LOX-1 augments oxLDL uptake by lysoPC-stimulated murine macrophages but is not required for oxLDL clearance from plasma

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Abstract **Oxidized LDL (oxLDL) promotes lipid accumulation as well as growth and survival signaling in macrophages. OxLDL uptake is mainly due to scavenger receptors SR-AI/II and CD36. However, other scavenger receptors such as lectin-like oxLDL receptor-1 (LOX-1) may also play a role. We used mice with targeted inactivation of the LOX-1 gene to define the role of this receptor in the uptake of oxLDL and in activation of survival pathways. There was no difference in uptake or degradation of ^{125}I-oxLDL in unstimulated macrophages from wild-type and LOX-1 knockout mice and no difference in the rate of clearance of oxLDL from plasma in vivo. However, when expression of LOX-1 was induced with lysophosphatidylcholine, oxLDL uptake and degradation increased 2-fold in wild-type macrophages but did not change in LOX-1 knockout macrophages. Macrophages lacking LOX-1 showed the same stimulation of PKB phosphorylation and enhancement of survival by oxLDL as wild-type cells. These data show that LOX-1 does not alter the uptake of oxLDL in unstimulated macrophages and is not essential for the pro-survival effect of oxLDL in these cells. However, LOX-1 expression is highly inducible by lysophosphatidylcholine and pro-inflammatory cytokines, and if that occurred in macrophages within atheromas, LOX-1 could substantially increase oxLDL uptake by lesion macrophages.**—Schaeffer, D. F., M. Riazy, K. S. Parhar, J. H. Chen, V. Duronio, T. Sawamura, and U. P. Steinbrecher. **LOX-1 augments oxLDL uptake by lysoPC-stimulated murine macrophages but is not required for oxLDL clearance from plasma.** ***J. Lipid Res.*** **2009.** 50: **1676–1684.**

Supplementary key words apoptosis • lysophosphatidycholine • macrophage • oxidized low density lipoprotein • scavenger receptor

The lectin-like oxidized LDL receptor (LOX-1) is a 50 kDa type II membrane receptor belonging to the C-type lectin family. It is expressed in vascular smooth muscle cells, macrophages, fibroblasts, and platelets (1, 2). It is reported to be upregulated in pathological conditions including hypertension, diabetes, and atherosclerosis. The potential role of LOX-1 in atherosclerotic process has been based on a number of observations. LOX-1 has the capability to bind, internalize, and degrade oxLDL (3). The activation of LOX-1 by oxLDL induces endothelial dysfunction and apoptosis (4–6). Furthermore LOX-1 is present in atheroma-derived cells and has been identified in animal and human atherosclerotic lesions in vivo (7, 8). Mehta et al. recently reported that in LDL receptor knockout mice, LOX-1 gene inactivation reduces the extent of atherosclerosis (9). The LOX-1 receptor has also been shown to mediate intracellular cell signaling events. For example, the binding of oxLDL to LOX-1 is reported to activate signaling via ERK that modulates the Bax/Bcl2 ratio in vascular smooth muscle cells and to activate NF- B in endothelial cells (10).

LOX-1 is a member of the scavenger receptor family of multiligand receptors that are thought to play a role in innate immunity (11). Other members of this family expressed in macrophages include SRAI/II, CD36, macrosialin/CD68, macrophage receptor with collagenous structure (MARCO), scavenger receptor expressed in endothelial cells (SREC), and a transmembrane chemokine and receptor for phosphatidylserine and oxLDL, SR-PSOX/CXCL16 (11). Previous studies indicate that 30–40% of the degradation of extensively oxidized LDL by

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Abbreviations: acLDL, acetylated low density lipoprotein; BMDM, bone marrow derived macrophages; lysoPC, lysophosphatidycholine; M-CSF, macrophage colony-stimulating factor; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; oxLDL, oxidized low density lipoprotein.

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mouse peritoneal macrophages is mediated by SR-AI/II and that CD36 accounts for an additional 35% of oxLDL degradation (12, 13). In peritoneal macrophages from mice lacking both SRAI/II and CD36, degradation of oxLDL was reduced by 75% (13). It has been suggested that LOX-1 may also be an important receptor for oxLDL in macrophages (14), but to date no studies have provided a quantitative estimate of the contribution of LOX-1 to oxLDL uptake by macrophages.

We recently showed that oxidized LDL blocks apoptosis in macrophages through activation of the phosphatidylinositol 3-kinase/protein kinase B (PI3K/PKB) cascade, translocation of NF- B to the nucleus, and induction of the anti-apoptotic factor $BclX_L$ (15, 16). Our preliminary results in SR-AI/II macrophages (17) and CD36 knockout macrophages (unpublished) suggest that the anti-apoptotic effect of oxLDL does not require either of these two scavenger receptors. LOX-1 was deemed an attractive candidate because its much higher affinity for oxidized LDL compared with acetyl LDL or native LDL is congruent with the finding that oxLDL (but not acetyl LDL or native LDL) can inhibit apoptosis in macrophages and because it has been shown to be involved in survival signaling and NF- B activation (4, 6, 18).

In the present study we describe results of experiments using LOX-1 knockout mice to examine the role the LOX-1 receptor plays in mediating uptake of, and signaling by, ox-LDL in macrophages.

MATERIALS AND METHODS

Materials

Carrier-free Na ^{125}I was purchased from Perkin Elmer (Woodbridge, Ontario). DMEM, αMEM medium, propidium iodide, and RNase A were obtained from Invitrogen (Burlington, ON, Canada). Defined fetal bovine serum (FBS) was from HyClone (Logan, UT). Promega (Madison, WI) supplied 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS). Phenazine methosulfate (PMS) and 1-stearoyl lysophosphatidylcholine were from Sigma Aldrich (St. Louis, MO). Centriplus 20 ultrafilters were purchased from Amicon (Beverly, MA). Molecular Probes (Eugene OR) provided 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI). Anti-CD68 was purchased from Serotec (Raleigh, NC). Primary antibody against p85 subunit of PI3K was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against total and phosphorylated PKB (Ser470) were from Cell Signaling Technology (Beverly, MA).

Animals

Inactivation of the single-copy murine LOX-1 receptor gene by homologous recombination was performed in the laboratory of Dr. T. Sawamura (9). Offspring with the inactivated gene in germine cells were backcrossed 8 times onto a C57bl/6 background. LOX-1 genotypes were verified by PCR analysis of tail DNA in all breeding animals, as well as in selected experimental animals. DNA was extracted using a Qiagen DNeasy kit. The forward primer for the LOX-1 deletion was 5′-CAGCGAACACAGCTCCGTCTTGAAGG-3′ and the reverse primer was 5′-GGCCAACCATGGCTTGGGAGAATGG-3′. The forward primer for the neomycin-resistance cassette was 5′-CAACGCTATGTCCTGATAGCGGTCC-3′ and the reverse primer was 5′-CGTGTTCCGGCTGTCAGCGCAGG-3′. Wild-type C57bl/6 mice were purchased from The Jackson Laboratory. All animal procedures were in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of British Columbia.

Lipoprotein isolation and labeling

LDL (d = 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation of EDTA-anticoagulated fasting plasma obtained from healthy normolipidemic volunteers (19). Radioiodination was performed using a modification of the iodine monochloride method of MacFarlane (20). The specific activity was between 100 and 150 cpm/ng LDL protein. Iodination was performed before oxidation or acetylation of LDL. Lipoprotein-deficient serum (LPDS) was prepared from the d > 1.25 fraction.

LDL modification

LDL was oxidized by incubating 200 μg/ml LDL in PBS containing 5 μmol/L $CuSO_4$ at 37°C for 20 h (21). OxLDL was then washed and concentrated to about 1 mg/ml with Centriplus 20 ultrafilters (Millipore, Bedford, MA). Agarose gel electrophoresis typically showed a 4-fold increase in electrophoretic mobility for oxidized LDL compared with native LDL. LDL was acetylated by adding four 1-μl aliquots of acetic anhydride at 10 min intervals to 2 mg of LDL in 600 μl of ice-cold 50% saturated sodium acetate (22).

Macrophage isolation and culture

Peritoneal macrophages were obtained from wild-type or LOX-1 knockout C57/bl mice by peritoneal lavage with ice-cold Ca^{2+}-free Dulbecco's PBS. Macrophages were suspended in α-minimal essential medium (α-MEM) with 10% fetal bovine serum (FBS) and plated in 12-well plastic culture plates. Nonadherent cells were removed by medium exchange after 1 h. Adherent macrophages were cultured overnight, washed with α-MEM, and then used for experiments. Bone marrow cells were isolated from the femurs of 6–8-week-old female C57/bl mice as described previously (22). Cells were plated for 24 h in RPMI 1640 containing 10% FBS and 10% L-cell conditioned medium as the source of M-CSF. The nonadherent cells were removed and cultured in the above medium for 5–7 days until 80% confluence was reached.

Reverse transcription polymerase chain reaction analysis

RNA was isolated from BMDM and peritoneal macrophages (PM) from wild-type or LOX-1 knockout mice using Trizol (Invitrogen, Burlington, ON). Total RNA was then used as a template for first strand cDNA synthesis using M-MLV reverse transcriptase from Promega (Madison, WI) according to the manufacturer's instructions. The resulting cDNA was amplified using the forward primer 5′-CGTGTTCCGGCTGTCAGCGCAGG-3′ and the reverse primer 5′-CAACGCTATGTCCTGATAGCGGTCC-3′. These sequences were obtained from the genomic sequence using the algorithm Primer 3 (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). PCR was performed with Taq DNA polymerase (Invitrogen, Burlington, ON) in a 25 μl reaction volume containing 10 pmol of each primer. Amplification was performed for 35 cycles at 94°C for 2 min, 94°C for 40s, 60°C for 1 min, and 72°C for 1 min. The final elongation phase was done at 72°C for 10 min. The PCR products were electrophoresed on a 1.5% agarose gel and visualized using ethidium bromide under UV light.

Fluorescent labeling

Fluorescent labeling of oxLDL was performed by adding 100 μg DiI in 60μl dimethyl sulfoxide to 2 mg of oxLDL in the pres-

ence of d > 1.21g/ml plasma fraction as a source of lipid transfer activity (24). Following an 8 h incubation period at 37°C, the labeled oxLDL was reisolated by ultracentrifugation at d = 1.1. This procedure typically resulted in an incorporation of 5–15 μg of DiI per mg of LDL protein.

Assays of LDL uptake and degradation in cultured cells

Macrophages were incubated at 37°C with varying concentrations of radioiodinated native, oxidized, or acetylated LDL in α-MEM with 2.5% lipoprotein deficient serum. After 4 h, the medium was removed and the cells were washed. LDL degradation products were quantified by mixing 1 ml of the supernatant medium with 350 μl of 50% trichloroacetic acid and 350 μl of 7.5% $AgNO_3$. The mixture was centrifuged at 2500 rpm for 15 min, and the supernatant was counted on a LKB 1282 γ counter. The cells were removed from the plates with Teflon cell lifters and counted to determine cell-associated LDL.

Uptake of DiI labeled oxLDL by macrophages

Peritoneal macrophages were incubated for 5 h at 37°C with 10 μg/ml DiI-labeled oxidized LDL in α-MEM containing 2.5% LPDS. Cells were washed with PBS, fixed with 2% formaldehyde, and mounted in 90% glycerol, 9.75% PBS, and 0.25% 1,4-diazabicyclo[2,2,2]octane. Cells were examined with a Zeiss Axioskop fluorescence microscope in epifluorescent mode with a fluorescein filter set.

In vivo clearance of LDL

Clearance of oxLDL or native LDL was measured as previously described (25). Briefly, wild-type or LOX-1 deficient mice were anesthetized with 2% isoflurane. The external jugular vein was exposed with the aid of a Zeiss operating microscope (Carl Zeiss Canada Ltd., Don Mills, Ontario) and cannulated with a PE 10 polyethylene catheter (outer diameter 0.61 mm; VWR Canlab), positioned with its tip in the superior vena cava. ^{125}I oxLDL (1-2 million cpm) in 200 μl of 150 mM NaCl was drawn into a 1ml syringe. The syringe was counted before and after the injection to allow an accurate determination of the amount of radioactivity administered. Five or six 20 μl blood samples were collected over 20 min. A second injection was then performed with a similar amount of native LDL, and four or five 20 μl samples were collected over 20 min. For oxLDL, the concentration at zero time was calculated using the injected dose of oxLDL and the volume of distribution of native LDL.

Cell viability assay

Macrophage survival was determined by the MTS-formazan method. This assay is based on the cellular bioreduction of MTS by mitochondrial dehydrogenase enzymes in metabolically active cells. The quantity of formazan product formed was measured by the absorbance at 490 nm and is directly proportional to the number of viable cells in culture (26). MTS-PMS solution (20 μl/well) was added to wells containing 100 μl of culture medium in 96 well plates for 3 h before terminating the experiment. This resulted in final MTS and PMS concentrations of 333 μg/ml and 25 μM, respectively. After 3 h incubation at 37°C in a humidified 5% CO_2 atmosphere, the absorbance at 490 nm was recorded with an ELISA plate reader.

Apoptosis assay

Propidium iodide staining and FACS analysis was used to quantitate the subdiploid population. At the end of 24 hr incubation, BMDM from wild-type or LOX-1 knockout mice were fixed and permeabilized with ethanol 70% (v/v) for 1 h at 4°C and washed twice in PBS containing 0.01% glucose. Cells were then resuspended in the same buffer plus RNase A (final concentration 0.1 mg/ml) and propidium iodide (final concentration 0.12 mg/ml). Fluorescence was measured with a BD FACS Canto. The data was analyzed with FCS Express Pro Software Version 3 (De Novo Software, Thornhill, Canada) with gating to exclude debris and cellular aggregates.

Immunoblotting

BMDM were harvested as described above and lysed in ice-cold homogenization buffer [20 mM morpholinepropanesulfonic acid, pH 7.2, 1% Triton X-100, 50 mM β-glycerol phosphate, 5 mM EGTA, 2 mM EDTA, 1 mM sodium vanadate, 25 μM β-methyl aspartic acid, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, aprotinin (10 μg/ml), and leupeptin (10 μg/ml)]. Lysates were centrifuged at 14,000 rpm for 10 min, and the protein content of supernatants was quantified using the Bradford protein assay. Fifty micrograms of protein from each sample was loaded and separated by SDS-PAGE using a 10% separating gel. Gels were calibrated with prestained SDS-PAGE low molecular weight standards (Bio-Rad, Hercules, CA). Proteins were then transferred to nitrocellulose membranes which were then blocked for 1 h with 4% skim milk, 0.01% NaN_3 in TBS-0.1% Tween 20. The membranes were cut at about the 70 kDa point, and the bottom section was then incubated with anti-phospho(Ser473) PKB antibody overnight in TBS-0.1% Tween 20 at 4°C, washed three times, and then incubated with horseradish peroxidase-conjugated secondary antibody at a 1:5,000 dilution for 1 h. The bands were then visualized by enhanced chemiluminescence. This blot was then stripped in Tris buffer containing SDS (2%) and β-2-mercaptoethanol (100 mM) at 50°C for 20 min, washed, and reprobed with antibody to PKB. The top section of the membrane was incubated with antibody against the p85 subunit of PI3K, and bands were visualized as above.

Analytic procedures

LDL and cell protein was assayed by the method of Lowry (27) in the presence of 0.05% sodium deoxycholate with BSA as the standard. Lipoprotein electrophoresis was done using the Titan gel lipoprotein electrophoresis system (Intermedico, Markham, Ontario). Lipoprotein bands were visualized by staining with Fat red.

Statistical analysis

Statistical analysis was done using ANOVA or Student's *t*-test as appropriate. $P < 0.05$ was taken as significant.

RESULTS

LOX-knockout mice

Homozygous LOX-1 deficient animals had no overt phenotype and were fertile. The absence of LOX-1 mRNA was verified by RT-PCR (**Fig. 1**).

Uptake of fluorescently labeled oxLDL by macrophages

The lipophilic fluorophore diI crosses lysosomal membranes very slowly and so serves as a cumulative marker of the quantity of labeled LDL that is internalized by cells (24, 28). To obtain a morphologic comparison of the internalization of oxLDL in control and LOX-1 deficient macrophages, we incubated peritoneal macrophages grown on coverslips for 5 h with diI-labeled oxLDL. Fluorescence micrographs indicate that there was no apparent difference in the intensity or distribution of fluorescence between wild-type and LOX-1 deficient cells (**Fig. 2**).

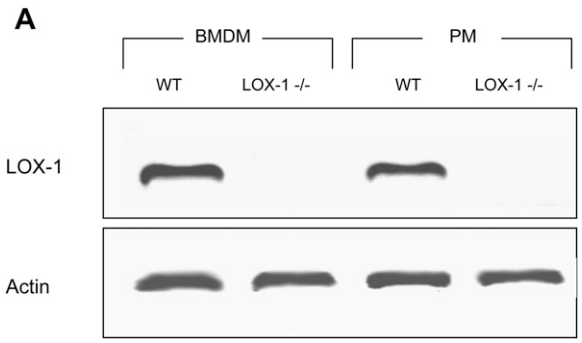

Fig. 1. Absence of LOX-1 mRNA in macrophages harvested from LOX-1 knockout mice. Absence of LOX-1 mRNA in knockout animals was verified by RT-PCR of total RNA from bone marrow derived macrophages (BMDM) or peritoneal macrophages (PM). An amplicon with migration consistent with the predicted nucleotide sequence is seen in the wild-type cells, whereas no band is seen in knockout cells.

Uptake and degradation of modified LDLs in wild-type and LOX-1 deficient peritoneal macrophages

To quantify the relative rates of oxLDL uptake, we incubated macrophages for 5 h with radioiodinated native LDL, oxLDL, or acetyl LDL and then measured rates of LDL uptake and degradation. There was no difference in the rate of uptake of native LDL or either modified LDL in wild-type compared with LOX-1 knockout peritoneal macrophages (**Fig. 3**).

Uptake and degradation of oxLDL in wild-type and LOX-1 deficient peritoneal macrophages

Different scavenger receptors are known to have different affinities for minimally, moderately, or extensively oxidized LDL. We therefore tested the uptake and degradation of radioactively labeled oxLDL in LOX-1 deficient and wild-type macrophages. There was no difference between wild-type and LOX-1 deficient macrophages in uptake or degradation of minimally, moderately, or extensively oxidized LDL (**Fig. 4**).

Effect of induction of LOX-1 gene expression on oxLDL uptake and degradation in wild-type macrophages

LOX-1 has been shown to mediate uptake and degradation of oxLDL in endothelial cells, smooth muscle cells, and in transfected cell lines. Hence, it seemed possible that the failure to detect a difference between wild-type and LOX-1 deficient macrophages might be because a high level of expression of SRAI/II and CD36 masked the contribution of LOX-1. To test this, we took advantage of the observation that lysophosphatidylcholine causes a marked and selective (up to 5-fold) induction of LOX-1 in endothelial cells and smooth muscle cells (29, 30). Cultured peritoneal macrophages from wild-type and LOX-1 deficient mice were preincubated for 12 h with varying concentrations of lysophosphatidylcholine, and then incubated for 5 h with ^{125}I oxLDL or ^{125}I LDL (**Fig. 5A**, 5B). Lysophosphatidylcholine caused a concentration-dependent increase in oxLDL uptake and degradation in wild-type macrophages but had no effect on uptake and

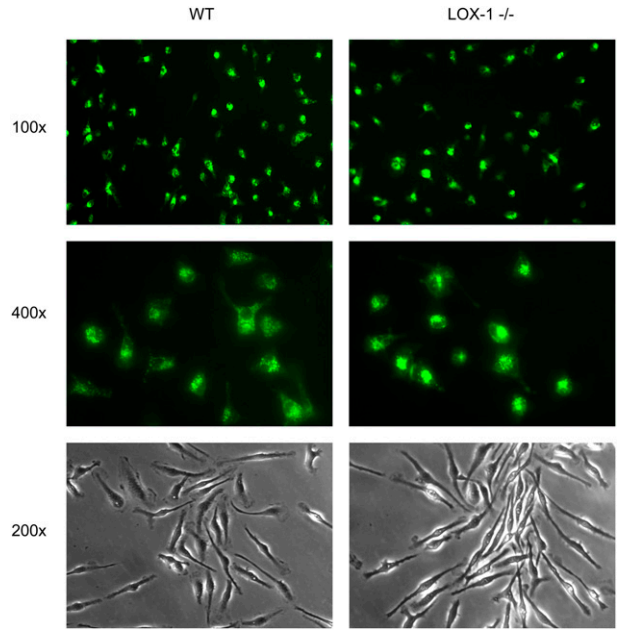

Fig. 2. Uptake of oxLDL in peritoneal macrophages. Resident peritoneal macrophages from wild-type (WT) and LOX-1 knockout mice were incubated for 5 h with 10μg/ml DiI-labeled oxidized LDL, and then examined using a Zeiss Axioskop fluorescence microscope. No difference in fluorescence intensity is seen.

degradation in LOX-1 deficient cells. These concentrations of lysophosphatidylcholine are the same as those reported to induce LOX-1 expression in smooth muscle cells and endothelial cells (29). The upregulation of LOX-1 mRNA by these concentrations of lysophosphatidylcholine was confirmed by RT-PCR (Fig. 5C).

In vivo clearance of oxLDL

To directly assess the role of LOX-1 in oxLDL clearance in vivo, we compared the rates at which oxLDL was removed from plasma from wild-type and LOX-1 deficient mice. Animals were injected first with ^{125}I-oxLDL and then

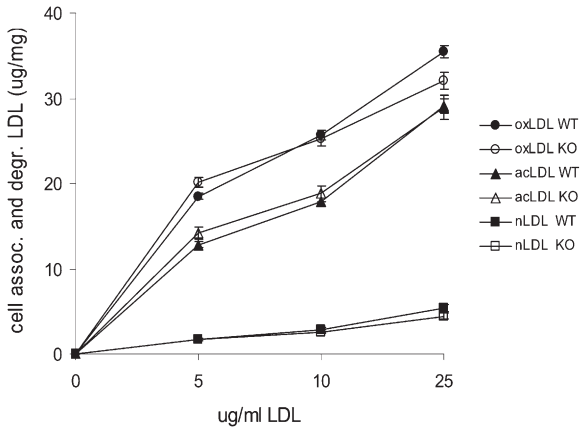

Fig. 3. Uptake and degradation of native, oxidized, and acetylated LDL in macrophages. Cultured peritoneal macrophages from wild-type mice (solid symbols) or knockout mice (open symbols) were incubated with the indicated concentration of ^{125}I-labeled native LDL, oxLDL, or acetyl LDL.

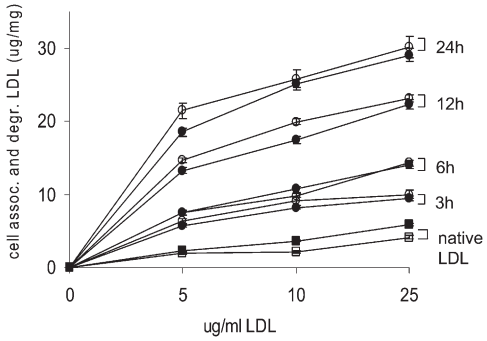

Fig. 4. Uptake and degradation of LDL oxidized to various degrees in wild-type and LOX-1 deficient peritoneal macrophages. Cultured peritoneal macrophages from wild-type mice (solid symbols) or knockout mice (open symbols) were incubated with the indicated concentration of native ^{125}I-LDL (squares) or ^{125}I-LDL oxidized by exposure to 5μM Cu^{2+} for 3 h, 6 h, 12 h, or 24 h (circles). The electrophoretic mobility of oxLDL was 1.0 (3 h), 1.3 (6 h), 2.2 (12 h) or 3.8 (24 h) times that of native LDL. After 5 h, cells and media were assayed for uptake and degradation of labeled lipoprotein. Results are the sum of cell-associated and degraded LDL expressed as μg LDL per mg cell protein. Values are the means +/− SEM of triplicates.

with native ^{125}I-LDL and serial blood samples were assayed for radioactivity. The rates of plasma clearance of oxLDL were very rapid and indistinguishable between wild-type and LOX-1 deficient animals (**Fig. 6**).

Role of LOX-1 in the ability of oxLDL to increase survival of BMDM

We have shown that oxLDL promotes growth and cytokine-independent survival in macrophages at least in part by activating the PI3K/PKB pro-survival pathway (15–17 26). We previously found that oxLDL promoted survival in SRAI/II-deficient macrophages (31) and in CD36-deficient macrophages (unpublished), so we hypothesized that LOX-1 might be the receptor required for PKB activation. To address this, we compared the ability of oxLDL to promote survival in cytokine-deprived BMDM from wild-type and LOX-1 deficient mice. After 24 h there was no significant difference in the viability of wild-type and LOX-1 deficient cells treated with oxLDL (**Fig. 7A**). There was no significant difference between wild-type and LOX-1 BMDM in the percentage of sub-diploid cells after incubation with oxLDL (Fig. 7B). Deficiency of LOX-1 did not abolish the ability of oxLDL to promote PKB phosphorylation in BMDM (**Fig. 8**).

DISCUSSION

The studies described in this report address two different aspects of the interaction of oxLDL with LOX-1 in macrophages. The first issue is the role of LOX-1 in uptake and degradation of oxLDL by macrophages. LOX-1 clearly has the ability to bind and internalize LDL in endothelial cells and in CHO cells transfected with LOX-1 receptor constructs (3). LOX-1 has a similar ligand specificity as SR-AI/II and CD36, although its affinity for ox-

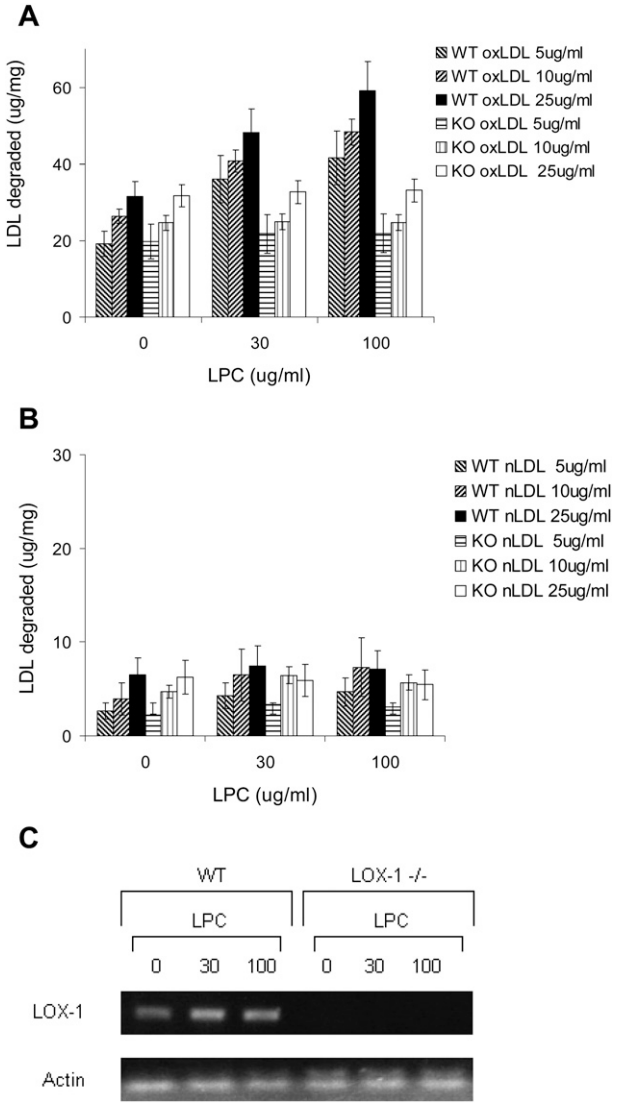

Fig. 5. Effect of induction of LOX-1 with lysophosphatidylcholine on oxLDL uptake and degradation. Cultured peritoneal macrophages from wild-type and LOX-1 knockout mice were preincubated for 12 h with 0, 30, or 100 ug/ml lysophosphatidylcholine. Cells were then washed and incubated for 5 h with varying concentrations of ^{125}I-oxLDL (A) or ^{125}I-LDL (B). Values shown are means ± SD of the sum of cell-associated and degraded oxLDL, reflecting total oxLDL uptake during the incubation. (C) RT-PCR blot showing that LOX-1 mRNA expression was increased in wild-type macrophages by these concentrations of lysophosphatidylcholine.

LDL is substantially higher than its affinity for acetyl LDL (31). We previously showed that about 70% of the uptake of oxLDL in macrophages was not due to SRAI/II (12). Competition experiments indicated that polyinosinic acid, but not acetyl LDL or native LDL, blocked the SRAI/II-independent uptake of oxidized LDL (12). Although CD36 has been shown to contribute to oxLDL uptake in macrophages, our preliminary findings in CD36 −/− macrophages showed no reduction in the anti-apoptotic effect of oxLDL. Hence it seemed possible that some of the SRAI/II and CD36-independent uptake might be due to a receptor such as LOX-1. This receptor has a reported

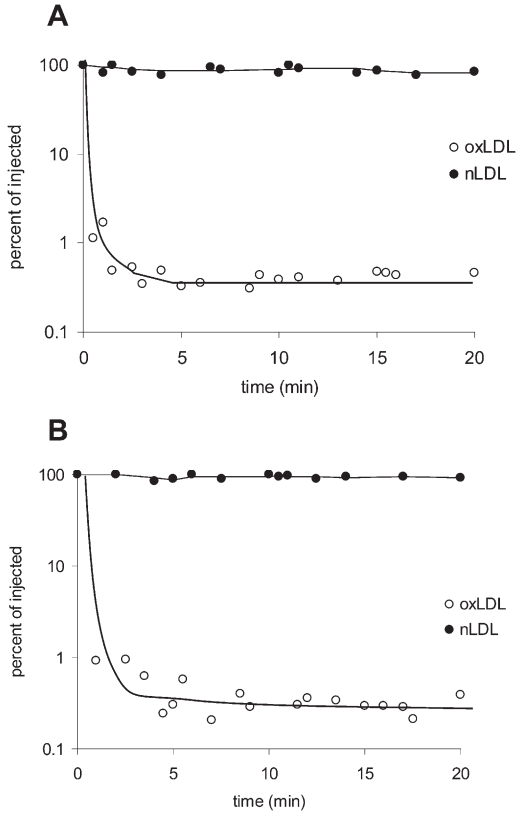

Fig. 6. In vivo plasma clearance of oxidized and native LDL in wild-type and LOX-1 deficient mice. Wild-type mice (A, n = 3) or LOX-1 knockout mice (B, n = 3) were anesthetized with 2% isoflurane. Oxidized ^{125}I LDL (open circles) or native ^{125}I LDL (solid circles) was injected through a cannula placed in the internal jugular vein. Serial 20 μl blood samples were withdrawn at the indicated time points and radioactivity was measured. Points for all three animals in each case are plotted together. The electrophoretic mobility of oxLDL in this experiment was 4.1-fold that of native LDL.

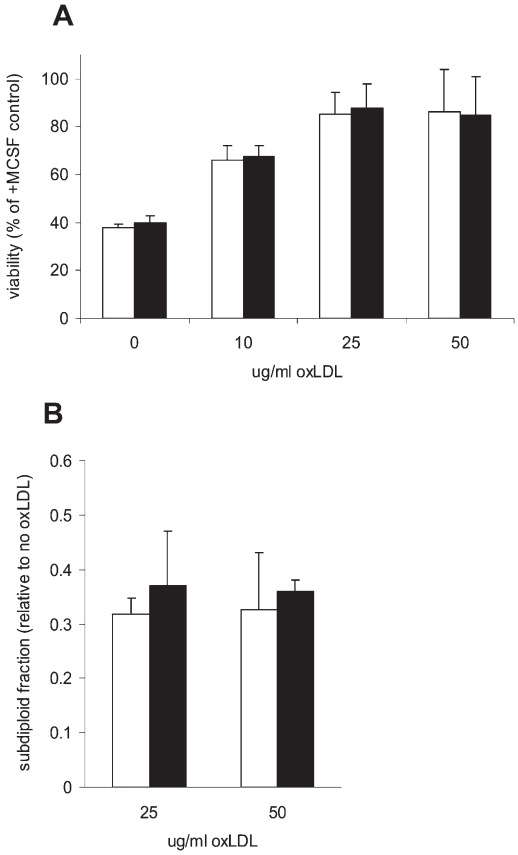

Fig. 7. LOX-1 deficiency does not impair the ability of oxLDL to inhibit macrophage apoptosis. (A) BMDM from wild-type mice (open bars) or LOX-1 −/− mice (solid bars) were seeded at 3 × 10^3 cells/well in 96 well plates and cultured for 24 h. They were then washed and incubated for 24 h without M-CSF but with the indicated concentrations of ox-LDL. Viability was then measured by the bioreduction of MTS (described in Materials and Methods) and expressed as a percentage of that in control cells treated with M-CSF. (B) BMDM were plated at 10^6 cells/well in six well plates and incubated for 24 h in medium containing indicated concentration of oxLDL but no M-CSF. The sub-diploid population was measured by flow cytometry after propidium iodide staining. Results are expressed relative to control cells incubated without CSF, which typically amounted to about 40% of cells. In both panels, results are the means ± SD of pooled data from three independent experiments. None of the differences between wild-type and LOX-1 −/−. macrophages was significant.

ligand specificity that is congruent with the ligand specificity that we observed in SRAI/II-deficient macrophages (12). It has been shown that LOX-1 is expressed in monocyte-derived macrophages (14) and in macrophages in atherosclerotic lesions (7). Furthermore, a recent report indicates that LOX-1 accounts for at least half the uptake of oxLDL in PMA-activated HL-60 macrophages (32). However, in the present studies there was no effect of LOX-1 gene inactivation on oxLDL uptake by BMDM or peritoneal macrophages.

One assumption that is implicit in the interpretation of our results is that there is no compensatory increase in the activity of other scavenger receptors in LOX-1 −/− mice and macrophages that exactly balances the effect of the inactivation of LOX-1 gene. A minor contribution of LOX-1 to oxLDL uptake might be masked if there was a high level of nonsaturable uptake mediated by phagocytosis or pinocytosis. However, we have previously shown that at the relatively low concentration of labeled oxLDL used in the present studies, more than 80% of the uptake and degradation of the labeled oxLDL can be competed by 10-fold excess of unlabeled oxLDL or by polyinosinic acid, indicating that the uptake is mediated by a high-affinity saturable mechanism, presumably a receptor(s) (12, 33). Overall, our finding that LOX-1 gene inactivation does not significantly alter oxLDL uptake in unstimulated murine macrophages indicates that LOX-1 accounts for at most 5–10% of oxLDL uptake by these cells. On the other hand, when LOX-1 was upregulated in macrophages by preincubation with lysophosphatidylcholine, internalization of oxLDL increased by more than 40%. It has been shown that pro-inflammatory cytokines such as TNFα (34) and TGFβ (35) upregulate LOX-1 and downregulate SR-AI/II and CD36. Hence, it is possible that in microenvironments where these cytokines are relatively abundant, notably in atherosclerotic lesions, LOX-1 might play a significant role in oxLDL uptake. It is also possible that endothelial cells and smooth muscle cells might rely on LOX-1 as a recep-

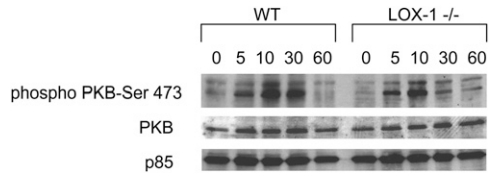

Fig. 8. LOX-1 is not essential for induction of PKB phosphorylation by oxLDL in BMDM. Bone marrow-derived macrophages from wild-type (WT) or LOX-1 knockout mice were incubated with or without 25 μg/ml oxLDL for 0–60 min. Cell lysates were then analyzed by immunoblotting for phosphorylation of PKB at serine 473. Immunostaining for the p85 subunit of PI3K as well as for total PKB was used to monitor loading.

tor for the internalization of oxLDL or for mediating oxLDL-induced signal transduction.

The second issue addressed by these studies was the potential role of LOX-1 in survival signaling. The importance of macrophage survival has been recently underlined by a series of studies in different animal models. Currently it is believed that at least in early stages of atherosclerosis, inhibition of macrophage apoptosis or induction of macrophage growth enhances lesion progression (36). It has been shown that oxLDL activates p42/44 MAPK (15), p38MAPK (37), PKC (38), and PI3K/PKB (15, 26). Activation of PI3K/PKB by oxidized LDL promotes growth and survival of macrophages (15, 26). The upstream steps by which oxLDL activates these signaling pathways have not yet been defined. As LOX-1 has been shown to activate PKCα (39), PKCβ (40), p38MAPK (41), and p42/44MAPK (42), it seemed appropriate to determine if it accounted for part of the anti-apoptotic signaling of oxLDL. However, we found no difference in the anti-apoptotic effect of oxLDL in LOX-1 knockout compared with wild-type macrophages.

In contrast to our findings in BMDM, previous studies in smooth muscle cells (10), cultured endothelial cells (43), and chondrocytes (44) reported a pro-apoptotic effect of oxLDL acting at least in part via LOX-1. There are a number of explanations for these apparently discrepant results. In the study by Kataoka et al. in smooth muscle cells (10), oxLDL only induced apoptosis at concentrations of 40 μg/ml or higher, and Fig. 1A in that paper actually shows increased viability of smooth muscle cells at lower concentrations of oxLDL. Also, they incubated cells in medium containing only 1% fetal bovine serum. We have reported a similar biphasic effect of oxLDL in macrophages, with concentrations higher than 100 μg/ml associated with toxicity in macrophages incubated in 10% fetal bovine serum (15). In macrophages incubated in serum-free medium, the threshold for toxicity of oxLDL was much lower, around 20 μg/ml (15). The study by Imanishi and colleagues found that oxLDL upregulated Fas expression and potentiated the effect of an agonistic anti-Fas antibody on apoptosis in endothelial cells (43). This study did not investigate apoptosis induced by oxLDL, only its effect on apoptosis induced by anti-Fas. In this study, endothelial cells were cultured in medium containing only 1% FBS, which would have made them more vulnerable to toxic effects of oxLDL. The study by Nakagawa and coworkers showed that in chondrocytes, oxLDL at concentrations of 40 μg/ml or higher induced apoptosis. Chondrocytes were incubated in serum-free medium, which may explain why the toxic threshold for oxLDL was relatively low. Also, their oxLDL preparations may not have been dialyzed after oxidation, and we have previously shown that this results in a high concentration of toxic water-soluble aldehydes in the oxLDL mixture (45) Although, it appears that LOX-1 plays a role in oxLDL-mediated toxicity in these cells, our results in macrophages provide no evidence that LOX-1 is involved in the anti-apoptotic effect of low concentrations of oxLDL.

Other receptors that are capable of binding oxLDL or some of its components could play a role in pro-survival signaling, including the phosphatidylserine receptor (46), PPARα (47, 48), PPARγ (49–51), TLR4 (52), and FcγRII (53). Studies are under way to determine if any of these play a role in the pro-survival effect of oxLDL in macrophages.

Dr. Osamu Cynshi provided LOX-1 knockout mice from a colony maintained at Chugai Pharmaceutical Corp., Tokyo.

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ERRATA

The authors of the article "LOX-1 augments oxLDL uptake by lysoPC-stimulated murine macrophages but is not required for oxLDL clearance from plasma" (*J. Lipid Res.* 2009. 50: 1676–1684) have advised the *Journal* that the primer sequences shown for the amplification of LOX-1 cDNA are incorrect. The correct primer sequences are forward primer 5′-CATGTTGGCCTTGACATCTG -3′ and the reverse primer 5′-GTTGGTTGGGAGACTTTGGA -3′.

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