FEBS 30134 FEBS Letters 579 (2005) 6537–6542

Impaired ATP-binding cassette transporter A1-mediated sterol efflux from oxidized LDL-loaded macrophages

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Received 6 October 2005; accepted 21 October 2005

Available online 2 November 2005

Edited by Sandro Sonnino

Abstract **We investigated the interaction of oxidized low density lipoprotein (OxLDL) with the ATP-binding cassette A1 (ABCA1) pathway in J774 macrophages. Cellular efflux to apolipoprotein AI (apo-AI) of OxLDL-derived cholesterol was lower than efflux of cholesterol derived from acetylated low density lipoprotein (AcLDL). ABCA1 upregulation by 8-(4-chlorophenylthio)adenosine 3′:5′-cyclic monophosphate (cpt-cAMP) or 22 (R)-hydroxycholesterol (22-OH) and 9-*cis* retinoic acid (9cRA) increased the efflux to apo-AI of cellular sterols derived from AcLDL, but not of those from OxLDL. AcLDL, but not OxLDL, induced ABCA1 protein content and activity in J774. However, OxLDL did not influence J774 ABCA1 upregulation by cpt-cAMP or 22-OH/9cRA. We conclude that sterols released to cells by OxLDL are available neither as substrate nor as modulator of ABCA1.**

Keywords: ATP-binding cassette A1; Macrophages; Oxidized low density lipoprotein; Cholesterol efflux; Atherosclerosis; Foam cells

1. Introduction

A number of evidences suggests a role for low density lipoprotein (LDL) oxidation in the development of atherosclerosis. This includes the identification of lipid oxidation products in human and animal atheroma and plasma [1] and the observation that various cells of the arterial intima can oxidize LDL [2]. Since macrophages are unable to down-regulate scavenger receptors and therefore the inflow of cholesterol, removal (efflux) of excess cellular cholesterol from these cells is of particular importance to prevent foam cell formation [3]. Efflux of cellular cholesterol is a complex and heterogeneous process promoted by many factors and requiring the presence of extracellular cholesterol acceptors [4].

ATP-binding cassette A1 (ABCA1) is crucial for the mobilization of cholesterol from tissues via its interaction with apolipoproteins or lipid-poor particles [5]; consistently ABCA1 expression is upregulated by macrophages in atherosclerotic lesions, presumably in response to cholesterol enrichment [6]. Consistently, ABCA1 gene expression is highly responsive to liver-X-receptor (LXR) agonists, such as 22-hydroxycholesterol (22-OH) and 27-hydroxycholesterol in combination with retinoic-X-receptor (RXR) ligands such as 9-*cis*-retinoic acid (9cRA) [7,8]. Expression of ABCA1 in macrophages is also induced by cAMP stimulation and most importantly by cholesterol enrichment [9,10].

Although it has been reported that oxidized low density lipoprotein (OxLDL)-loaded cells release cholesterol less efficiently than do cells loaded with acetylated LDL (AcLDL) [11], the impact of OxLDL on ABCA1-mediated pathway remains to be clarified. In the present study we investigated the ABCA1-mediated efflux of sterols accumulated from OxLDL in murine macrophages, as well as the effect of OxLDL loading on ABCA1 cellular function and content.

2. Materials and methods

2.1. Materials

Fetal calf serum (FCS), bovine serum albumin (BSA), 3′,5′-cyclic monophosphate (cpt-cAMP), 9cRA, 22-OH were purchased from Sigma (St. Louis, MO, USA). Apo-AI was kindly donated by Dr. Laura Calabresi (University of Milan). [1,2-^{3}H]cholesterol and [methyl-^{3}H] -choline chloride were from Amersham Biosciences (Uppsala, Sweden). Rabbit-anti-mouse ABCA1 antibody was purchased from Novus Biological (Cambridge, UK).

2.2. Lipoprotein preparation

Human LDL (d = 1.019–1.063 g/ml) were isolated from plasma of healthy volunteers by sequential ultracentrifugation (Beckman L5-50, Palo Alto, CA) [12]. LDL were acetylated according to Basu et al. [13]. Chemical oxidation was performed under sterile condition, by incubating LDL at 37°C for 24 h, at 0.2 mg protein/ml phosphate buffer

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Abbreviations: ABCA1, ATP-binding cassette A1; AcLDL, acetylated low density lipoprotein; apo-AI, apolipoprotein AI; BSA, bovine serum albumin; 9cRA, 9-*cis* retinoic acid; cpt-cAMP, 8-(4-chlorophenylthio)adenosine 3′:5′-cyclic monophosphate; CE, cholesteryl ester; FCS, fetal calf serum; FC, free cholesterol; LDL, low density lipoprotein; 22-OH, 22 (R)-hydroxycholesterol; MPM, mouse peritoneal macrophages; PBS, phosphate buffer saline; PMA, phorbol, 12-myristate, 13-acetate; OxLDL, oxidized low density lipoprotein; 7K, 7-ketocholesterol

0014-5793/$30.00
doi:10.1016/j.febslet.2005.10.042

saline (PBS) + 20 μM $CuSO_4$. Oxidation was blocked in ice, with the addition of BHT 40 μM. Modification of lipoproteins was tested by non-denaturing gel electrophoresis [11]. AcLDL and OxLDL were radiolabeled with [^{3}H]-cholesteryl linoleate (Amersham, Buckinghamshire, UK) by incubation with serum containing the cholesteryl ester (CE) transfer protein [14]. Specific activity ranged from 711 to 1926 cpm/μg protein for OxLDL and from 789 to 1637 cpm/μg protein for AcLDL, respectively.

2.3. Cell cultures

J774 mouse macrophages were cultured in RPMI1640 supplemented with 10% FCS. Mouse peritoneal macrophages (MPM) were obtained from peritoneum of Swiss mice as previously described [15] and were plated in Dulbecco's minimum essential medium supplemented with 10% FCS. Human monocytes-derived macrophages THP-1 were cultured in RPMI1640/HEPES supplemented with 1 mM Na pyruvate, 50 μM β-mercaptoethanol, 2.5 mg/ml glucose and 10% FCS. THP-1 differentiation was obtained adding 50 ng/ml phorbol, 12-myristate, 13-acetate (PMA).

2.4. Sterol and phospholipid efflux

80% confluent cells were [^{3}H]sterol loaded by incubation with 30 μg/ml [^{3}H]-AcLDL or 30 μg/ml [^{3}H]-OxLDL for 24 h in medium with 0.2% BSA. In separated experiments cellular cholesterol was radiolabeled by adding 2–3 μCi/ml [1,2-^{3}H]cholesterol in medium supplemented with 1% FCS and 30 μg/ml AcLDL or 30–60 μg/ml OxLDL for 24–48 h. Efflux was promoted for 4–18 h by incubation with 20 μg/ml apolipoprotein AI (apo-AI) in a medium supplemented with 0.2% BSA and results corrected for efflux in absence of apo-AI. Time zero (T_0) cells wells were harvested before incubation with apo-AI. The percent efflux of lipid was calculated as: (cpm in medium/cpm To) × 100 [5].

Phospholipid efflux was evaluated in J774 labeled with 4 μCi/ml [methyl-^{3}H]choline chloride for 48 h in RPMI medium with 1% FCS in the presence of 30 μg/ml AcLDL or 30 μg/ml OxLDL. After this time cells were treated with 20 μg/ml apo-AI for 18 h in a medium supplemented with 0.2% BSA. Efflux media were extracted by the Bligh and Dyer method [16].

2.5. Western blot analysis

Cell monolayers were lysed in 1% Triton X-100, 0.5% NP-40, 10 mM tris buffer containing 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.2 mM PMSF and homogenized through a 27-gauge needle. Protein were separated on 5% Tris–acetate gels and immunoblotted with anti-ABCA1 polyclonal antibody.

2.6. Analysis of ABCA1 mRNA by real time quantitative RT-PCR

Total RNA was prepared using NucleoSpin RNA II Flyer (Macherey-Nagel) according to the instructions given by the manufacturer. Real time quantitative PCR analysis was used to determine the levels of ABCA1 in J774 upon treatment with 30 μg/ml AcLDL or OxLDL in medium supplemented with 0.2% BSA. Reverse transcription and PCRs were performed according to the manufacturer's instructions (Applied Biosystems). Sequence-specific amplification was detected with an increasing fluorescent signal of FAM (reporter dye) during the amplification cycle on an ABI Prism 7000 (Applied Biosystems).

2.7. [^{3}H]-Sterol thin layer chromatography (TLC)

Cells and lipoproteins were extracted, respectively, by isopropanol or chloroform:methanol (1:1). TLC was performed with a mobile phase of 170 ml hexanes, 20 ml ethyl-ether and 10 ml methanol. The recovery of TLC procedure ranged from 70% to 80%.

2.8. Intracellular cholesterol mass

Macrophages were incubated for 32 h with either AcLDL (30 μg/ml) or OxLDL (30–60 μg/ml) in RPMI supplemented with 1% FCS. Lipids were extracted from the washed monolayers with hexane: isopropanol (3:2, v/v). Total cholesterol was determinated following the instructions of Cholesterol Clonital Kit (Amplimedical). Cellular proteins were quantitated according to Lowry assay.

2.9. Statistical analysis

Results were presented as means ± S.D. of data obtained from triplicate wells. Significant differences were established by Student's *t*-test using Graph Pad Prism (GraphPad Software, Inc).

3. Results

3.1. Cellular efflux of sterols derived from [^{3}H]-AcLDL or [^{3}H]-OxLDL

TLC analysis indicated that more than 90% of radioactivity was present as CE in [^{3}H]-AcLDL, while in [^{3}H]-OxLDL the radioactivity was equally distributed among CE, free cholesterol (FC) and a fraction that migrate as standard 7-ketocholesterol (7K) (Table 1). Calculations based on specific activity indicate that the amounts of [^{3}H]-sterols incorporated by cells were 66.16 ± 7.4 and 79.33 ± 5.9 μg/mg after 24 h incubation with either 30 μg/ml of [^{3}H]-AcLDL or [^{3}H]-OxLDL respectively, indicating no difference in total [^{3}H]-sterol cellular uptake. About 90% of total radioactive sterols of cells incubated with [^{3}H]-AcLDL was associated with cholesterol. In [^{3}H]-OxLDL-loaded cells cholesterol represented 70% and 30% of radioactivity migrated with 7K (Table 1). Colorimetric enzymatic assay indicated that cellular sterol mass increased from 85.9 to 139.5 μg/mg and 130.9 μg/mg after incubation with 30 μg/ml AcLDL or 30 μg/mg OxLDL, respectively.

Efflux experiments indicate that [^{3}H]-OxLDL-loaded cells released less sterols to apo-AI than cells incubated with [^{3}H]-AcLDL (Fig. 1). In [^{3}H]-AcLDL loaded cells, induction of ABCA1 by cpt-cAMP or 22-OH/9cRA increased the percent of [^{3}H]-sterol efflux to apo-AI by about 2- and 3-fold, respectively. The same treatments failed to stimulate radioactivity release in cell exposed to [^{3}H]-OxLDL (Fig. 1). Confirmatory results were observed in MPM. The [^{3}H]-sterol efflux evaluated in MPM treated with 22-OH/9cRA increased 2.9 times in [^{3}H]-AcLDL and only 1.2 in [^{3}H]-OxLDL loaded cells.

3.2. Effect of AcLDL or OxLDL on cellular ABCA1 activity and expression

AcLDL loading increased phospholipid efflux from J774 by about 4-fold, while none stimulatory effect was observed upon OxLDL treatment (Fig. 2A). Similarly to phospholipid, cholesterol efflux to apoA-I was induced by incubation of cells with AcLDL, but not with OxLDL (Fig. 2B). Similar results were obtained evaluating cholesterol efflux after a shorter incubation time with apo A-I. The efflux to apo A-I after 4 h was 1.2% ± 0.08, 2.2% ± 0.27 and 1.3% ± 0.14 in control, AcLDL and OxLDL pre-treated cells, respectively.

Table 1
[^{3}H]Sterol distribution in [^{3}H] lipoproteins and in [^{3}H] lipoprotein-loaded J774 macrophages

	CE	FC	Oxysterols
	Percent of total sterols in lipoproteins		
[^{3}H]-AcLDL	91.5 ± 3.1	4.2 ± 0.8	1.3 ± 0.3
[^{3}H]-OxLDL	33.9 ± 1.5	30.7 ± 1.2	29.4 ± 1.1
	Percent of total sterols in loaded cells		
J774+[^{3}H]-AcLDL	35.1 ± 2.1	58.1 ± 2.3	1.8 ± 0.3
J774+[^{3}H]-OxLDL	14.3 ± 1.2	46.8 ± 1.6	33.9 ± 1.3

FC, cholesterol; CE, cholesteryl esters.

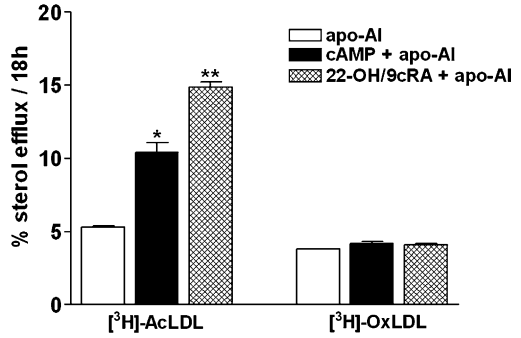

Fig. 1. Efflux of [^{3}H]-sterols from [^{3}H]-AcLDL or [^{3}H]-OxLDL-loaded J774. Cell monolayers were incubated with 30 μg/ml [^{3}H]-AcLDL or 30 μg/ml [^{3}H]-OxLDL for 24 h in RPMI medium containing 0.2% BSA. 20 μg/ml apo-AI was added with or without 0.3 mM cpt-cAMP or 5 μg/ml 22-OH and 10 μM 9cRA for 18 h. Data are from a representative experiment of four with triplicate wells ($n = 3$). Values are expressed as means ± S.D. $^*P < 0.001$, $^{**}P < 0.0001$ compared with apo-AI alone.

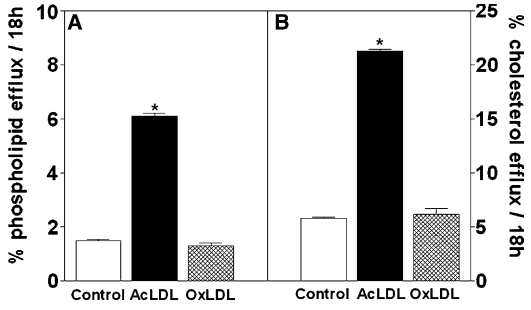

Fig. 2. Efflux of phospholipids and cholesterol to apo-AI from AcLDL or OxLDL-loaded J774 under basal condition. Cells were labeled with either 4 μCi/ml [methyl-^{3}H]-choline chloride for 48 h (A) or 3 μCi/ml [^{3}H]-cholesterol for 24 h (B) in RPMI medium plus 1% FCS in the presence of 30 μg/ml AcLDL or 30 μg/ml OxLDL. After this time cells 20 μg/ml apo-AI were added for 18 h. Data are from a representative experiment of six with triplicate wells ($n = 3$). Values are expressed as means ± S.D. $^*P <$ 0.0001, compared with control, (A) and (B).

We next evaluated the effect of AcLDL or OxLDL on phospholipid efflux in cells expressing ABCA1 upon stimulation by cpt-cAMP (Fig. 3A) or by 22-OH/9cRA (Fig. 3B). Phospholipid efflux was not affected by OxLDL and was further stimulated by AcLDL. Similar results were obtained evaluating cholesterol efflux (data not shown). To evaluate whether the inability of OxLDL to influence lipid efflux could depend on time of incubation with cells we performed a time course experiment. As depicted, throughout the incubation times with the modified lipoproteins, cholesterol efflux to apoA-I was induced by incubation of cells with AcLDL, but not with OxLDL (Fig. 4). Similar results were obtained with an higher concentration of OxLDL (60 μg/ml) (data not shown). In MPM and in human THP-1 after 48 h of incubation with modified lipoproteins cholesterol efflux increased almost 2 times in AcLDL loaded cells and was unchanged following OxLDL loading (Fig. 5).

Accordingly to the results on lipid efflux, in J774 cells AcLDL and not OxLDL loading induced ABCA1 expression while cpt-cAMP treatment increased the ABCA1 protein levels

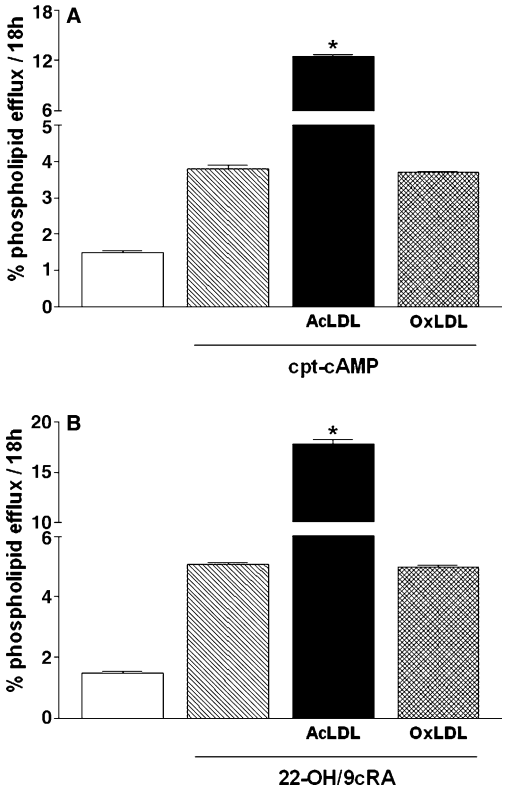

Fig. 3. Efflux of phospholipids to apo-AI from AcLDL or OxLDL-loaded J774 expressing ABCA1. J774 macrophages were labeled with 4 μCi/ml [methyl-^{3}H]-choline chloride for 48 h in RPMI medium with 1% FCS in the presence of 30 μg/ml AcLDL or 30 μg/ml OxLDL. After this time cells were treated with 20 μg/ml apo-AI in the presence or absence of either 0.3 mM cpt-cAMP (A) or 5 μg/ml 22-OH and 10 μM 9cRA (B) for 18 h. Data are from a representative experiment of three with triplicate wells ($n = 3$). Values are expressed as means ± S.D. $^*P < 0.0001$, compared with cpt-cAMP alone (A) or 22-OH/9cRA alone (B).

in the presence of both modified LDL (Fig. 6). Consistently, mRNA levels of ABCA1 in J774 were increased by about 2-fold by incubation with AcLDL but not by OxLDL (Fig. 7).

4. Discussion

OxLDL have a defective cellular catabolism [17] that include a reduced capacity of OxLDL-loaded macrophages to efflux their cholesterol and oxysterol content to an extracellular acceptor [11,18]. One of the major pathway of cholesterol release from macrophages involves the process of lipid efflux mediated by ABCA1, however no information is available on OxLDL and ABCA1 pathway interactions. Purposes of the present study were: (1) to investigate in cultured macrophages the effect of stimulation of ABCA1 activity on cellular efflux of sterols derived from OxLDL; (2) to evaluate in the same cells the effect of OxLDL on cellular ABCA1 expression and activity.

For the first aim LDL were labeled with [^{3}H]-CE and subsequently either acetylated or copper-oxidized. As previously described, 24 h copper modification of LDL was appropriate for comparison with AcLDL [19]. As reported by others [11,20], in our present study the amount of sterols released to cells was

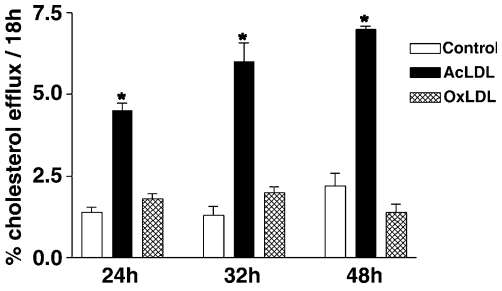

Fig. 4. Cholesterol efflux to apo-AI in J774 exposed to AcLDL or OxLDL for increasing periods of time. Monolayers were labeled with 2 μCi/ml [^{3}H]-cholesterol in RPMI medium plus 1% FCS in the presence of 30 μg/ml AcLDL or 30 μg/ml OxLDL for 24, 32 and 48 h. Cells were then incubated for 18 h with RPMI containing 20 μg/ml lipid free apo-AI. Data are from a representative experiment of three with triplicate wells ($n = 3$). Values are expressed as means ± S.D. $^*P < 0.0001$, compared with control cells.

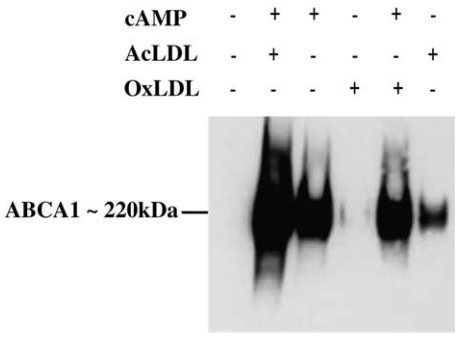

Fig. 6. ABCA1 protein expression in AcLDL or OxLDL-loaded J774 macrophages. J774 macrophages were treated with RPMI + 0.2% BSA alone, in the presence of 30 μg/ml AcLDL or OxLDL. After 24 h cells were incubated in the presence or absence of 0.3 mM cpt-cAMP. After this incubation cells were washed with PBS and solubilized for Western blot analysis as described in Section 2.

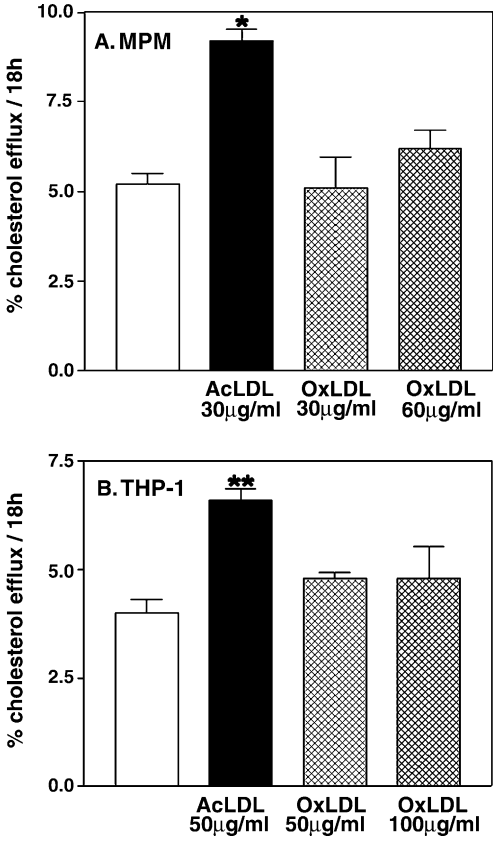

Fig. 5. Efflux of cholesterol to apo-AI from AcLDL or OxLDL-loaded MPM or THP-1. THP-1 were treated with 50 ng/ml of PMA for 72 h. Monolayers were then labeled with 2 μCi/ml [^{3}H]-cholesterol for 48 h in RPMI medium plus 1% FCS in the presence of AcLDL or OxLDL. Cells were then incubated for 18 h with RPMI containing 20 μg/ml lipid free apo-AI. Data are from a representative experiment of four with triplicate wells ($n = 3$). Values are expressed as means ± S.D. $^*P < 0.0001$, $^{**}P < 0.001$ compared with control cells.

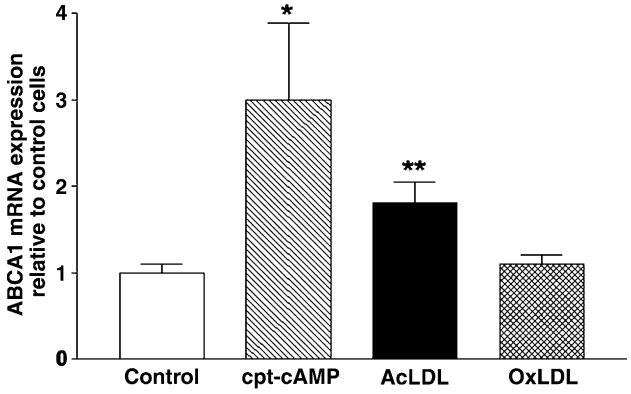

Fig. 7. ABCA1 mRNA levels in AcLDL or OxLDL-loaded J774 macrophages. Cells were treated as described on Fig. 5. After this incubation cells were washed with PBS and solubilized for RT-PCR analysis as described in Section 2. Values are expressed as means ± S.D. $^*P < 0.05$, $^{**}P < 0.01$ compared with control cells.

comparable in [^{3}H]-OxLDL and [^{3}H]-AcLDL loaded cells. The oxidation procedure induced the appearance of 30% of [^{3}H]-sterols that migrate in the TLC as standard 7K and an other 30% that migrate as standard FC. These results are completely consistent with those reported by Kritharides showing by high performance liquid chromatography (HPLC) that about 30% of sterols content in their OxLDL preparation was constituted by 7K and an other 30% by FC [11,21]. Analysis of [^{3}H]-sterol content of cells incubated with our preparation of [^{3}H]-OxLDL indicated that only 15% of cholesterol was in the esterified form. This result is in agreement with previous studies showing that CE released to macrophages by OxLDL could be hydrolyzed, but not esterified in these cells [22].

Stimulation of the ABCA1 pathway in macrophages could induce the release of sterols derived from [^{3}H]-AcLDL, but not those from [^{3}H]-OxLDL. 7K is not substrate for efflux to apoA-I [23,24], this might explain the lack of increase in ABCA1-mediated sterol efflux derived from OxLDL in our experiments. However, since stimulation of ABCA1 did not produce any increase of efflux of radioactive sterols, we must conclude that also the portion of radioactive cholesterol delivered to cells by OxLDL was not available for ABCA1.

For the second aim we tested the effect of unlabeled OxLDL or AcLDL on ABCAI expression and activity in the J774 macrophages. Our results indicate that AcLDL could stimulate ABCA1 expression as well as phospholipid and cholesterol ef-

flux to apo-AI. No effect was observed upon OxLDL incubation, consequently phospholipid and cholesterol efflux to apo-AI was higher in AcLDL-loaded cells than in cells exposed to OxLDL. The apparent discordance between relative mRNA and protein level in our experiments (see Figs. 6 and 7) has been previously reported and it may involve a post-transcriptional regulation of ABCA1 expression [25]. The lack of ability of OxLDL to stimulate ABCA1 was not overcome by increasing the period of incubation with cells. These results might involve at least two mechanisms: first, cholesterol delivered by OxLDL is not sufficient or available for ABCA1 upregulation; second, the oxysterols present in these lipoproteins may exert an inhibitory effect on the ABCA1 pathway. The second hypothesis is in contrast with our results showing that OxLDL do not inhibit ABCA1 expression and activity induced in J774 by cpt-cAMP or 22-OH/9cRA. Gelissent et al. [24] reported that 7K cholesterol may reduce apo-AI mediated efflux, therefore, one would have expected an inhibitory effect on ABCA1 in our experimental conditions. However, in Gelissent work 7K was delivered to cells by 7K-enriched AcLDL [24]. Our present results suggest that OxLDL release cholesterol and oxysterols to cellular pools with a limited ability to modulate cell lipid metabolism, including ABCA1 activity. Consistently, it was demonstrated that OxLDL are trapped in an endolysosomial compartment and the released cholesterol was not available for esterification [18]. A reduced cholesterol efflux to apo-AI in OxLDL-loaded cells, as compared to cells exposed to AcLDL, has been previously reported by Kritharides et al. [11] in resident MPM. We suggest that this difference in cholesterol efflux may be explained, at least in part, by the ability of AcLDL, but not of OxLDL, to upregulate the ABCA1 pathway. Our results in J774 were confirmed evaluating the effect of OxLDL on cholesterol efflux in MPM and in THP-1 cells. In the latter cellular models stimulation of ABCA1 expression by OxLDL has been previously reported [26]. We do not have an obvious explanation for this apparent discrepancy, however it could be related to a different extent of cell differentiation.

In conclusion we demonstrated that ABCA1 stimulation fails to induce efflux of cholesterol released to cells by OxLDL. In addition we showed that OxLDL do not modify cellular ABCA1 protein expression. These functional impairments may amplify the atherogenic potential of OxLDL.

Acknowledgments: The work was supported by grants from Compagnia di San Paolo, the Istituto Nazionale Ricerche Cardiovascolari and the Italian Ministry of University and Scientific Research.

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