microglia. Since their anti-inflammatory effects are mimicked by oxysterols and at least in part by LXR agonists, we conclude that oxidative stress regulates inflammatory responses through oxidative modulation of LDL in an oxidation-dependent manner in brain microglia.

We prepared oxLDL according to the standard method of AAPH-mediated oxidation. Oxidative modification of LDL was essential for the observed anti-inflammatory effects, as no inhibitory effects were noted with native LDL and AcLDL. Moreover, the inhibitory effects of AAPH-oxLDL showed a positive correlation with oxidative stress. However, we did not observe any apparent inhibitory effects using Cu-oxLDL, which showed a significant increase in MDA as measured by TBARS assay, but not in direct measurements of LPO (Fig. 2B and Table 2). This finding is consistent with a previous report that the LPO content of oxLDL is responsible for its induction of cellular damage [37].

The biological effects of oxLDL are largely mediated by products formed during oxidation of the lipid component of LDL. Since the composition of oxLDL might be altered during the oxidation process and each component of oxLDL triggers its own intracellular responses, the degree of LDL oxidation might be one of the most important factors in determining its diverse biological functions. Oxidation of LDL is a complex reaction that generates various

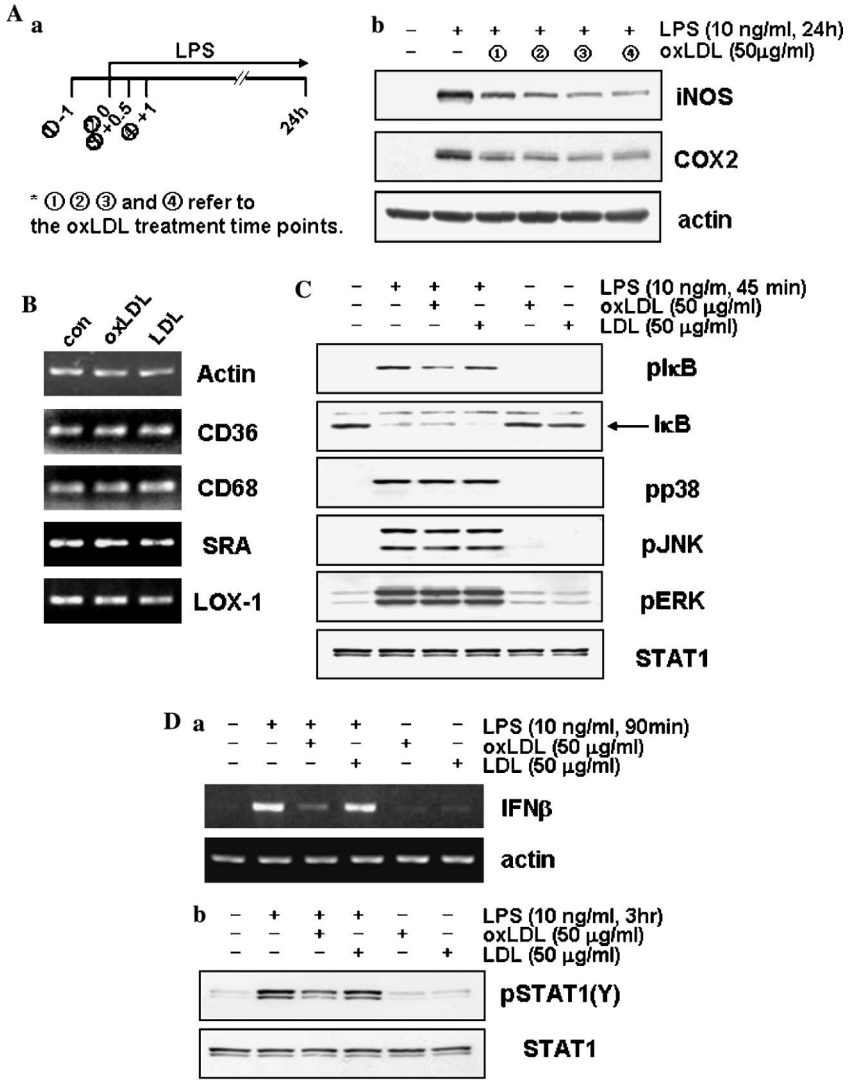

Fig. 4. oxLDL inhibits LPS-mediated downstream signaling mediators. (A) To rule out the possibility that the inhibitory effects of oxLDL are achieved by simple interference with LPS binding, we added oxLDL at several time points as indicated in (a) and stimulated with LPS for 24 h. Cell lysates were then obtained and subjected to Western blot analysis using antibodies against iNOS and COX-2 (b). (B) Primary microglia were stimulated with LDL (50 μg/ml) or oxLDL (50 μg/ml) for 8 h. Total RNA was isolated and RT-PCR was then performed for detection of transcripts of known oxLDL receptors. Actin was used a loading control. (C) Microglia were pretreated with LDL or oxLDL for 1 h and stimulated with LPS (10 ng/ml) for 45 min. Cell lysates were obtained and subjected to Western blot analysis using antibodies as noted in the figure. STAT1 was used as a loading control. (D) Cells were pretreated with LDL or oxLDL for 1 h and stimulated with LPS for 90 min or 3 h. Expression of IFN-β (a) and phosphorylation of STAT (b) were analyzed by RT-PCR and Western blot analyses, respectively. Data shown are representative of three independent experiments.

lipid mediators, and the functional complexity of oxLDL is likely to reflect the relative level of these mediators in the particle. Among these lipid mediators, lysophosphatidylcholine (LPC), and oxysterols are of special interest because they are among the major constituents and display opposite effects on inflammation. As previously elucidated by other groups, we also observed a pro-inflammatory, not anti-inflammatory, effect in LPC-treated cells (data not shown). The anti-inflammatory effects of oxLDL were simulated by two oxysterols, 7KC and 22(R)-HC. Oxysterols, which are mono-oxygenated derivatives of cholesterols, may be formed by either spontaneous or enzyme-mediated processes. The spontaneous oxidation of cholesterol is a well-recognized phenomenon, generally termed auto-oxidation, which can be initiated by compounds including various radical species, lipid peroxides, and divalent metal cations such as copper ions. In general, oxysterols are known to be more toxic than cholesterol itself, and we also observed that oxysterols exhibit cytotoxic effects at higher doses but dramatic anti-inflammatory effects at the relatively low dose used in our experiments. Thus, the anti-inflammatory effects of AAPH-oxLDL are thought to be mediated by its oxysterol components.

Because oxysterols are natural ligands for LXRs, we tested whether LXRs were involved in the anti-inflammatory mechanism of oxLDL/oxysterols. A recent study showed that LXRs play critical roles in inflammation in addition to cholesterol metabolism and fatty acid homeostasis. Two LXR isoforms (LXR-α and LXR-β), encoded by distinct genes, have been identified. LXRs form

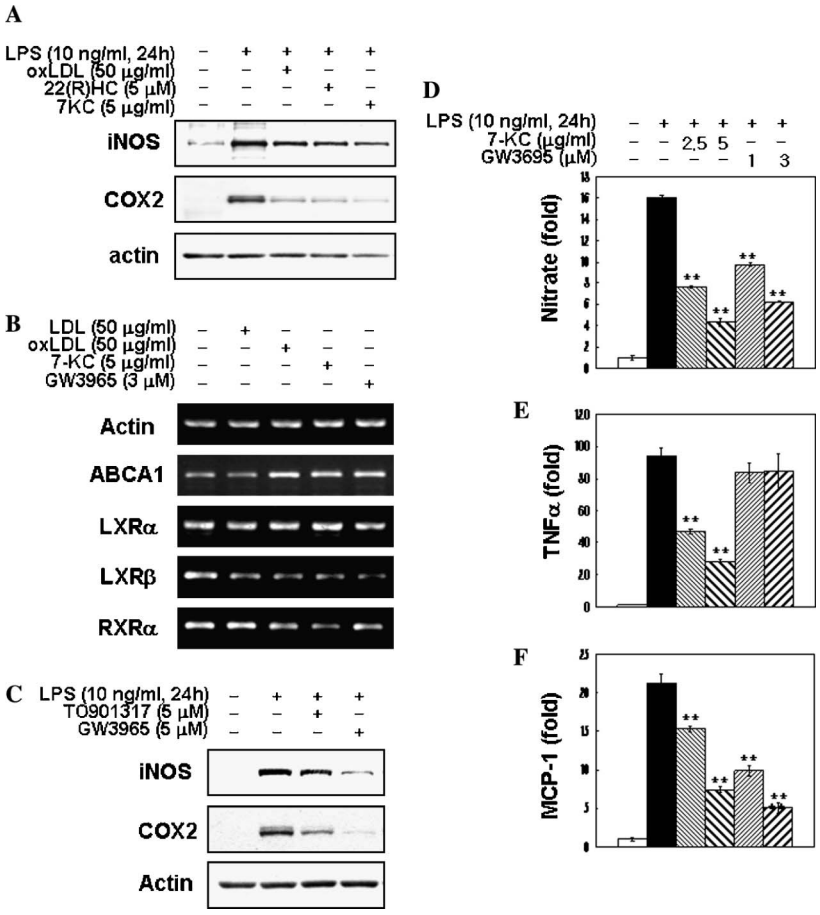

Fig. 5. Oxysterol/LXR agonists mimicked anti-inflammatory effects of oxLDL. Primary microglia were pretreated with the indicated doses of drugs for 1 h and stimulated with LPS for 24 h. Cell lysates were obtained and subjected to Western blot analysis using antibodies against iNOS and COX-2 (A,C). Extracted total RNA was subjected to RT-PCR analysis using primers described in Table 1 (B). Actin was loaded as a control. Media were obtained from cultured cells under the same conditions in (A–C). The release of nitrate, TNF-α, and MCP-1 was measured as described in Fig. 3 (D–F). Significant difference from the LPS group by Dunnett's multiple range test, $^{**}P < 0.01$.

obligate heterodimers with RXRs, which are members of the nuclear receptor superfamily that can be regulated by 9-*cis*-retinoic acid (9cRA) and the synthetic RXR agonist MA. To gain insight into the mechanism responsible for the anti-inflammatory effects elicited by oxLDL/oxysterols, we tested whether LXR activation mimicked the anti-inflammatory effects of oxLDL/oxysterols in LPS-activated microglia using the synthetic LXR agonists TO901318 and GW3965. While NO and MCP-1 release was suppressed by treatment with oxLDL, oxysterols, and LXR agonists, TNF-α release was suppressed by oxLDL and oxysterols, but not by LXR agonists. The potentiation of inhibitory responses by MA was not observed in TNF-α release, further suggesting the existence of an LXR-independent mechanism. Activators of PPARγ, another nuclear receptor that forms heterodimers with RXR, also antagonize inflammatory responses in both receptor-dependent and -independent manners [38–40]. Moreover, we noted that MCP-1 and TNF-α are differentially regulated, with activator protein (AP)-1 signaling performing a critical role in expression of MCP-1, but not in TNF-α (Lee and Jou, unpublished data). Altogether, these results show that oxLDL/oxysterols are likely to have LXR-independent effects.

How do oxLDL/oxysterols suppress inflammatory mediator release in LXR-dependent and -independent manners in LPS-stimulated microglia? We excluded the possibility that oxLDL interferes with LPS binding to its receptors extracellularly by adding oxLDL at several time points before and after LPS treatment. The suppressive effects of oxLDL on cellular responses such as inflammation [41] and apoptosis [42] primarily occur through alteration in gene expression. In our system, however, inhibitory effects on inflammation were still observed even when oxLDL was added after LPS treatment. Thus, we suggest that oxLDL interferes with LPS-induced signal transduction. LPS activates a Toll-like receptor (TLR), which causes activation of NF-κB. And then, the gene encoding IFN-β is transcribed and translated, and the secreted product binds to interferon receptor (IFNR), which results in STAT1 phosphorylation (pSTAT1). Activation of MAPK-mediated pathways by LPS may represent an additional mechanism for STAT1 phosphorylation. NF-κB, pSTAT1, and MAPK act in concert to

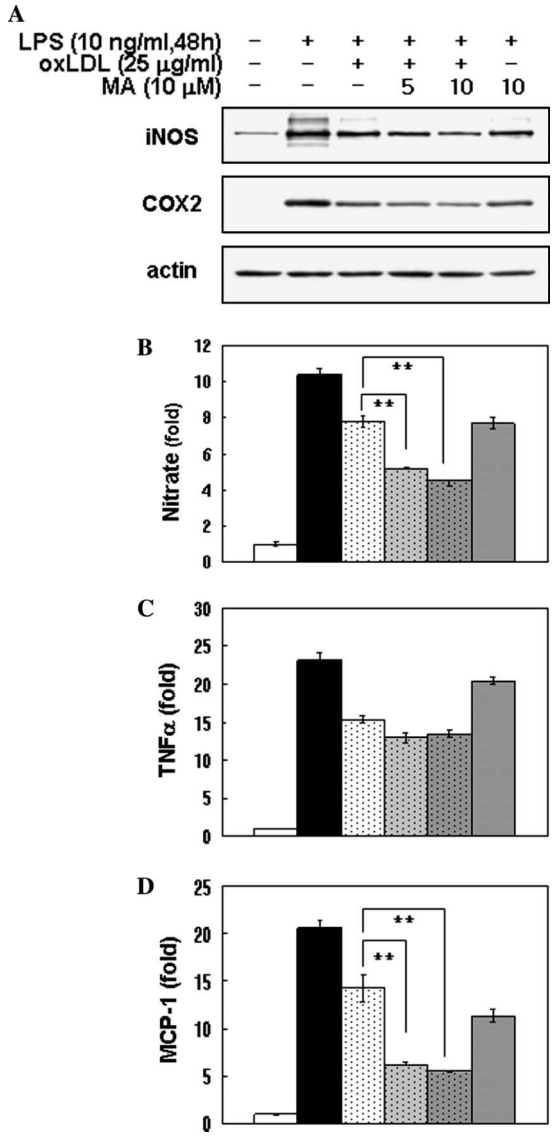

Fig. 6. RXR agonist potentiates the anti-inflammatory effects of oxLDL in LPS-stimulated microglia. Cells were pretreated with RXR agonist (MA, 10 μM) for 30 min before oxLDL treatment. They were then treated with oxLDL (25 μg/ml) for 1 h and stimulated with LPS (10 ng/ml) for 24 h. Western blots were then performed as described in Fig. 1 (A). The release of NO (B), TNF-α (C), and MCP-1 (D) was measured as described in Fig. 3. Significant differences between the marked groups by Dunnett's multiple range test, $^{**}P < 0.01$.

promote expression of inflammatory genes [32], but in our experimental conditions, oxLDL inhibited LPS-induced signal transduction pathways via suppression of IFN-β, NF-κB, or pSTAT1, but not MAPK signaling.

In spite of numerous reports that oxidative stresses are harmful to cellular physiology and thereby result in the pathogenesis responsible for diverse human diseases, anti-oxidant strategies have shown limited success in treating human disease. The present study shows that AAPH-oxLDL suppresses inflammatory mediator release in LPS-activated microglia and that these inhibitory effects can be simulated by oxysterols, particularly in doses lower than those that cause cytotoxicity. Oxysterols residing in biological membranes as well as lipoprotein are normally present in trace amounts; thus, it is biologically relevant that they can act at these lower doses. Our study also reveals that oxLDL/oxysterols may have opposing cellular functions depending on the degree of oxidation. Most importantly, the present data reveal that oxidative stresses act indirectly through regulation of inflammatory responses, which may help explain why anti-oxidant strategies have thus far underperformed in clinical situations. Thus, the relationship between oxidative stress and inflammation needs to be further clarified and the signaling pathways involved need to be fully dissected for these molecules to be developed into effective treatments for various neurodegenerative diseases.

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