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Regulation of MSR-1 and CD36 in macrophages by LOX-1 mediated through PPAR-γ

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ABSTRACT

We observed uniform sustained Dil-ox-LDL uptake in macrophages in the presence or absence of ox-LDL receptor-1 (LOX-1). We wondered if the deficiency of LOX-1 modulates the expression of two other scavenger receptors, macrophage scavenger receptor-1 (MSR1) and CD36, on macrophages to account for the unaltered ox-LDL uptake. Macrophages were isolated from wild-type (WT) and LOX-1 knockout (KO) mice and stimulated with ox-LDL. Dil-ox-LDL uptake and expression of MSR1 and CD36 examined. Abrogation of LOX-1 did not significantly change Dil-ox-LDL uptake by macrophages. LOX-1 KO macrophages showed a significant decrease in CD36 at baseline as well as after ox-LDL stimulation and a marked almost 100% increase in the expression of MSR1, both at mRNA and protein levels (all $p < 0.05$ vs. WT macrophages). Further, we observed a reduction in the expression of PPAR-γ in LOX-1 KO macrophages. To ascertain the role of PPAR-γ in the altered expression of MSR1 and CD36, LOX-1 KO macrophages were treated with troglitazone, a PPAR-γ agonist. Activation of PPAR-γ by troglitazone reversed the increased expression of MSR1 as well as the decreased expression of CD36 in LOX-1 KO macrophages. LOX-1 abrogation induces MSR1 and inhibits CD36 expression. The increase in MSR1 most likely accounts for sustained Dil-ox-LDL uptake despite LOX-1 abrogation. The alterations in CD36 and MSR1 occur through a decrease in PPAR-γ.

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1. Introduction

Oxidative modified LDL plays a key role in atherogenesis [1]. Macrophages internalize ox-LDL through different scavenger receptors, and transform into foam cells which release growth factors and cytokines. These are believed to be important steps in atherogenesis [2].

The scavenger receptors MSR1, CD36 and LOX-1 are three main receptors for ox-LDL on macrophage membrane [3]. MSR1 belongs to scavenger receptor A family, and has been implicated in many processes, such as atherosclerosis, Alzheimer's disease, and host defense [4–6]. CD36 is a member of the class B scavenger receptor family and its activation has been implicated in foam cell formation [7]. It appears that MSR1 and CD36 account for greater than 90% of the lipid accumulation in macrophages exposed to oxidized LDL [7]. LOX-1 is another important scavenger receptor; it belongs to the C-type lectin superfamily, and promotes growth and migration of macrophages and induces their transformation into foam cells [8].

PPAR-γ, also known as the glitazone receptor, has been shown to regulate fatty acid storage and glucose metabolism [9]. Several studies show that ox-LDL stimulates PPAR-γ and subsequently enhances CD36 and inhibits MSR1 in macrophages [10–13].

Our laboratory has been working on the distribution, regulation and therapeutic implications of LOX-1 for over a decade. Our recent studies show that LOX-1 abrogation has the potential to reduce atherogenesis, myocardial ischemic injury and hypertensive response to angiotensin II [14–16]. The status of MSR1 and CD36 in the absence of LOX-1 is unknown. These studies were conducted to address this question.

2. Materials and methods

2.1. Animals

C57BL/6 (also called wild-type or WT) mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Homozygous LOX-1 KO mice were generated as described previously [14–16]. After

Abbreviations: Ox-LDL, oxidized low density lipoprotein; LOX-1, lectin-like scavenger receptor for ox-LDL; MSR1, macrophage scavenger receptor 1; PPAR-γ, peroxisome proliferator-activated receptor gamma; LOX-1 KO, LOX-1 Knockout; Dil-ox-LDL, 1,1′-dioctadecyl-3,3,3′3′-tetramethyl-indocarbocyanine perchlorate labeled oxidized low-density lipoprotein; WT, wild type; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells.

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backcrossing 8 times with C57BL/6 strain, the genetic background was replaced. By brother–sister mating, C57BL/6 and LOX-1 KO mice were bred and housed in the breeding colony at the local institution. Male mice, 6–8 weeks of age, were utilized in the present studies. All experimental procedures were approved by the Institutional Animal Care and Usage Committee.

2.2. Genotyping

Genomic DNA was isolated from the tails of WT and LOX-1 KO mice using DNeasy Blood-Tissue Kit according to the manufacturer's protocol [17].

2.3. Macrophages harvesting and preparation

Mice were injected intraperitoneally with 1 ml 4% brewer modified thioglycollate medium (BD Biosciences, San Jose, CA) to stimulate accumulation of macrophages. Three days later, 5 ml cold 1× PBS was injected intraperitoneally, and macrophages were harvested. Isolated cells were then cultured in the 1640 medium (ATCC, Manassas, VA) along with 10% FBS for 2 h. Macrophages were then treated with ox-LDL (TBARS 50.00 ± 0.54 nmol/mg) (Biomedical Technologies Inc., Stoughton, MA).

2.4. Dil-ox-LDL uptake

For visualization of ox-LDL uptake, macrophages format were incubated with Dil-ox-LDL 5 μg/ml (Biomedical Technologies Inc., Stoughton, MA) for 2 h at 37 °C. After incubation, cells were gently washed with sterile 1× PBS for 3 times and imaged by fluorescent microscopy as described previously [17]. Number of fluorescence cells was counted manually and expressed as percentage of all cells.

2.5. RT-real-time quantitative PCR

Total RNA was extracted using TRIZOL reagent (Invitrogen, Grand Island, NY). After reverse transcription, the resulting cDNA (100 ng) was amplified using 200 nM primers (Invitrogen, Grand Island, NY). Primer sequences are shown in Table 1. Each mRNA was detected by GoTaq® qPCR Master Mix kit (Promega, Madison, WI). Quantitative PCR was performed using the Applied Biosystems 7900 real-time PCR system as described previously [17]. The reaction system and standard cycling condition followed with supplied protocols. The comparative threshold cycles values were normalized for GAPDH reference genes.

2.6. Western blot

The antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Abcam (Cambridge, MA). The relative expression of proteins was normalized with β-actin as described previously [17].

2.7. Statistical analysis

All experiments were performed in at least triplicates. Values were analyzed by using one-way ANOVA (multiple means) or the Newman–Keuls-Student's t test. All results were presented as means ± SD. A P value <0.05 was considered significant.

3. Results

3.1. Confirmation of LOX-1 gene knock-out and absence of LOX-1 does not change Dil-ox-LDL uptake in macrophages

LOX-1 DNA was completely absent in all LOX-1 KO mice as shown in Fig 1A.

To assess ox-LDL uptake in the presence or absence of LOX-1, macrophages from WT and LOX-1 KO mice were incubated with Dil-ox-LDL. As shown in Fig 1B, there was no difference in Dil-ox-LDL uptake in macrophages obtained from WT or LOX-1 KO mice.

3.2. Ox-LDL and CD36 and MSR1 gene expression

As shown in Fig 1C, low concentrations (⩽5 μg/ml) of ox-LDL significantly enhanced LOX-1, CD36 and MSR1 gene expression ($P < 0.05$ vs. Control), while 10 μg/ml concentration of ox-LDL decreased LOX-1, CD36 and MSR1 gene expression ($P < 0.05$ vs. 5 μg/ml ox-LDL) in macrophages from WT mice. Maximal stimulation of LOX-1, CD36 and MSR1 was seen after 3–6 h of incubation with ox-LDL; the longer duration of incubation decreased the expression of LOX-1, CD36 and MSR1, reflecting cell death.

3.3. Effect of LOX-1 abrogation on CD36 and MSR1 expression

To elucidate the effect of LOX-1 abrogation on CD36 and MSR1 expression, we measured CD36 and MSR1 expression (mRNA and protein) in macrophages from WT and LOX-1 KO mice. As shown in Fig. 2A and B, CD36 expression in LOX-1 KO mice macrophages decreased by almost 50% and that of MSR1 increased by almost 100% as compared with WT group. These changes in CD36 and MSR1 expression were seen both at mRNA and protein levels, and at baseline ($P < 0.05$) as well as after ox-LDL stimulation with ox-LDL ($P < 0.05$).

3.4. LOX-1 abrogation and PPAR-γ and NF-κB expression

To determine the intracellular mechanism of regulation of CD36 and MSR1 expression, we measured PPAR-γ and NF-κB expression in WT and LOX-1 KO mice macrophages. As shown in Fig 3A, PPAR-γ mRNA expression was markedly decreased in these cells ($P < 0.05$ vs. WT macrophages). Treatment of LOX-1 KO mice with ox-LDL significantly increased PPAR-γ gene expression, but it was still lower than in the WT macrophages stimulated with ox-LDL. Interestingly, NF-κB, a target of PPAR-γ action, mRNA was not affected by LOX-1 abrogation.

Table 1
Primer sequences for real-time PC.

Primer sequences	Forward primer	Reverse primer
LOX-1	5′-CTGGATTGGATTGCATCGGAA-3′	5′-CAGCTCCGTCTTGAAGGTATG-3′
CD36	5′-ATGGGCTGTGATCGGAACTG-3′	5′-GTCTTCCCAATAAGCATGTCTCC-3′
MSR1	5′-TGAACGAGAGGATGCTGACTG-3′	5′-TGTCATTGAACGTGCGTCAAA-3′
PPAR-γ	5′-TCGCTGATGCACTGCCTATG-3′	5′-GAGAGGTCCACAGAGCTGATT-3′
NF-κB	5′-CAGGTCCACTGTCTGCCTCT-3′	5′-TGTCACTATCCCGGAGTTCA-3′
GAPDH	5′-AGGTCGGTGTGAACGGATTTG-3′	5′-TGTAGACCATGTAGTTGAGGTCA-3′

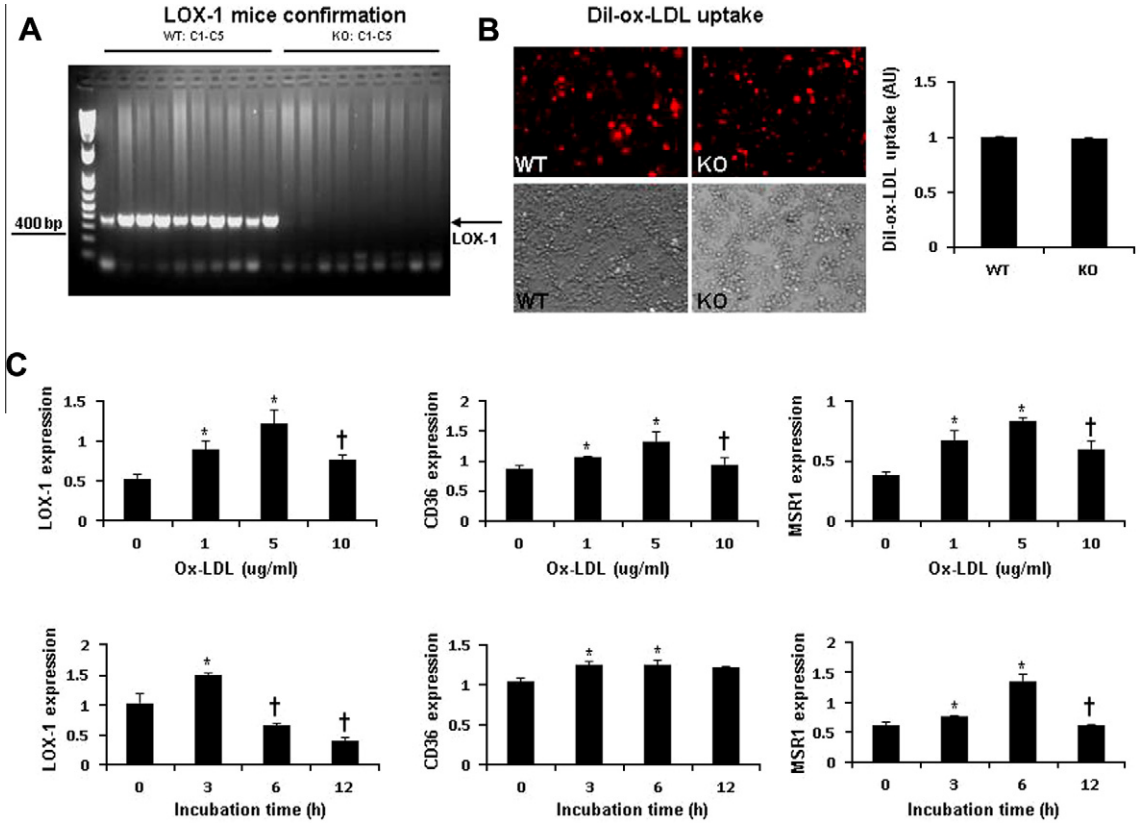

Fig. 1. (A) LOX-1 gene KO confirmation. WT:C1-C5 represents WT mice LOX-1 DNA. KO:C1-C5 represents LOX-1 KO mice LOX-1 DNA. LOX-1 gene is completely abrogated. (B) Unchanged Dil-ox-LDL uptake in macrophages from WT and LOX-1 KO mice. (C) Upper panel shows that small concentration of ox-LDL (⩽5 μg/ml) increase CD36, MSR1 and LOX-1 mRNA, while 10 μg/ml decreases it (*$P < 0.05$ vs. Control, †$P < 0.05$ vs. 5 μg/ml ox-LDL). Lower panel shows the increase at 3–6 h of incubation, but a decrease at 12 h. (*$P < 0.05$ vs. Control, †$P < 0.05$ vs. ox-LDL 3–6 h).

To examine the role of PPAR-γ in the regulation of CD36 and MSR1, we incubated LOX-1 KO mice macrophages with troglitazone (10 μM), a PPAR-γ agonist, followed by treatment with ox-LDL (5 μg/ml). As expected, PPAR-γ expression increased with troglitazone treatment ($P < 0.05$ vs. without troglitazone) (Fig 3B). Importantly, diminished CD36 expression rebounded to near normal level, and the enhanced MSR1 level fell (both $P < 0.05$ vs. without troglitazone).

4. Discussion

In this study, our first major observation was that the expression of all three major receptors LOX-1, CD36 and MSR1 on mice macrophages increased when cells were stimulated with modest concentration of ox-LDL (⩽5 μg/ml). Further, the increased expression in response to ox-LDL occurred within 3–6 h and then began to fall, perhaps a reflection of cytotoxicity of ox-LDL.

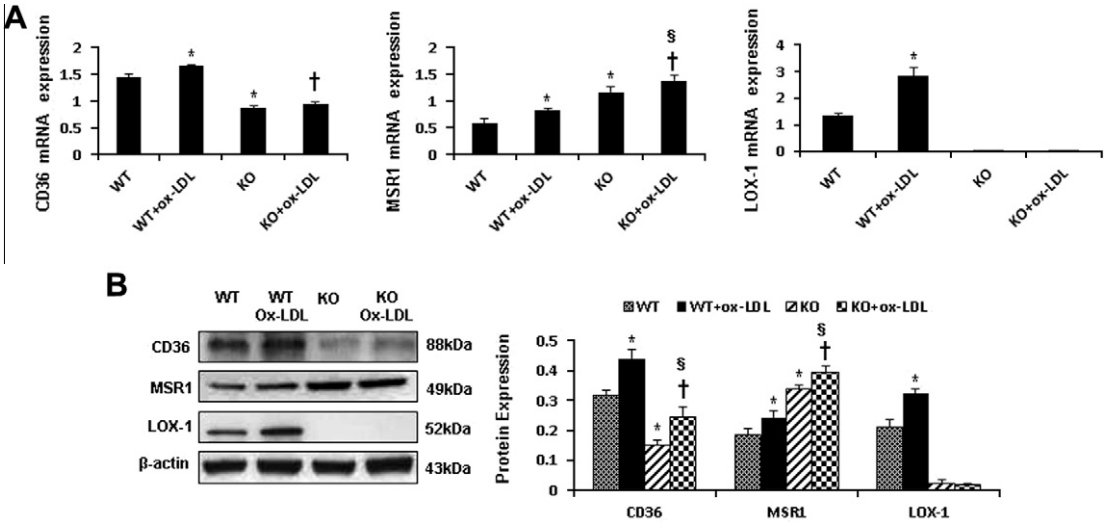

Fig. 2. Effect of LOX-1 abrogation on CD36 and MSR1 expression. (A) CD36 mRNA expression is decreased while MSR1 increased in LOX-1 KO macrophages, both at baseline and after ox-LDL stimulation ($P < 0.05$). (B) CD36 protein expression is decreased while MSR1 increased in LOX-1 KO macrophages, both at baseline and after ox-LDL stimulation ($P < 0.05$). Bar graphs represent data in mean ± SD, *$P < 0.05$ vs. WT, †$P < 0.05$ vs. WT + ox-LDL, §$P < 0.05$ vs. LOX-1 KO.

Our second major observation was sustained Dil-ox-LDL uptake in mouse macrophages despite LOX-1 abrogation. This prompted us to study the influence of LOX-1 abrogation on two other major scavenger receptors, CD36 and MSR1. We observed a significant decrease in the expression of CD36 and a dramatic almost 100% increase in the expression of MSR1. These alterations in CD36 and MSR1 were seen both at transcriptional and translational levels. The alterations in CD36 and MSR1 were present at baseline as well as after ox-LDL stimulation. We believe that the sustained ox-LDL uptake in LOX-1 KO mouse macrophages most likely reflects a marked increase in MSR1.

Our third major observation was a marked increase in PPAR-γ in macrophages treated with ox-LDL. The PPAR-γ expression was much less in LOX-1 KO mouse macrophages than in WT mouse macrophages. Previous studies from our laboratory show that PPAR-γ activators reduce LOX-1 expression [18]. Now we show that LOX-1 abrogation reduces PPAR-γ expression. These observations suggest a negative feedback loop between LOX-1 and PPAR-γ expression.

The changes in CD36 and MSR1 in LOX-1 KO mice macrophages may be attributed to the decrease of PPAR-γ, a type II nuclear receptor which regulates lipid and glucose metabolism.

A number of studies have shown that PPAR-γ regulates CD36 promoter and serves to increase CD36 expression and reduce MSR1 expression [10–13]. Based on the known role of PPAR-γ in the regulation of scavenger receptors and the presently described interaction between LOX-1 and PPAR-γ, it is logical to postulate that LOX-1 abrogation reduces CD36 and increases MSR1 expression via PPAR-γ (Fig. 4).

Our studies provide a strong evidence for this postulate since stimulation of PPAR-γ with troglitazone promptly and almost completely reversed the changes in the expression of CD36 and MSR1 to the same levels as seen in WT mouse macrophages.

As scavenger receptors, CD36 and MSR1, are expressed at higher level than LOX-1 in macrophages [19]. Originally identified by their role in mediating the uptake and internalization of modified lipoproteins like ox-LDL, CD36 and MSR1 have been thought to be essential for foam cell formation. However, recent work suggests

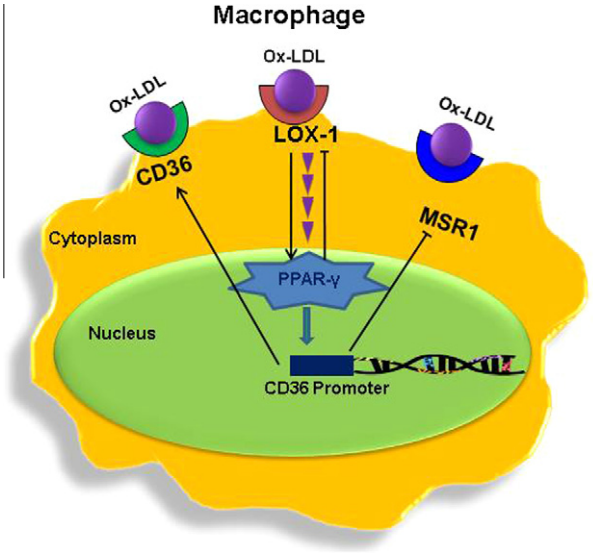

Fig. 4. A proposed interaction between LOX-1, CD36 and MSR1 in macrophages. Ox-LDL via LOX-1 induces PPAR-γ expression in a negative feedback mechanism. The elevated PPAR-γ then stimulates CD36 promoter which results in an increase in CD36 and a decrease in MSR1 gene/protein. The abrogation of LOX-1 would have an opposite effect on CD36 and MSR1.

that deficiency of CD36 or MSR1 does not prevent macrophage foam cell formation [20]. Another study reports that combined deficiency of CD36 and MSR1 does not reduce atherosclerosis [21]. On the other hand, LOX-1 is the predominant scavenger receptor on endothelial cells [22], and its abrogation significantly reduces atherogenesis in LDLR KO mice given high cholesterol diet [14]. We have attributed reduction in atherogenesis to a reduction in oxidant stress and inflammatory response and to the preservation of endothelium-dependent relaxation. The absence of change in ox-LDL uptake in LOX-1 KO macrophages suggest that foam cell formation may not be a critical factor in inhibition of atherogenesis in mice missing LOX-1.

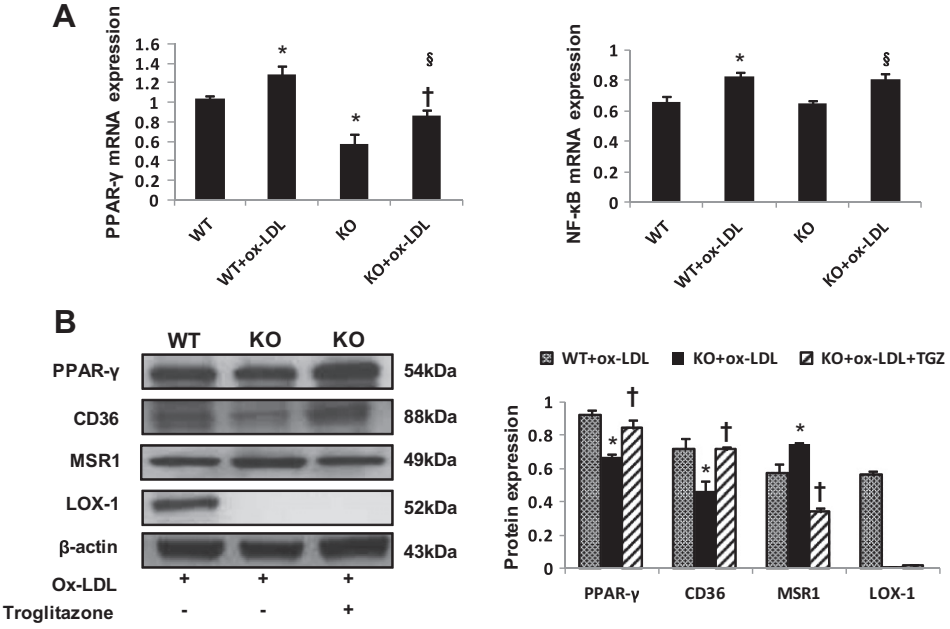

Fig. 3. (A) PPAR-γ mRNA expression is decreased in LOX-1 KO macrophages, both at baseline and after ox-LDL stimulation ($P < 0.05$). NF-kB p65 mRNA is similar in WT and LOX-1 KO macrophages, and the expression increases similarly after ox-LDL stimulation. (B) PPARγ expression increase after treatment with triglitazone (TGZ), CD36 expression also increases markedly after TGZ treatment, and MSR1 expression decreases after TGZ treatment. Bar graphs represent data in mean ± SD, $^{*}P < 0.05$ vs. WT, $^{\dagger}P < 0.05$ vs. WT + ox-LDL, $^{\S}P < 0.05$ vs. LOX-1 KO.

Collectively these observations imply that LOX-1 abrogation does not affect ox-LDL uptake in macrophages. Enhancement of MSR1 via inhibition of PPAR-γ appears to be the most likely basis of sustained ox-LDL uptake in the absence or presence of LOX-1.

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