

Liver X receptors inhibit human monocyte-derived macrophage foam cell formation by inhibiting fluid-phase pinocytosis of LDL

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Abstract Liver X receptors (LXRs) are ligand-activated transcription factors involved in the control of lipid metabolism and inflammation. Several studies have recently shown that LXRs promote reverse cholesterol transport and inhibit atherosclerosis. Our study investigated whether LXRs affect macrophage uptake of LDL by human monocyte-derived macrophages. We have previously shown that human monocytes differentiated into macrophages with macrophage-colony-stimulating factor (M-CSF) constitutively take up large amounts of native LDL by receptor-independent, fluid-phase pinocytosis. In the research reported here, human monocytes were differentiated to macrophages in the presence of M-CSF with or without the LXR agonists T0901317 or 22(R)-hydroxycholesterol. Then, macrophages were incubated with native ¹²⁵I-LDL to determine LDL uptake. T0901317 and 22(R)-hydroxycholesterol inhibited ¹²⁵I-LDL uptake by 68 ± 1% and 69 ± 2%, respectively, and decreased pinocytotic vacuoles in the macrophages. ¹²⁵I-BSA uptake, a measure of fluid-phase pinocytosis, and ¹²⁵I-LDL uptake were the same, and T0901317 treatment inhibited uptake of both to the same degree. T0901317 did not affect receptor-mediated uptake of acetylated LDL, showing that the LXR effect is specific for fluid-phase pinocytosis of lipoproteins. Our results show that LXRs downregulate macrophage pinocytosis of LDL. The findings reveal an additional new mechanism by which LXR agonists may inhibit macrophage cholesterol accumulation and atherosclerosis, namely, by inhibiting macrophage uptake of LDL.—Buono, C., Y. Li, S. W. Waldo, and H. S. Kruth. Liver X receptors inhibit human monocyte-derived macrophage foam cell formation by inhibiting fluid-phase pinocytosis of LDL. *J. Lipid Res.* 2007. 48: 2411–2418.

Supplementary key words atherosclerosis • cholesterol • endocytosis

Macrophage uptake of lipoproteins, especially LDL, is considered a critical process in the development of atherosclerosis, causing transformation of the macrophages

into foam cells, thus promoting cholesterol accumulation and inflammation in the atherosclerotic plaque (1, 2). Heretofore, researchers believed that macrophages could internalize only modified lipoproteins, typically through a variety of scavenger receptors (3, 4). In recent studies, we have demonstrated an alternative mechanism for macrophage foam cell formation that does not depend on LDL modification or macrophage receptors (5–7). In this mechanism of foam cell formation, macrophages take up LDL through receptor-independent, fluid-phase pinocytosis. Receptor-independent fluid-phase uptake of ¹²⁵I-LDL does not show saturation of uptake with increasing ¹²⁵I-LDL concentrations and cannot be inhibited by excess amounts of unlabeled LDL (5, 7). Pinocytosis of fluid and solute such as LDL contained in the fluid can occur either through uptake of fluid by micropinocytosis within small vesicles (micropinosomes) less than 0.1 μm in diameter, or by macropinocytosis within large vacuoles (macropinosomes) usually greater than 1 μm (8, 9). Macropinosomes form from extended plasma membrane folds that fuse back with the plasma membrane, trapping extracellular fluid with the intracellular vacuoles that are formed.

Macrophage pinocytosis of LDL is either constitutive or activated through a protein kinase C-dependent signaling pathway, depending on the macrophage differentiation phenotype. Differentiation of human monocytes in the presence of FBS and macrophage-colony-stimulating factor (M-CSF) produces an elongated macrophage phenotype that shows uptake of large amounts of LDL and accumulation of high levels of cholesterol by constitutive pinocytosis (7). This is the macrophage foam cell model we have studied in this report. Alternatively, human monocytes can be differentiated with human serum or FBS and granulocyte/macrophage-CSF, producing a rounded “fried egg” macro-

Abbreviations: DPBS, Dulbecco's phosphate-buffered saline; LXR, liver X receptor; M-CSF, macrophage-colony-stimulating factor; PPAR, peroxisome proliferator-activated receptor.

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phage phenotype that shows LDL uptake and cholesterol accumulation following stimulation of pinocytosis by protein kinase C activation (5, 6, 10). The pinocytosis of fluid that occurs with both macrophage phenotypes is accompanied by vigorous macropinocytosis, suggesting that this mechanism of fluid uptake contributes to the total pinocytotic fluid uptake observed in these macrophages.

Several studies have indicated that liver X receptors (LXRs) are important transcription factors involved in the control of lipid metabolism and inflammation (as reviewed in Ref. 11). It has been shown that LXRs inhibit atherosclerosis in experimental mouse studies. Treatment of atherosclerosis-prone LDL receptor- and apolipoprotein E (apoE)-deficient mice with synthetic LXR agonists reduces the development of atherosclerosis (12, 13). Other studies, using macrophage-selective LXR-deficient mice created by bone marrow transplantation, have further established that this reduction in atherosclerosis is dependent on LXR activity in macrophages (14, 15). One possible mechanism by which LXRs inhibit atherosclerosis is by promoting macrophage cholesterol efflux via induction of the genes encoding apoE and ATP binding cassette transporters A1 and G1 in macrophages (16–25). Our study has shown another possible mechanism by which LXRs could also affect atherosclerosis, by decreasing macrophage receptor-independent, fluid-phase pinocytosis of LDL.

MATERIALS AND METHODS

Culture of human monocyte-derived macrophages

Human monocytes were purified with counterflow centrifugal elutriation of mononuclear cells obtained by monocytopheresis of normal human donors. M-CSF monocyte-derived-macrophage cultures were begun with the elutriated human monocytes suspended in RPMI 1640 medium (MediaTech) with 10% FBS (GIBCO) seeded in 6-well plates (Corning Cellbind) at a density of 2×10^5 monocytes/cm². After 2 h incubation in a cell culture incubator with 5% CO₂/95% air at 37°C, the monocyte cultures were rinsed three times with RPMI 1640 medium. Then, the monocytes were differentiated into macrophages by culturing with RPMI 1640 medium containing 10% FBS, 100 ng/ml M-CSF (PeproTech), and 25 ng/ml IL-10 (PeproTech) (26) with or without the synthetic LXR agonist T0901317 (5 μM) (Cayman Chemical) or the natural LXR agonist 22(R)-hydroxycholesterol (5 μM) (Sigma-Aldrich). In one experiment, peroxisome proliferator-activated receptor (PPAR) agonists clofibrate (100 nM) (Calbiochem), L165,041 (5 μM) (Sigma-Aldrich), or ciglitazone (5 μM) (Cayman Chemical) were added to the macrophage differentiation medium. The medium was replaced with fresh medium after 6 days of culture, and monocyte-derived macrophages were used for experiments the following day.

For experiments, monocyte-derived macrophage cultures (about 0.2 mg protein/well) were first rinsed three times with serum-free RPMI 1640 medium. Cultures were incubated in serum-free RPMI 1640 medium with the indicated concentration of LDL and the reagents specified in each experiment. Experimental incubations with these monocyte-derived macrophages were carried out in the presence of the same concentration of cytokines and agonists used during differentiation of monocytes into macrophages, unless noted otherwise.

Preparation of lipoproteins for use in experiments

Before use, human LDL (Intracel) was dialyzed against 1 l of 0.15 M sodium chloride and 0.3 mM EDTA (pH 7.4) for 12 h at 4°C, then against RPMI 1640 medium (two changes, 1 l/each change) for 24 h. Human ¹²⁵I-LDL and ¹²⁵I-acetylated LDL (Bio-medical Technologies) were dialyzed against 0.15 M sodium chloride and 0.3 mM EDTA (pH 7.4) over 36 h at 4°C (three changes, 1 l/each change). All dialysis was carried out with Pierce Slide-A-Lyzer cassettes (10,000 molecular weight cutoff). After dialysis, lipoproteins were sterilized by passage through a 0.45 μm (pore size) low-protein binding filter (Gelman Acrodisc). ¹²⁵I-LDL specific activity was adjusted to 2.25×10^{-5} μCi/ng by adding unlabeled LDL dialyzed similar to the ¹²⁵I-LDL. ¹²⁵I-acetylated LDL was used at the original specific activity of 37.7×10^{-5} μCi/ng, as provided by the manufacturer. Lipoprotein concentrations are expressed in terms of protein.

Assay of ¹²⁵I-LDL and ¹²⁵I-acetylated LDL cell association and degradation

Macrophage cell association and degradation of ¹²⁵I-LDL and ¹²⁵I-acetylated LDL were determined according to the methods of Goldstein and Brown (27). Lipoprotein degradation was quantified by measurement of trichloroacetic acid-soluble organic iodide radioactivity in supernatants of culture media samples that were centrifuged at 15,000 g for 10 min. Values obtained in the absence of cells were <5% of those values obtained with cells.

Cell-associated ¹²⁵I-LDL and ¹²⁵I-acetylated LDL were determined by rinsing macrophages three times with Dulbecco's phosphate-buffered saline (DPBS) plus Ca²⁺, Mg²⁺, and 0.2% BSA, followed by three times with DPBS plus Ca²⁺ and Mg²⁺, all at 4°C. Then, macrophages were dissolved overnight in 0.1 N NaOH at 37°C. Aliquots of cell samples were assayed for protein content and ¹²⁵I radioactivity with a γ counter (28).

Determination of fluid-phase pinocytosis

¹²⁵I-BSA (MP Biomedicals) cell association and degradation by macrophages was determined similar to ¹²⁵I-LDL described above and served as a means to determine fluid-phase uptake by the macrophages (7, 27). Before incubations, ¹²⁵I-BSA-specific activity was adjusted to 2.25×10^{-5} μCi/ng by adding unlabeled BSA. Macrophages were incubated with 250 μg/ml ¹²⁵I-BSA.

Depletion of macrophage cholesterol with methyl-β-cyclodextrin

Macrophage cholesterol content was decreased by treating macrophages with methyl-β-cyclodextrin (Sigma-Aldrich) as previously described (29). Monocytes were cultured for 7 days in differentiation medium with M-CSF and then used for two parallel experiments to determine the effect of methyl-β-cyclodextrin treatment on macrophage cholesterol content and fluid-phase pinocytosis. Macrophages were first incubated for 1 h without or with methyl-β-cyclodextrin (5 mM) in RPMI 1640 without serum. Then, the macrophages were incubated for 5 h in fresh media without or with methyl-β-cyclodextrin and either unlabeled BSA (250 μg/ml) for determination of cholesterol content as previously described (30) or with ¹²⁵I-BSA (250 μg/ml) for assessment of fluid-phase pinocytosis.

Statistical analysis

All data are presented as the mean ± standard error of the mean. The means were determined from three culture wells for each data point. Statistical comparisons of means were made using Student's *t*-statistic (unpaired). A *P* value ≤0.05 was considered significant.

RESULTS

LXR agonist decreases macrophage macropinosomes

In a previous study (7), we found that M-CSF-differentiated macrophages contain macropinosome vacuoles that function in constitutive pinocytosis. The constitutive pinocytosis mediates fluid-phase uptake of LDL, causing high levels of macrophage cholesterol accumulation and foam cell formation. In the current study, we observed a noticeable reduction of macropinosome vacuoles in the macrophages when macrophages were differentiated with either T0901317 (Fig. 1), or the natural LXR agonist 22(R)-hydroxycholesterol (not shown). The LXR agonist effect of decreasing macrophage vacuoles suggested a potential inhibitory effect of LXR agonists on macrophage pinocytosis of fluid and consequently, receptor-independent fluid-phase uptake of LDL.

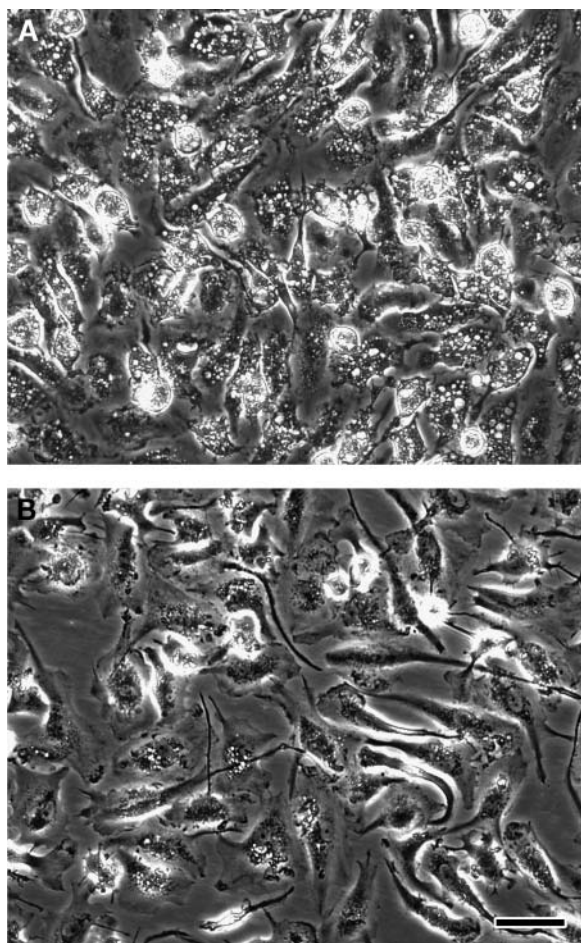


Fig. 1. T0901317 treatment decreases macropinosomes in macrophages. Shown are phase photo-micrographs of differentiated monocyte-derived macrophages following 7 day culture of monocytes in differentiation medium either without T0901317 (A), or in differentiation medium containing T0901317 (5 μ M) (B). There are fewer macropinosome vacuoles in T0901317-treated macrophages compared with control macrophages. The bar is 50 μ m and applies to both A and B.

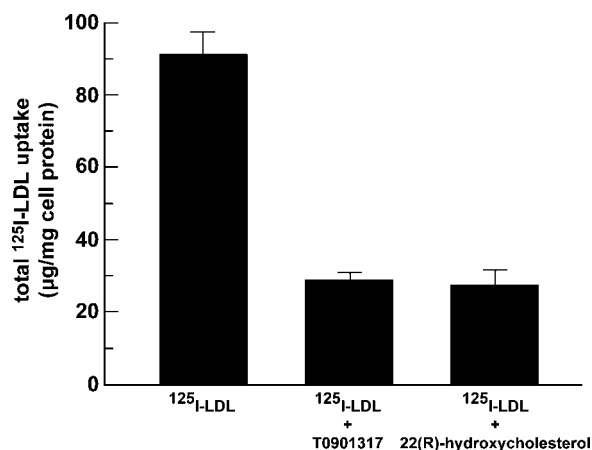


Fig. 2. Liver X receptor (LXR) agonists decrease macrophage uptake of ^{125}I -LDL. Monocytes were cultured for 7 days in differentiation medium without any LXR agonist or with T0901317 (5 μM) or 22(R)-hydroxycholesterol (5 μM). Then, the differentiated monocyte-derived macrophages were incubated for 1 day in serum-free differentiation medium containing the indicated LXR agonist treatment and 500 $\mu\text{g}/\text{ml}$ ^{125}I -LDL. Total uptake of the ^{125}I -LDL was determined as the sum of cell-associated and degraded ^{125}I -LDL as described in Materials and Methods. Error bars indicate \pm SEM.

LXR agonist decreases macrophage uptake of LDL

To learn whether LXR agonist treatment affected macrophage uptake of LDL, monocytes were differentiated to macrophages in the presence of T0901317 or 22(R)-hydroxycholesterol for 7 days and were then incubated for 1 day with 500 $\mu\text{g}/\text{ml}$ of ^{125}I -LDL. Total uptake of ^{125}I -LDL was reduced 68 \pm 1% and 69 \pm 2% in T0901317- and 22(R)-hydroxycholesterol-treated macrophages, respectively, compared with untreated control macrophages (Fig. 2). Treatment of macrophages with T0901317 did not affect the percentage of ^{125}I -LDL that was degraded by the macrophages following uptake. Macrophages without and with T0901317 treatment degraded similar amounts of ^{125}I -LDL in three experiments, 90 \pm 1% and 88 \pm 1%, respectively.

As expected, T0901317 inhibition of ^{125}I -LDL uptake was paralleled by a decrease in macrophage cholesterol accumulation. Control macrophages showed a large increase in cholesterol content after LDL incubation (96 \pm 5 to 463 \pm 22 nmol/mg/cell protein), whereas T0901317-treated (5 μM) macrophages showed a much smaller increase in their cholesterol content after LDL incubation (47 \pm 7 to 156 \pm 6 nmol/mg/cell protein) (Fig. 3). Thus, T0901317 inhibited macrophage cholesterol accumulation by 70 \pm 1% compared with controls ($P < 0.05$). Treatment of macrophages with 10 μM T0901317 rather than 5 μM did not result in greater inhibition of cholesterol accumulation (data not shown).

In an additional experiment, T0901317 was added to differentiating monocytes at different time points following initiation of the culture, resulting in 1, 3, 5, or 7 days of treatment (7 days indicating treatment from the beginning of culture). Then, as in the above experiment, after

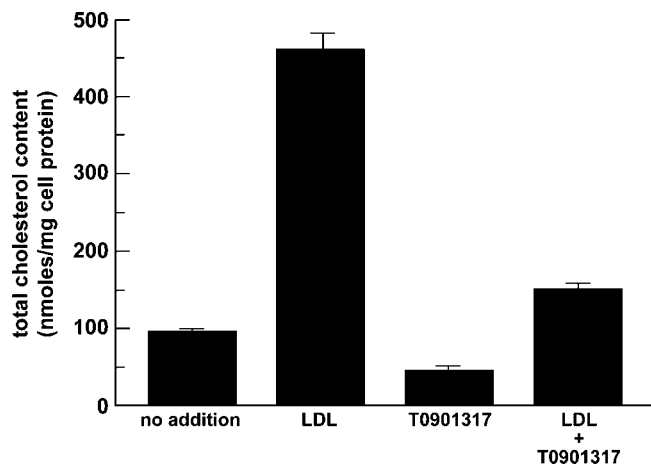


Fig. 3. T0901317 decreases cholesterol accumulation when monocytes are differentiated with this LXR agonist. Monocytes were cultured for 7 days in differentiation medium without or with 5 μ M T0901317. Then, the differentiated monocyte-derived macrophages were incubated for 1 day in serum-free differentiation medium without any addition, with 2 mg/ml LDL, with 5 μ M T0901317, or with LDL and T0901317 together. Following incubations, macrophages were harvested for determination of their total cholesterol contents. All the cholesterol was unesterified for conditions incubated without LDL. There was no significant difference in the percent of cholesterol that was esterified, 50% and 42%, respectively, for macrophages incubated with LDL or LDL + T0901317. Error bars indicate \pm SEM.

7 days of culture, the monocyte-derived macrophages were incubated for 1 day with 500 μ g/ml 125 I-LDL. Macrophage total uptake of 125 I-LDL was progressively reduced as the time of T0901317 treatment was increased (**Fig. 4**). Maximal T0901317 inhibition ($58 \pm 1\%$) of 125 I-LDL uptake occurred when the drug was administered from the beginning of the culture (i.e., 7 days of treatment). Only some inhibition ($17 \pm 2\%$) of 125 I-LDL uptake occurred when T0901317 was added to macrophages 1 day before incubation of macrophages with LDL. Likewise, maximal T0901317 inhibition ($72 \pm 1\%$) of cholesterol accumulation occurred when the drug was administered from the beginning of the culture, whereas only some inhibition ($28 \pm 2\%$) of cholesterol accumulation occurred when T0901317 was added 1 day before incubation of macrophages with LDL.

PPAR agonists do not decrease macrophage uptake of LDL

PPARs α , β/δ , and γ are members of the nuclear receptor superfamily that regulate gene expression in response to the binding of fatty acids and their metabolites. PPARs have been shown to affect macrophage cholesterol metabolism, usually by modulation of LXRs (17, 21, 31). For this reason, we tested whether differentiation of human monocytes into macrophages in the presence of PPAR agonists would also affect macrophage uptake of LDL similar to LXR agonists. Monocytes differentiated to macrophages over 7 days with either no agonist, the LXR agonist T0901317, the PPAR α agonist clofibrate, the PPAR β/δ agonist L165,041, or the PPAR γ agonist ciglitazone

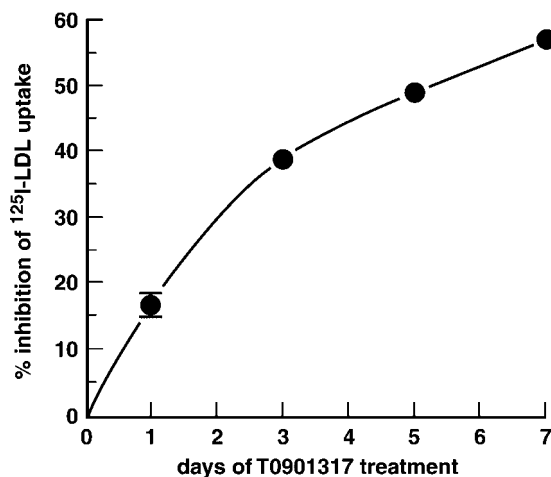


Fig. 4. Effect of T0901317 treatment time on macrophage uptake of 125 I-LDL. T0901317 (5 μ M) was added to differentiating monocytes at different time points following initiation of the culture, resulting in 1, 3, 5, or 7 days of treatment (7 days indicating treatment from the beginning of culture). Then, after 7 days of culture, the monocyte-derived macrophages were incubated for 1 day with 500 μ g/ml 125 I-LDL and T0901317, except for the control, which received no T0901317 treatment. Following incubations, total uptake of 125 I-LDL (the sum of cell-associated and degraded 125 I-LDL) by the T0901317-treated macrophages compared with control macrophages was determined. The percent T0901317 inhibition of macrophage 125 I-LDL uptake was calculated (y axis).

zone were incubated with 500 μ g/ml 125 I-LDL for 24 h. Only T0901317 inhibited 125 I-LDL uptake by macrophages, whereas the PPAR agonists did not affect 125 I-LDL uptake (**Table 1**).

Decrease in macrophage fluid uptake induced by LXR agonist accounts for decrease in LDL uptake

In our earlier study, 125 I-LDL uptake by M-CSF-differentiated macrophages was linear, showing no evidence of saturation with 125 I-LDL concentrations up to as much as

TABLE 1. PPAR agonists do not affect macrophage uptake of 125 I-LDL

Agonist	Total 125 I-LDL Uptake μ g/mg cell protein
No addition	160 \pm 12
T0901317	79 \pm 18 ^a
Clofibrate	142 \pm 50
L165,041	165 \pm 21
Ciglitazone	143 \pm 11

Monocytes were cultured for 7 days in differentiation medium containing either no addition of agonists, liver X receptor agonist T0901317 (5 μ M), PPAR α agonist clofibrate (100 nM), PPAR β/δ agonist L165,041 (5 μ M), or PPAR γ agonist ciglitazone (5 μ M). Then, the differentiated monocyte-derived macrophages were incubated for 24 h in serum-free medium containing the indicated agonist treatment and 500 μ g/ml 125 I-LDL. Total uptake of the 125 I-LDL was determined as the sum of cell-associated and degraded 125 I-LDL, as described in Materials and Methods. Results are expressed as means \pm SEM. PPAR, peroxisome proliferator-activated receptor.

^a $P = 0.003$ when T0901317-treated macrophage uptake of 125 I-LDL is compared with controls.

250 $\mu\text{g/ml}$ (7). ^{125}I -LDL uptake could not be inhibited by anti-LDL receptor antibody or even with an excess of unlabeled LDL. These findings showed that the mechanism of LDL uptake was fluid-phase pinocytosis rather than receptor-mediated endocytosis. In the present study, we compared the amount of fluid uptake by control and T0901317-treated macrophages to learn whether T0901317 inhibited macrophage fluid uptake, and if so, whether the inhibition of fluid uptake could account for the decrease in ^{125}I -LDL uptake.

Macrophages take up BSA by fluid-phase pinocytosis, and so we used this probe to measure macrophage fluid uptake, as was done previously (7, 27). One set of T0901317-treated and control macrophages was incubated for 24 h with 250 $\mu\text{g/ml}$ ^{125}I -BSA, and another set of macrophages for each experimental condition was incubated with 250 $\mu\text{g/ml}$ ^{125}I -LDL. As we showed previously (7), the entire amount of ^{125}I -LDL uptake could be accounted for by fluid-phase pinocytosis, inasmuch as the total macrophage uptake of ^{125}I -LDL and ^{125}I -BSA incubated at the same concentrations was similar (Table 2). The amount of ^{125}I -BSA uptake was reduced by about 60% in T0901317-treated macrophages compared with controls, and this inhibition was similar to T0901317 inhibition of ^{125}I -LDL uptake (Table 2). Thus, LXR agonist-mediated decrease in macrophage fluid-phase pinocytosis accounted for the decrease in ^{125}I -LDL uptake.

LXR agonist affects predominantly fluid-phase pinocytosis rather than receptor-mediated endocytosis

To investigate whether the effect of T0901317 is specific for fluid-phase pinocytosis, we examined whether T0901317 has an inhibitory effect on receptor-mediated endocytosis of ^{125}I -acetylated LDL, known to be taken up by macrophage scavenger receptors (32–34). First, we examined the concentration dependence of ^{125}I -acetylated LDL uptake by M-CSF-differentiated macrophages. In contrast to the nonsaturable uptake of ^{125}I -LDL that we reported previously (6, 7), ^{125}I -acetylated LDL uptake showed saturation at 25–50 $\mu\text{g/ml}$, consistent with receptor-mediated endocytosis (data not shown). T0901317-treated and control macrophages were incubated with 25 $\mu\text{g/ml}$ ^{125}I -acetylated LDL and a 10-fold higher concentration of ^{125}I -LDL for 24 h to examine receptor-mediated uptake

TABLE 2. Effect of T0901317 on fluid-phase pinocytosis

Addition	Total ^{125}I -LDL Uptake		Inhibition
	– T0901317	+ T0901317	
	$\mu\text{g/mg cell protein}$		%
^{125}I -LDL	67 \pm 6	27 \pm 4	60
^{125}I -BSA	70 \pm 17	27 \pm 12	61

Monocytes were cultured for 7 days in differentiation medium without or with 5 μM T0901317. Then, T0901317-treated and control macrophages were incubated for 1 day with 250 $\mu\text{g/ml}$ ^{125}I -LDL or 250 $\mu\text{g/ml}$ ^{125}I -BSA as a measure of fluid-phase pinocytosis. Total uptake was determined as the sum of the cell-associated and degraded ^{125}I -LDL or ^{125}I -BSA, as described in Materials and Methods. Results are expressed as means \pm SEM.

TABLE 3. Effects of T0901317 on receptor-mediated endocytosis and fluid-phase pinocytosis

Condition	Cell-associated	Degraded	Total
	^{125}I -lipoprotein	^{125}I -lipoprotein	
	$\mu\text{g protein/mg cell protein}$		
^{125}I -LDL	7.5 \pm 0.2	91.8 \pm 0.7	99.3 \pm 0.6
^{125}I -LDL + T0901317	3.2 \pm 0.1 ^a	41.6 \pm 0.3 ^a	44.8 \pm 0.6 ^a
^{125}I -acetylated LDL	12.9 \pm 0.8	90.9 \pm 5.1	103.8 \pm 6.1
^{125}I -acetylated LDL + T0901317	13.9 \pm 1.1	84.6 \pm 6.4	98.6 \pm 7.3

Monocytes were cultured for 7 days in differentiation medium without or with 5 μM T0901317. Then, T0901317-treated and control macrophages were incubated for 1 day with 25 $\mu\text{g/ml}$ ^{125}I -acetylated LDL or 250 $\mu\text{g/ml}$ ^{125}I -LDL. Following incubations, media were collected, and then macrophages were rinsed and harvested to measure their protein, cell-associated ^{125}I -lipoprotein, and the ^{125}I -lipoprotein degraded by the macrophages (i.e., trichloroacetic acid-soluble ^{125}I -lipoprotein in the media). Total ^{125}I -lipoprotein uptake was calculated as the sum of the cell-associated and degraded ^{125}I -lipoprotein. Results are expressed as means \pm SEM.

^aSignificantly different from untreated macrophages incubated with ^{125}I -lipoprotein alone ($P < 0.05$).

of ^{125}I -acetylated LDL and receptor-independent, fluid-phase uptake of ^{125}I -LDL (Table 3). Despite a similar total uptake of ^{125}I -LDL and ^{125}I -acetylated LDL in untreated macrophages, T0901317 did not inhibit receptor-mediated ^{125}I -acetylated LDL uptake, but did inhibit receptor-independent, fluid-phase uptake of ^{125}I -LDL by 55% compared with untreated macrophages. This showed that T0901317 inhibition is specific for fluid-phase pinocytosis of lipoproteins and does not affect receptor-mediated endocytosis of lipoproteins.

Cyclodextrin-induced decrease in macrophage cholesterol content does not regulate pinocytosis

A previous study showed that cholesterol depletion (presumably in the plasma membrane) induced by methyl- β -cyclodextrin inhibits phorbol ester-induced macropinocytosis in the human epithelial carcinoma cell line A421 (29). We observed that T0901317 treatment significantly decreased basal cholesterol levels in the macrophages by about 50% (Fig. 3). Thus, we considered the possibility that T0901317-induced depletion of macrophage cholesterol was the mechanism by which T0901317 decreased pinocytosis. To test this idea, macrophage cholesterol content was decreased by treating macrophages with methyl- β -cyclodextrin (5 mM) for 1 h. Then, macrophages were incubated with unlabeled BSA (250 $\mu\text{g/ml}$) for 5 h before determination of macrophage cholesterol content. After the 1 h incubation with methyl- β -cyclodextrin, macrophage cholesterol content decreased 44% compared with untreated control macrophages, and decreased further to 25% of control macrophage values after a 5 h incubation with BSA. The cholesterol content in all experimental conditions for these unloaded macrophages was $\geq 95\%$ unesterified. In a parallel experiment, methyl- β -cyclodextrin-pretreated and -untreated control macrophages were incubated with ^{125}I -BSA (250 $\mu\text{g/ml}$) for 5 h to measure fluid uptake. It was determined that macrophage cholesterol depletion

TABLE 4. Effect of macrophage cholesterol content on fluid-phase pinocytosis

Parameter Measured	Treatment	
	– Methyl- β -cyclodextrin	+ Methyl- β -cyclodextrin
Total cell cholesterol content	<i>nmol/mg cell protein</i>	
Before treatment	117 \pm 2	117 \pm 2
After 1 h treatment	121 \pm 6	53 \pm 1
After 1 h treatment followed by additional 5 h treatment	108 \pm 2	29 \pm 2
Total 125 I-BSA uptake	<i>μg/mg cell protein</i>	
After 1 h treatment followed by additional 5 h treatment	20 \pm 1	23 \pm 2

Monocytes were cultured for 7 days in differentiation medium. Then, the macrophages were pretreated for 1 h with and without methyl- β -cyclodextrin (5 mM) added to serum-free differentiation medium. This was followed by 5 h incubation with and without methyl- β -cyclodextrin and either unlabeled BSA (250 μ g/ml) or 125 I-BSA (250 μ g/ml) for determination of cholesterol content and assessment of fluid-phase pinocytosis, respectively, as described in Materials and Methods. Results are expressed as means \pm SEM.

did not change macrophage fluid uptake (Table 4), indicating that it was unlikely that T0901317-induced depletion of macrophage cholesterol affected pinocytosis in M-CSF-differentiated macrophages.

DISCUSSION

It is believed that LXR upregulation of cholesterol efflux genes underlies the anti-atherosclerotic effects of LXR activation observed in mouse models of atherosclerosis (as reviewed in Refs. 35, 36). Promoting cholesterol efflux from macrophage foam cells in atherosclerotic plaques could initiate removal of cholesterol from atherosclerotic plaques. However, macrophage cholesterol accumulation depends also on the uptake of cholesterol as well as the compensatory efflux of excess cholesterol. Our findings show for the first time that LXRs can regulate cholesterol accumulation not only by regulating cholesterol efflux but also by regulating macrophage uptake of LDL.

The culture model used for the research reported here is characterized by human monocyte-derived macrophages that constitutively take up large amounts of native LDL by fluid-phase pinocytosis (7). The widely accepted hypothesis for foam cell formation involves receptor-mediated endocytosis of modified or aggregated LDL (3, 4). Pinocytosis of fluid-phase rather than bound LDL is a novel mechanism of macrophage cholesterol accumulation (5–7). We have shown here that both synthetic and natural LXR agonists, T0901317 and 22(R)-hydroxycholesterol, downregulated macrophage LDL uptake by inhibiting fluid-phase pinocytosis of native LDL. T0901317 did not affect receptor-mediated endocytosis of acetylated LDL, showing that LXR specifically inhibits lipoprotein uptake mediated by fluid-phase pinocytosis.

PPAR α , β/δ , and γ also regulate genes involved in the control of lipid metabolism and inflammation, and it has

been suggested that there is “cross talk” between PPARs and LXRs during regulation of the cholesterol efflux pathway in macrophages (35). It has been reported that PPAR agonists can increase expression of LXR α but not LXR β in mouse macrophages and human monocyte-derived macrophages (17, 37). In the research reported here, we found that PPAR agonists targeting each of the three classes of PPARs failed to modulate LDL uptake. Only macrophages differentiated in the presence of LXR agonists (but not PPAR agonists) showed significant reduction of LDL uptake compared with control macrophages. It remains to be determined why PPAR agonists that regulate LXR expression in other macrophage systems showed no effect on the LXR-mediated downregulation of pinocytosis in these human macrophages.

LXR agonist-induced modulation of macrophage pinocytosis showed a progressively increasing inhibition of pinocytosis during the 7 day treatment with T0901317. This finding that maximal inhibition required 7 days of treatment with T0901317 suggests the possibility that genes indirectly regulated by LXRs may have mediated the inhibition of pinocytosis. However, we have observed that some genes in macrophages previously shown to be directly regulated by LXR nevertheless require more than 4 days of T0901317 treatment before maximal gene expression occurs (Y. Li, C. Buono, S. W. Waldo, and H. S. Kruth, unpublished observation).

Pinocytosis in our macrophage culture system was mediated in part by macropinocytosis, which, as we have shown previously, occurs in these human M-CSF-differentiated monocyte-derived macrophages. Macropinocytosis is a mechanism of fluid-phase pinocytosis described under certain conditions in other cell types, such as fibroblasts, epithelial cells, and dendritic cells. In dendritic cells, macropinocytosis functions in antigen processing (8, 38). In the human epithelial carcinoma cell line A431, macropinocytosis induced by activation of PKC with phorbol ester 12-O-tetradecanoylphorbol 13-acetate is regulated by the cholesterol content of the plasma membrane (29). When A431 epithelial cells were pretreated with methyl- β -cyclodextrin to extract cholesterol, the phorbol ester was unable to induce increased fluid-phase pinocytosis. Because LXR agonist treatment decreased basal cholesterol levels in the macrophages (probably due to stimulation of cholesterol efflux as discussed above), we considered that the mechanism of reduced fluid-phase pinocytosis by LXR-treated macrophages might be due to diminished plasma membrane cholesterol content mediated by LXR-stimulated cholesterol efflux. However, this was not the case; we found no reduction of constitutive fluid-phase pinocytosis in macrophages depleted of cholesterol by treatment with methyl- β -cyclodextrin. Thus, an LXR agonist-induced decrease in macrophage cholesterol content is not the mechanism by which LXRs regulate pinocytosis in M-CSF-differentiated human macrophages.

On the other hand, increased macrophage cholesterol content due to macropinocytosis of LDL would be expected to generate endogenous oxysterol LXR agonists (39). This should tend to downregulate macropinocytosis

of LDL assuming that oxysterols are generated. However, we previously published that macropinocytotic uptake of LDL does not downregulate macropinocytosis over a 2 day period, despite substantial macrophage cholesterol accumulation (7). That finding is consistent with what we have shown here, in that LXR activation must occur for longer than 2 days before macropinocytosis is substantially downregulated.

In conclusion, we have shown that activation of LXR transcription factors may be atheroprotective by a novel mechanism that decreases macrophage receptor-independent, fluid-phase pinocytosis of atherogenic lipoproteins such as LDL. In addition, pinocytosis leads to fluid-phase uptake and degradation of the anti-atherogenic HDL [(7) and unpublished data]. Thus, LXR-mediated decrease in macrophage pinocytosis of HDL may also be atheroprotective by increasing plaque HDL that is available to mediate reverse cholesterol transport. It is well known that LXRs, as transcription factors, regulate expression of several genes in macrophages, and it is very likely that some of those are directly or indirectly involved in the inhibitor effect of LXRs on pinocytosis. It will be of interest in future research to learn which LXR-regulated genes affect pinocytosis. **LR**

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