

## Pharmacological regulation of cholesterol efflux in human monocyte-derived macrophages in the absence of exogenous cholesterol acceptors

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### Abstract

Cholesterol efflux from human monocyte-derived macrophages in the absence of exogenous acceptors has been described, but is unclear in mechanism. We investigated this process in relation to the expression of relevant genes, intracellular cholesterol storage and apoE secretion using drugs affecting different aspects of cholesterol metabolism. Both natural (22R-hydroxycholesterol/9-*cis*-retinoic acid) and synthetic (T0901317 and RO264456) LXR/RXR ligands increased *ABCA1* and *ABCG1* mRNAs in native macrophages and in cells loaded with acetylated LDL (acLDL). The ACAT inhibitor avasimibe increased only *ABCG1* mRNA, whereas no treatment affected *apoE* mRNA. Avasimibe, progesterone, and natural but not synthetic LXR/RXR ligands prevented cholesterol esterification after acLDL-loading. Cholesterol efflux into acceptor-free medium was increased only by synthetic LXR/RXR ligands and avasimibe in acLDL-loaded cells. ApoE secretion was reduced by drugs affecting cholesterol trafficking but enhanced by LXR/RXR ligands. Incubation with an anti-apoE antibody virtually removed immunodetectable apoE from the medium, significantly increasing cholesterol storage and decreasing efflux. These findings indicate that in human macrophages spontaneous cholesterol efflux: (i) is not necessarily promoted by increasing intracellular free cholesterol, (ii) is increased by compounds that activate *ABCA1* and, to a greater extent, *ABCG1* and (iii) is only partially correlated with secretion of endogenous apoE, which acted as a cholesterol acceptor.

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### 1. Introduction

Once recruited into the arterial wall, monocytes differentiate into macrophages that accumulate cholesterol, probably mainly by the uptake of modified lipoproteins via scavenger receptors [1,2]. This increased cholesterol influx activates

acyl-coenzyme A:cholesterol acyltransferase (ACAT) and leads to storage of large amounts of cellular cholesteryl esters (CE), which gives rise to the foam-cell phenotype, a typical feature of early atherosclerotic lesions [1]. Since cholesterol accumulation in foam cells is not regulated by limiting uptake, the only way for macrophages to avoid toxicity due to cholesterol accumulation is for them somehow to secrete the excess cholesterol into the extracellular space. This cholesterol efflux is the first step of reverse cholesterol transport [3] and represents a promising target for the pharmacological control of atherogenesis.

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Human macrophages secrete apolipoprotein E (apoE) [4], which has been shown to facilitate cholesterol efflux from these cells. In humans, the common apoE polymorphism is an important determinant of macrophage cholesterol metabolism [4,5], which may partly explain the influence of this polymorphism on atherosclerosis risk. Most importantly, human monocyte-derived macrophages, in contrast to all other primary macrophage cultures or cell lines, are endowed with the ability to secrete cholesterol in apoE-containing particles in the absence of a cholesterol acceptor in the culture medium [6,7]. This implies that efflux studies performed in different cell models may not be entirely relevant to the full understanding of the actual efflux pathways from human foam cells *in vivo*.

Another important mediator of cell cholesterol efflux is adenosine triphosphate-binding cassette transporter A1 (ABCA1), a defect in the gene for which causes Tangier disease. ABCA1 is thought to require an apolipoprotein partner for transporting substrates [8]. Recently, other members of the same family such as ABCG1 and ABCG4 have been shown to be regulated by sterol levels in macrophages [9–13] and to mediate cellular cholesterol efflux to high-density lipoproteins [14]. ABCA1 has also been implicated in the control of apoE secretion from human monocyte-derived macrophages [15]. Signaling pathways mediated by the nuclear liver X receptor (LXR) appear to control macrophage cholesterol efflux by regulating in coordinate fashion the expression of apoE, ABCA1 and ABCG1 genes in THP-1 cells [16,17]. The nuclear retinoid X receptor (RXR) forms permissive heterodimers with LXR and other receptor subtypes. Ligands for RXR have also been shown to modulate cholesterol efflux [18] and atherosclerosis development. Although the different pathways of cellular cholesterol efflux are fairly well understood [19], much uncertainty still surrounds the process of spontaneous cholesterol efflux in human macrophages.

The aim of this study was to investigate the pharmacological regulation of this process using two sets of compounds with well-established mechanisms of action. First, we used drugs known to affect different steps of intracellular cholesterol transport, such as progesterone, atorvastatin and the specific ACAT inhibitor avasimibe to ascertain if increased availability of intracellular free cholesterol enhances spontaneous cholesterol efflux. Second, we used compounds that activate ABC transporters via interaction with the nuclear receptors LXR or RXR to determine their impact on spontaneous cholesterol efflux. These latter compounds were the natural LXR ligand 22*R*-hydroxycholesterol (22OH) plus the natural RXR ligand 9-*cis*-retinoic acid (9RA) and a synthetic LXR ligand, T0901317 [20] plus a synthetic RXR ligand, RO264456. The effects of pharmacological treatments on: (i) the accumulation of cell cholesterol, (ii) the expression of the cholesterol transporters ABCA1 and ABCG1, (iii) the efflux of cholesterol into acceptor-free medium, and (iv) the expression and secretion of apoE were assessed in macrophages with different cholesterol loading status.

## 2. Materials and methods

### 2.1. Cell culture

Human monocytes were obtained from 20 healthy volunteers by leukapheresis and elutriation as previously described [21]. Cells were plated in 25-cm<sup>2</sup> or 75-cm<sup>2</sup> flasks in Roswell Park Memorial Institute (RPMI) 1640 medium (Bio-Whittaker, Verviers, Belgium) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO, Eggenstein, Germany), 1 mM sodium pyruvate, 1% non-essential amino acid solution (both from GIBCO) and 20% pooled human serum (PAA, Linz, Austria). The cells were grown for 12 days to allow differentiation into macrophages. Since the apoE phenotype affects cholesterol homeostasis in human monocyte-derived macrophages [4], only donors with the apoE3/3 phenotype were selected for this study.

### 2.2. Isolation of lipoproteins

LDL was obtained from human plasma from healthy volunteers by sequential ultracentrifugation ( $d = 1.019$ – $1.063$ ). AcLDL was obtained by treatment of LDL with acetic anhydride [22].

### 2.3. Test compounds

The compounds affecting cholesterol transport used in the present study were: progesterone, which inhibits intracellular transport of lysosomal cholesterol [23]; atorvastatin, which blocks cholesterol esterification and endocytosis of modified lipoproteins [24] and avasimibe, a direct ACAT inhibitor [25]. The LXR and RXR agonists activating ABC transporters were 22*R*-hydroxycholesterol and 9-*cis*-retinoic acid, respectively (physiological ligands) as well as T0901317 and RO264456, respectively (synthetic ligands). Progesterone, 22*R*-hydroxycholesterol and 9-*cis*-retinoic acid were purchased from Sigma (Deisenhofen, Germany). Parke-Davis (Morris Plains, NJ, USA) kindly provided atorvastatin and avasimibe. Dr. Michael Pech at Hoffmann-La Roche (Basel, Switzerland) kindly provided the synthetic LXR agonist T0901317 and the synthetic RXR agonist RO264456. Compounds were tested at a single concentration selected on the basis of preliminary experiments or previously published studies.

### 2.4. Measurement of intracellular and secreted cholesterol

Cells were washed twice with serum-free RPMI 1640 and loaded with cholesterol by incubation with 80 µg/ml acLDL for 24 h. All cells were then washed three times with RPMI 1640 and incubated for a further 24 h in the same medium. Drugs were added either during both incubations (total of 48 h; experimental design A) or only during the incubation subsequent to cholesterol loading (24 h; experimental design

B) as indicated. At the end of the second 24-h incubation period, media were collected and centrifuged at 1500 rpm for 10 min to remove cell debris, while the cell monolayer was washed twice with PBS and harvested in 1 ml 1 M NaCl. All samples were frozen at  $-20^{\circ}\text{C}$  unless processed immediately. For cholesterol efflux measurement, 4 ml medium were processed using high performance liquid chromatography (HPLC) as previously described [26]. For the cell cholesterol assay, the protein concentration of the cell suspension was determined by the bicinchonic acid assay (Pierce Biotechnology, Rockford, IL). The volume of cell suspension containing 1 mg protein was then analysed by HPLC [26]. Results were expressed as  $\mu\text{g}$  cholesterol (or CE) per mg cell protein.

### 2.5. Secretion of apoE

ApoE was determined in medium samples by sandwich enzyme-linked immunosorbent assay (ELISA) essentially as previously described [5]. Briefly, a goat polyclonal anti-human apoE antibody (Roche Diagnostics, Mannheim, Germany) was used as the capture antibody, the immunoglobulin G (IgG) fraction from a laboratory rabbit anti-human apoE antiserum was used as the second antibody and a pig peroxidase-labeled anti-rabbit IgG (Dako, Hamburg, Germany) was used as the detection antibody. Ortho-phenylenediamine (Sigma) was selected as a peroxidase substrate for the chromogenic reaction.

### 2.6. Quantitative real-time polymerase chain reaction assay

Total RNA was extracted from macrophages using QIAshredder columns, RNeasy mini kit and RNase-free DNase set (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Five micrograms of total RNA were reverse-transcribed using Superscript II RT (Invitrogen, Karlsruhe, Germany). We quantified gene expression using the qPCR-core kit using SYBR-Green with ROX as a passive control and uracil-*N*-glycosylase (Eurogentec, Herstal, Belgium). Measurements were performed with a GeneAmp 5700 system (Applied Biosystems, Weiterstadt, Germany). The following primer pairs were used (forward and reverse, respectively): glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5'-tca aga agg tgg tga agc ag-3', 5'-tcg ctg ttg aag tca gag ga-3'; ABCA1: 5'-ccc tgt gga atg tac cta tgt g-3', 5'-gag gtg tcc caa aga tgc aa-3'; ABCG1: 5'-cag tcg ctc ctt agc acc a-3', 5'-tcc atg ctc gga ctc tct g-3'; apoE: 5'-ggt cgc ttt tgg gat tac ct-3', 5'-ctc agt tcc tgg gtg acc tg-3'; HMG-CoA reductase (HMG-CoA): 5'-ctg tca ttc cag cca agg tt-3', 5'-tgg cag agc cca ctaaat tc-3'.

### 2.7. Statistical analysis

Values at individual drug treatment groups and the values in non-loaded and acLDL-loaded cells were compared by

using *t* tests. All *p*-values are two-tailed. *p*-Values  $<0.05$  were taken to be significant.

## 3. Results

### 3.1. Expression of genes involved in cholesterol metabolism

We first investigated the expression of genes involved in macrophage cholesterol metabolism by measuring the levels of the relevant mRNA after 24 h incubations with the selected agents. In the non-loaded state, the level of *apoE* mRNA was unaffected by the different compounds compared with untreated cells (Fig. 1). In contrast, the level of *ABCA1* mRNA increased  $4.9 \pm 0.7$ -fold ( $p < 0.01$ ) in the non-loaded cells treated with the natural LXR/RXR ligands 22OH/9RA and even more so ( $8.1 \pm 1.3$ -fold,  $p < 0.01$ ) in the cells treated with the synthetic LXR/RXR ligands T0901317/RO264456. The level of *ABCG1* mRNA was increased  $6.3 \pm 1.0$ -fold by avasimibe and  $57 \pm 6$ -fold and  $66 \pm 8$ -fold by 22OH/9RA and T0901317/RO264456, respectively. The changes in *HMG-CoA* mRNA levels seen on treatment with atorvastatin ( $+2.8 \pm 0.5$ -fold) and avasimibe ( $-0.5 \pm 0.04$ -fold) did not reach statistical significance (Fig. 1). T0901317/RO264456 showed no effect. On cholesterol-loading of the cells, the

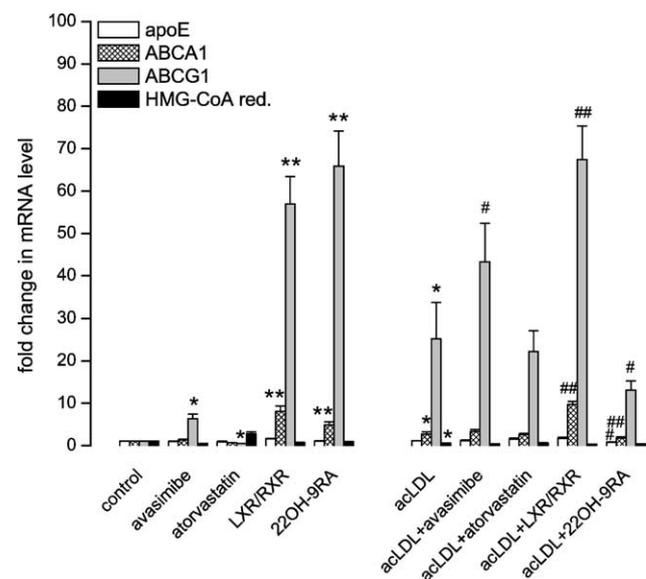


Fig. 1. Expression of genes involved in cholesterol metabolism in human monocyte-derived macrophages treated with pharmacological agents affecting intracellular cholesterol metabolism. Cells were incubated for 24 h in serum-free medium with avasimibe ( $2 \mu\text{M}$ ), the natural LXR and RXR agonists 22R-hydroxycholesterol (22OH) and 9-*cis*-retinoic acid (9RA) (both  $10 \mu\text{M}$ ), the synthetic LXR and RXR agonists T0901317 and RO2644569 ( $1 \mu\text{M}$  and  $100 \mu\text{M}$ , respectively; LXR/RXR), or atorvastatin ( $5 \mu\text{M}$ ) as indicated in the presence or absence of  $80 \mu\text{g/ml}$  acLDL. The mRNA levels were measured by quantitative real-time PCR and normalized using the housekeeping gene GAPDH. Results are expressed as mean + standard error of the mean (S.E.M.) of three to eight independent experiments performed in duplicate. \*  $p < 0.05$ , \*\*  $p < 0.01$  vs. control; #  $p < 0.05$ , ##  $p < 0.01$  vs. acLDL.

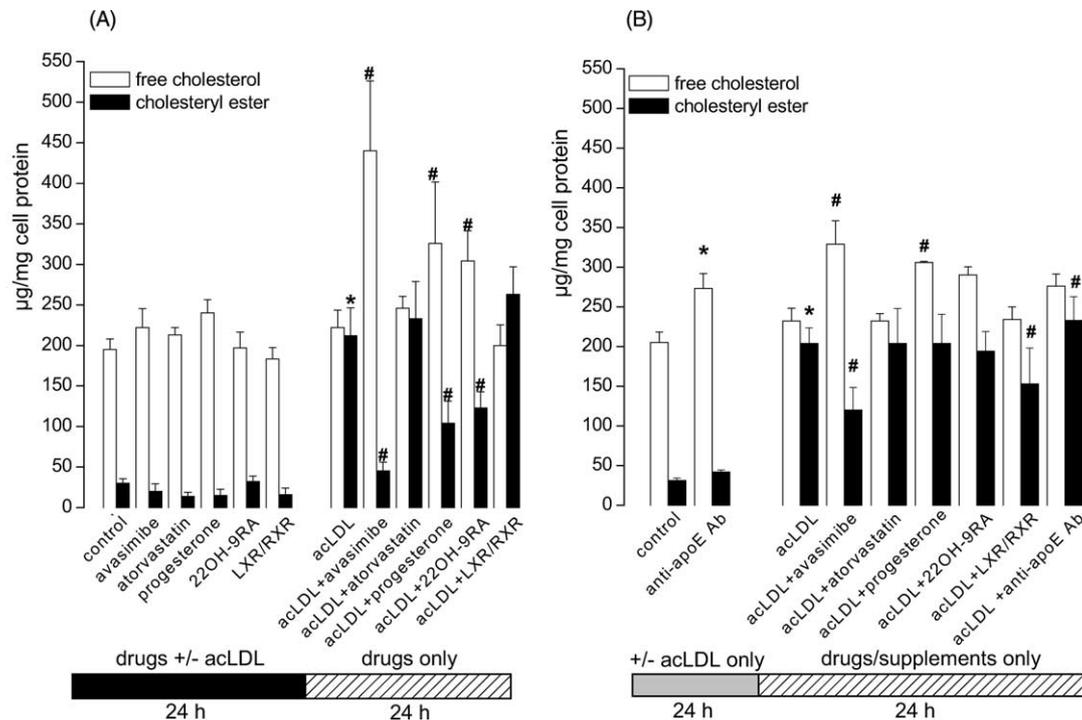


Fig. 2. Intracellular accumulation of free and esterified cholesterol in human monocyte-derived macrophages treated with drugs affecting intracellular cholesterol metabolism. Cells were incubated for 24 h in serum-free RPMI medium with or without addition of 80 µg/ml acLDL and in the presence (panel A) or absence (panel B) of avasimibe (2 µM), atorvastatin (5 µM), progesterone (30 µM), the natural LXR and RXR agonists 22R-hydroxycholesterol (22OH) and 9-*cis*-retinoic acid (9RA; both 10 µM) or the synthetic LXR and RXR agonists T0901317 and RO2644569 (LXR/RXR, 1 and 100 µM, respectively) as indicated. The cells were then washed and incubated for a further 24 h in serum-free RPMI medium with these compounds as indicated. At the end of this incubation, cells were harvested and intracellular FC and CE levels were measured by HPLC. Results are expressed as mean + S.E.M. of three to eight independent experiments performed in duplicate. \* $p < 0.05$  vs. control; # $p < 0.05$  vs. acLDL.

level of *apoE* mRNA remained unchanged, the level of *HMG-CoA* mRNA was halved, while the levels of *ABCA1* and *ABCG1* mRNAs increased  $2.7 \pm 0.6$ -fold and  $25 \pm 8$ -fold, respectively, compared with non-loaded cells. The level of *apoE* mRNA in the cholesterol-loaded cells changed little under treatment with any of the compounds used, including the LXR/RXR ligands, at variance with data obtained in other cell systems [17]. Treatment with avasimibe in cholesterol-loaded macrophages increased the mRNA levels of *ABCG1*. Treatment with 22OH/9RA blunted the increase in the levels of *ABCA1* and *ABCG1* mRNA triggered by cholesterol loading (*ABCA1*: 0.3-fold versus acLDL,  $p < 0.01$ ; *ABCG1*: 0.4-fold versus acLDL,  $p < 0.05$ ). By contrast, treatment with the synthetic LXR/RXR ligands in cholesterol-loaded cells had the opposite effect, and led to a surprising further increase in the mRNA levels for *ABCA1* (3.6-fold versus acLDL) and *ABCG1* (3.1-fold versus acLDL).

### 3.2. Effect of pharmacological agents on mass of free and esterified cholesterol

These experiments were designed to determine the effects of pharmacological agents on intracellular free cholesterol and CE levels in non-loaded, loaded and preloaded macrophages. After incubation with any pharmacological

agent for a total of 48 h (experimental design A), levels of free cholesterol and CE did not change in non cholesterol-loaded cells (Fig. 2A), as measured by HPLC [26]. As expected, cholesterol-loading markedly increased cellular CE ( $212 \pm 34$  versus  $30 \pm 5$  µg/mg cell protein,  $p < 0.01$ ) but did not change free cholesterol content ( $222 \pm 21$  versus  $195 \pm 13$  µg/mg cell protein,  $p > 0.05$ ). Atorvastatin did not affect cholesterol storage in the loaded cells, perhaps because statins reduce cholesterol esterification in macrophages only when added prior to cholesterol loading [27,28]. Avasimibe and, to a lesser extent, progesterone and 22OH/9RA significantly reduced cholesterol esterification in cholesterol-loaded cells. By contrast, cells treated with T0901317/RO2644569 had similar amounts of free cholesterol and, if anything, slightly more CE ( $263 \pm 34$  µg/mg cell protein) than untreated cholesterol-loaded cells (Fig. 2A), despite the marked increase in mRNA levels for the ABC transporters seen on treatment with these compounds (Fig. 1).

After incubation with any pharmacological agent for 24 h following cholesterol loading (experimental design B), most of the drugs produced the same effects already seen with experimental design A, albeit to a lesser extent. A surprising effect was observed with respect to the effects of the synthetic LXR/RXR ligands. Under experimental design A, these drugs, if anything, enhanced storage of CE (Fig. 2A).

With design B, however, they clearly reduced cellular CE content (Fig. 2B;  $153 \pm 35$  versus  $204 \pm 12$   $\mu\text{g}/\text{mg}$  cell protein,  $p < 0.05$ ). Addition of anti-apoE antibodies to the cell culture medium increased cellular cholesterol content by about 35% in cells preloaded or not with acLDL (Fig. 2B) while addition of the cholesterol acceptors apoA-I (25  $\mu\text{g}/\text{ml}$ ) and 4% cyclodextrin reduced it (data not shown).

### 3.3. Effect of pharmacological agents on cholesterol efflux

These experiments were performed to determine the effects of pharmacological agents on spontaneous cholesterol efflux from the macrophages under the above cholesterol-loading conditions. With experimental design A, the synthetic LXR/RXR agonists in the non-cholesterol loaded cells approximately doubled cholesterol efflux in the absence of exogenous cholesterol acceptors ( $71.8 \pm 9.4$  versus  $27.4 \pm 4.3$   $\mu\text{g}/\text{mg}$  cell protein,  $p < 0.05$ ) while the other agents had virtually no effect (Fig. 3A). Loading of the cells with acLDL increased efflux across the board, which was further enhanced by avasimibe (>45%), progesterone (>23%; both  $p < 0.05$  versus acLDL) and, to a greater extent, by the synthetic LXR/RXR ligands (>76%;  $p < 0.05$ ). When pharmacological agents were added only during the efflux phase (experimental design B), the synthetic LXR/RXR agonists in the acLDL-preloaded cells significantly promoted chole-

sterol efflux, while progesterone depressed it compared with untreated acLDL-preloaded cells. Incubation of the cells with anti-apoE antibodies reduced free cholesterol efflux by about 25% in acLDL-preloaded cells (Fig. 3B), whereas incubation with the inert cholesterol acceptor cyclodextrin increased free cholesterol efflux about eight-fold (data not shown).

### 3.4. Effect of pharmacological agents on apoE secretion

Finally, these experiments were performed to determine the effects of pharmacological agents on apoE secretion by the macrophages under the above cholesterol-loading conditions. Using experimental design A, avasimibe, atorvastatin and progesterone significantly reduced secretion of apoE by 30–80% in non-cholesterol-loaded cells (Fig. 4A). By contrast, both the natural and the synthetic LXR/RXR agonists increased apoE secretion more than two-fold. Cholesterol-loading with acLDL significantly increased apoE secretion ( $1.37 \pm 0.32$  versus  $1.08 \pm 0.21$   $\mu\text{g}$  apoE/mg cell protein/24 h) [4]. After cholesterol-loading, atorvastatin and 22OH/9RA lost their effects on apoE secretion, while avasimibe, progesterone, and the synthetic LXR/RXR agonists essentially retained the activity seen in the non cholesterol-loaded cells (Fig. 4A). When pharmacological agents were added only during the efflux phase (experimental design B), addition of anti-apoE antiserum markedly reduced the levels of immunodetectable apoE in the medium in cells preloaded

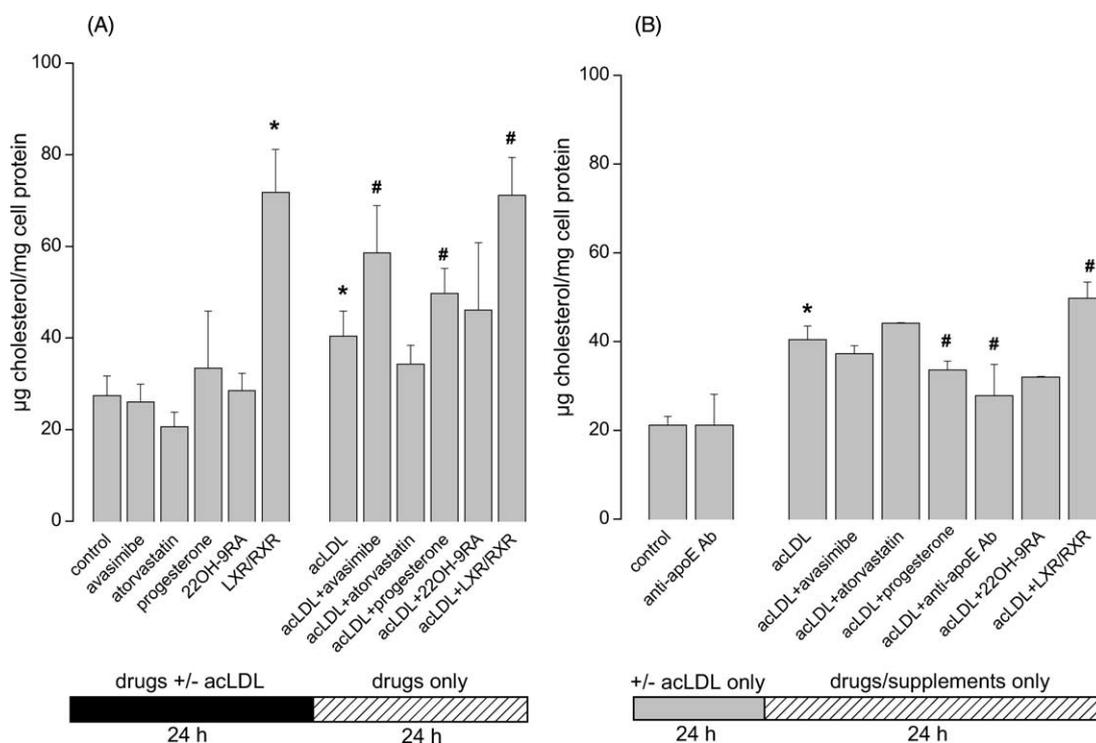


Fig. 3. Spontaneous cholesterol efflux from human monocyte-derived macrophages treated with drugs affecting cholesterol metabolism. Cells were incubated as described in the legend to Fig. 1. At the end of the experimental period, the media were collected, centrifuged and aliquots thereof assayed for free cholesterol secretion by HPLC. Results are expressed as mean  $\pm$  S.E.M. of three to eight independent experiments performed in duplicate. \* $p < 0.05$  vs. control; # $p < 0.05$  vs. acLDL.

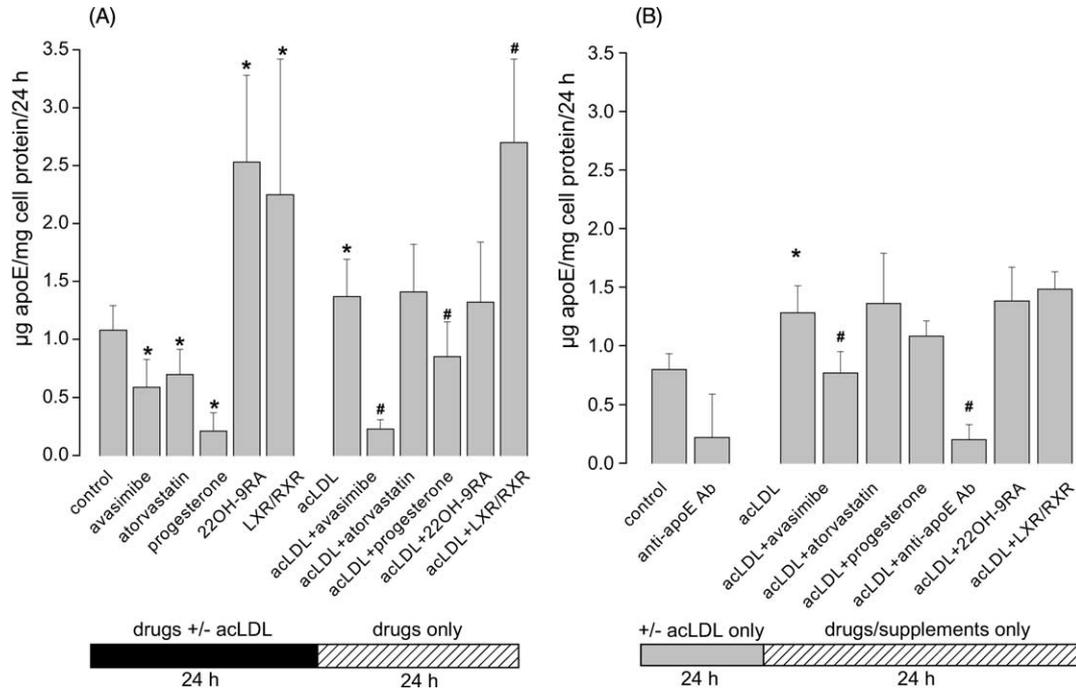


Fig. 4. ApoE secretion from human monocyte-derived macrophages treated with drugs affecting cholesterol metabolism. Cells were incubated as described in the legend to Fig. 1. At the end of the experimental period, the media were collected, centrifuged and aliquots thereof assayed for secreted apoE by ELISA. Results are expressed as mean + S.E.M. of three to eight independent experiments performed in duplicate. \*  $p < 0.05$  vs. control; #  $p < 0.05$  vs. acLDL.

or not with acLDL (Fig. 4B). In the cholesterol-preloaded cells, avasimibe reduced apoE secretion ( $0.70 \pm 0.11$  versus  $1.28 \pm 0.27$   $\mu\text{g}$  apoE/mg cell protein/24 h,  $p < 0.05$ ), while the other compounds had no effect (Fig. 4B). Incubation of cholesterol-loaded macrophages with apoA-I and cyclodextrin did not affect apoE secretion (data not shown).

#### 4. Discussion

Spontaneous cholesterol efflux from human monocyte-derived macrophages may be relevant within atherosclerotic lesions, where the availability of specific HDL subclasses as lipid acceptors is limited. In the present study, we investigated this poorly understood pathway using a pharmacological rather than biochemical approach.

A principal finding was that the cholesterol loading status of the macrophage affects spontaneous cholesterol efflux. For instance, in non-cholesterol-loaded cells, mRNA levels for the ABC transporters A1 and G1 were increased by both natural and synthetic LXR/RXR ligands. On loading the cells with cholesterol, the natural ligands no longer increased the mRNA levels for the ABC transporters, while the synthetic ligands continued to do so. We do not know the reason for this, although it has been suggested that the generation of specific oxysterols associated with cholesterol loading may affect interaction of 22OH, 9RA, or both, with the cognate receptor, thus impairing transactivation of ABC transporter genes [9,16,17,29]. Similarly, progesterone

stimulated cholesterol efflux if added already during the loading phase, but decreased efflux if treatment started after loading had been completed. In fact, progesterone-induced alteration of intracellular sterol homeostasis modulates the susceptibility of apoE to degradation [30], and consequently the efflux capacity of the cell.

Multiple regulatory lipid-sensitive mechanisms appear to drive cholesterol efflux in the absence of acceptors. Progesterone, avasimibe and 22OH/9RA reduced cholesterol esterification, whereas the synthetic LXR/RXR ligands T0901317 and RO2644569 did not (Fig. 2). However, only avasimibe [31] and T0901317/RO2644569 enhanced cholesterol efflux from cholesterol-loaded cells, the latter even in non-cholesterol-loaded macrophages (Fig. 3). Both avasimibe and progesterone are known to inhibit ACAT and both increase the content of free cholesterol in acLDL-loaded cells (Fig. 2). This may increase formation of intracellular oxysterols, in turn causing increased LXR/RXR-mediated efflux such as that induced by synthetic LXR/RXR ligands in acLDL-loaded macrophages in the present study, as noted above (Figs. 2 and 3). Treatment of fibroblasts with progesterone has been shown to cause accumulation of free cholesterol in a late endosomal/lysosomal compartment, and such accumulation is also seen in Niemann-Pick type C cells [32]. In both cases, inhibition of apoA-I-mediated cholesterol efflux was observed [33,34]. In macrophages, by contrast, inhibition of ACAT enhances apoA-I-mediated cholesterol efflux [35], an effect which we have now shown for non apoA-I-mediated efflux (Fig. 3). These findings indicate that the link

between impaired cholesterol esterification and targeting of free cholesterol to secretory pathways is of an indirect nature, because intracellular free cholesterol levels per se did not necessarily enhance spontaneous cholesterol efflux. Also, these mechanisms appear to differ between different cell types.

Our results support an important role for secreted apoE of endogenous origin in mediating spontaneous cholesterol efflux. In our studies, net efflux of cholesterol was independent of the rate of apoE accumulation—which presumably reflects the rate of apoE secretion—in the culture medium. Thus, the ACAT inhibitor avasimibe increased cholesterol efflux from cholesterol-loaded cells (Fig. 3) despite a marked reduction of apoE accumulation (Fig. 4), in contrast to other studies [36], and no effect on *apoE* mRNA levels (Fig. 1). We have previously shown that macrophages from apoE4/4 subjects display ineffective net cholesterol efflux despite the greatest apoE secretion rate, whereas the opposite was true in macrophages from apoE2/2 subjects [4]. It has been reported that, at high concentrations of extracellular apoE, ABCA1-dependent mechanisms are involved in sterol efflux from RAW 264.7 cells [37]. Moreover, we have shown that ABCA1 modulates apoE secretion from human monocyte-derived macrophages [15]. On the other hand, Huang et al. described that endogenous apoE expression and secretion at physiological levels facilitates sterol efflux from mouse J774 macrophages independent of ABCA1 expression [38], although it is not clear if cholesterol efflux in mouse macrophage cell lines occurs by the same mechanisms as in primary human macrophages.

In order to integrate our findings into a functional model, only selected intracellular cholesterol pools appear to be involved in cholesterol efflux in the absence of exogenous acceptors. Intracellular apoE can transfer such pools to ABC transporters. ABCA1 binds not only to apoA-I but also to apoE [39]. Since lipid-free apoE can interact efficiently with ABCA1 and induce lipid efflux, endogenous intracellular and/or secreted apoE may represent the ultimate lipid acceptor [40]. However, removal of secreted apoE from the medium by specific antibodies reduced but not abolished cholesterol efflux, demonstrating the presence of both an apoE-dependent and apoE-independent process. In fact, other lipid-free apolipoproteins of endogenous origin might compete with apoE for ABCA1 binding and cellular cholesterol release. In the present experiments, expression of ABCG1, rather than that of ABCA1 or apoE, was most closely associated with cholesterol efflux. On cholesterol-loading, the level of ABCG1 mRNA increased much more than that of ABCA1 mRNA, as reported previously by us [41], and in contrast to studies in mouse macrophages [42]. These correlations between mRNA regulation and efflux modulation do not necessarily imply causal relationships and their functional relevance is currently under investigation. On the other hand, a major role of ABCG1 has been demonstrated in HDL-mediated cholesterol efflux in macrophages [14]. Although no exogenous HDL was used in our experiments, the occurrence of lipid-poor

apoA-I-free but apoE-containing particles as acceptors in ABCG1-mediated cholesterol efflux cannot be ruled out.

In conclusion, cholesterol efflux from human monocyte-derived macrophages in the absence of exogenous acceptors: (i) was not necessarily promoted by enlarged intracellular free cholesterol pools secondary to impaired formation of cholesteryl esters; (ii) was increased by compounds that activate ABCA1 and, to a greater extent, ABCG1; (iii) only partially correlated with net apoE secretion. These findings extend our understanding of the mechanisms of cholesterol efflux operating in human foam cells.

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