

Cholesterol absorption inhibitor Ezetimibe blocks uptake of oxidized LDL in human macrophages

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Abstract

Ezetimibe belongs to a group of selective and very effective 2-azetidione cholesterol absorption inhibitors which act on the level of cholesterol entry into enterocytes. Recent data indicated that the drug prevents the formation of a heterocomplex consisting of annexin-2 and caveolin-1 and leads to specific inhibition of an NPC1L1-dependent cholesterol uptake pathway required for uptake of micellar cholesterol into enterocytes. Earlier studies have shown that caveolin-1 and annexin-2 are also expressed in human macrophages and we show in this study that human macrophages express NPC1L1. Moreover in human macrophages, Ezetimibe (SCH58235) and its analogue, SCH354909, are bound to specific cell surface receptors followed by endocytosis via the classical endocytic pathway. SCH58235 had no effect on uptake and/or processing of acetylated LDL (Ac-LDL). In contrast, the compound inhibited uptake of oxidized LDL (Ox-LDL) by ~50% in a dose-dependent manner. SCH58235 blocked the lipid-induced induction of LXR/RXR target genes ABCA1, ABCG1, and apolipoprotein E distinctively more effectively in macrophages loaded with Ox-LDL than in those loaded with Ac-LDL. Based on these findings, we presume that the caveolin-1-, annexin-2-, and NPC1L1-dependent cholesterol uptake system that is operating in enterocytes may also contribute to class B scavenger receptor-dependent uptake of Ox-LDL in human monocyte-derived macrophages.

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Specific inhibitors of intestinal cholesterol absorption are important drugs of the future to prevent atherosclerosis and cardiovascular disease [1]. As was shown by the Scandinavian Simvastatin Survival Study, coronary patients with low baseline ratios of serum cholestanol and plant sterols to cholesterol (indicating low cholesterol absorption) but not those with high ratios (high cholesterol absorption) experienced reduced recurrences of coronary events during simvastatin treatment. Simvastatin suppressed the synthesis of cholesterol markedly more effectively in subjects with high than with low

baseline synthesis but reduced respective serum cholesterol levels less markedly than synthesis due to individual differences of cholesterol absorption [2,3]. Thus, treatment with cholesterol absorption inhibitors, like Ezetimibe, preferentially in combination therapy with inhibitors of HMG CoA reductase, are ideal tools for primary and secondary prevention in subjects with high cholesterol absorption (“cholesterol hyperabsorbers”).

Ezetimibe analogues are selective and very effective 2-azetidione cholesterol absorption inhibitors which act on the level of cholesterol entry into enterocytes [4]. It could be demonstrated that the inhibition of cholesterol absorption effected by these agents is not mediated via changes in either the size or composition of the

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intestinal bile acid pool, or the level of mRNA expression of proteins that facilitate cholesterol efflux from the enterocyte, but rather may involve disruption of the uptake of luminal sterol across the microvillus membrane in a direct manner [5]. Moreover, it could be shown that Ezetimibe blocks the class B scavenger receptor SR-BI *in vitro* but results obtained from studies with SR-BI knockout mice excluded that SR-BI is essential for intestinal cholesterol absorption or the activity of Ezetimibe [6]. Recently, it could be shown that Ezetimibe-mediated inhibition of cholesterol entry into enterocytes depends on NPC1L1, an NPC1-related cholesterol transporter expressed in enterocytes, the liver, and other cell-types [7]. In addition, recent data indicated that the drug prevents the formation of a heterocomplex consisting of annexin-2 and caveolin-1, apparently required for uptake of micellar cholesterol into enterocytes [8]. Since the pharmacology of Ezetimibe is characterized by a considerable systemic uptake it must be considered that its action may not be restricted to the intestine alone. Therefore, important aims of the present study were to investigate whether NPC1L1 is expressed in human monocyte-derived macrophages and whether the drug may be associated with potentially anti-atherosclerotic effects that may result from its influence on foam cell formation.

Experimental procedures

Materials. SCH58235 Ezetimibe (1-(4-fluorophenyl)-(3*R*)-[3-(4-fluorophenyl)-(3*S*)-hydroxy-propyl]-(4*S*)-(4-hydroxyphenyl)-2-azetidinone) and its labelled analogue SCH354909 (for details see [6]) were kindly provided by Dr. H.R. Davis Jr. (Schering-Plough, Kenilworth, NJ, USA). LDL was prepared according to standard procedures by density gradient ultracentrifugation from pooled serum samples collected from normolipidaemic subjects.

Preparation of modified lipoproteins. Acetylated and oxidized LDL were prepared from native LDL according to [9].

Cell culture and lipid analyses. Human monocyte-derived macrophages were obtained and cultured as previously described [10]. Monocytes differentiated into macrophages by cultivation for 10 days. At day 11, cells were incubated in RPMI1640 containing antibiotics and the supplements for 24 h as indicated in the figure legends. Cells were washed twice with PBS and separate flasks were harvested either for lipid determination or RNA extraction. Quantization of non-esterified cholesterol and cholesterol esters was performed by high performance liquid chromatography followed by identification of unassigned cholesterol ester species by means of secondary ion mass spectrometry as described in [11,12]. All shown data represent results from at least three independent experiments each performed in triplicate. Statistical analysis was performed with Student's *t* test ($p < 0.05$ was considered statistically significant).

Gene expression studies. RNA was isolated from cells and subjected to real-time quantitative PCR employing an ABI-Prism 7900 HT instrument essentially as described in [10]. The following primer pairs were used for transcript amplification:

ABCA1, 5'-CCCTGTGGAATGTACCTATGTG-3'/5'-GAGGTGTCCAAAGATGCAA-3'; ABCG1, 5'-CAGTCGCTCCTTAGCAACA-3'/5'-TCCATGCTCGGACTCTCTG-3'; HMGCoAR, 5'-ACC AATAGAGGCTGCAGAGC-3'/5'-GAGTACAAGCACGTGGAA

G-3'; GAPDH, 5'-TCAAGAAGGTGGTGAAGCAG-3'/5'-TCGCTGTTGAAGTCAGAGGA-3'; Apo E, 5'-GGTCGCTTTTGGGATTACCT-3'/5'-CTCAGTTCCTGGGTGACCTG-3'; LXR α , 5'-AGCGTCCACTCAGAGCAAG-3'/5'-ACAGTCATTCGTGCACATCC-3'; and RXR α , 5'-GCCAATGAGAACATGAGCTG-3', 5'-ACCAGAACACCAAGGCAGAG-3'.

Statistical analyses. All shown data represent means based on at least three independent experiments each performed in triplicate. Statistical analysis was performed with Student's *t* test ($p < 0.05$ was considered statistically significant).

Results and discussion

Expression of NPC1L1 and interaction of SCH354909 and SCH58235 with human macrophages

Although Ezetimibe analogues act primarily in the intestine, it is possible that the drug influences cholesterol metabolism also in other tissues. Since it was shown that NPC1L1 plays a crucial role in its mode of action in the intestine, we first studied whether NPC1L1 is expressed in human macrophages. As shown in Fig. 1, NPC1L1 is expressed in this cell type, albeit at a lower level than in the intestine or the liver. A quantitative evaluation of the real-time PCR data provided a value of 0.3–0.5% of expression compared with human intestine or liver.

Since binding of the drug to the cell surface and/or internalization are crucial steps in its mode of action, we next incubated human monocyte-derived macrophages with the fluorescently labelled Ezetimibe analogue SCH354909. Incubation at 4°C for 15 min led to extensive cell surface binding which could be competed almost entirely with a 10-fold excess of the non-labelled

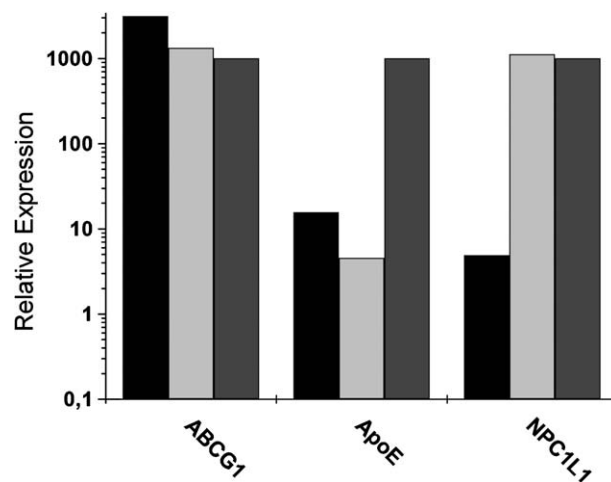


Fig. 1. Expression of NPC1L1 in human intestine, liver, and monocyte-derived macrophages. The expression of NPC1L1 was determined in human monocyte-derived macrophages (black columns) relative to its expression in the liver (dark grey columns, set as 1.000 arbitrary units) and intestine (light grey columns) by real time PCR as described in Experimental procedures. For comparison, data are also given for expression of ABCG1 and apolipoprotein E as indicated.

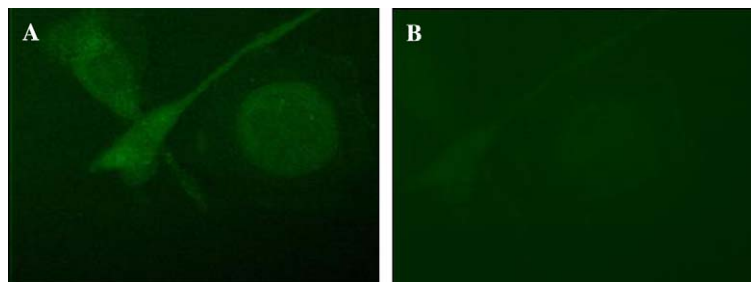


Fig. 2. Interaction of the fluorescently labelled Ezetimibe analogue SCH354909 with human monocyte-derived macrophages. Cells were incubated at 37°C for 24h with medium supplemented with 0.1 μM SCH354909 as described in Experimental procedures. The experiment shown in (B) was performed in the presence of 10-fold excess of unlabeled drug (SCH58235).

SCH58235 (not shown). As shown in Fig. 2A, SCH354909 was taken up very effectively by the cells. After 1h of incubation at 37°C, the fluorescently labelled drug was detected mainly in cytoplasmic vesicles which, according to their characteristic morphology and distribution, most likely represented endosomes and lysosomes. In contrast, cell surface binding was relatively weak under these conditions. Given the fact that co-incubation with a 10-fold excess of SCH58235 (non-labelled drug) repressed the uptake of the SCH354909 considerably (Fig. 2B), it seems highly likely that the compound enters the cell via a specific uptake mechanism (i.e., receptor-mediated endocytosis).

Effect of SCH58235 on receptor-mediated endocytosis of modified lipoproteins

Acetylated LDL (Ac-LDL) is a ligand to scavenger receptor class A (SR-A). In contrast, previous studies using LDL oxidized by various means showed that at least 11 different scavenger receptors which are collectively categorized as “scavenger receptor family” are involved in the uptake of Ox-LDL (reviewed in [13]). Advanced glycation endproducts (AGE) and their receptor systems have been studied independently until recent findings showed that AGE-proteins are also recognized as active ligands by scavenger receptors including SR-A, class B scavenger receptors (SR-B) such as CD36 and SR-BI, type D scavenger receptor (LOX-1), and FEEL-1/FEEL-2 [14]. In summary, the currently available data support that endocytic uptake of Ac-LDL by macrophages and macrophage-derived cells is mainly mediated by SR-A. Conversely, endocytic uptake of Ox-LDL is mediated mainly by SR-A and class B scavenger receptors, CD36 and, to a lower extent, SR-BI. This led to the conclusion that especially CD36 is a key receptor involved in foam cell formation in the early stage of atherosclerosis.

In order to identify uptake pathways that may be influenced by Ezetimibe analogues, we incubated human monocyte-derived macrophages for 24h with 80 μg/ml Ac-LDL or Ox-LDL in the presence or absence of

SCH58235. Foam cell formation was monitored by measuring cholesterol oleate by HPLC in cell homogenates. As shown in Fig. 3, concentrations up to 1 μM of the compound had no influence on cholesterol ester formation resulting from incubation with Ac-LDL. This result excludes SR-A as a major target for Ezetimibe. Conversely, the corresponding experiment performed with 80 μg/ml of Ox-LDL revealed that SCH58235 led to a dose-dependent repression of cholesterol ester formation. At a concentration of 0.1 μM, SCH58235 inhibited cholesterol ester formation by 30% ($p < 0,05$) whereas 1 μM resulted in 44% ($p < 0,001$) inhibition (Fig. 4). In contrast, the drug did not affect the concentration of non-esterified cholesterol (Fig. 4). Thus, SCH58235 caused a specific and effective inhibition of foam cell formation in vitro resulting from uptake Ox-LDL.

Given these data, we next studied whether the drug also affected the expression of genes involved in

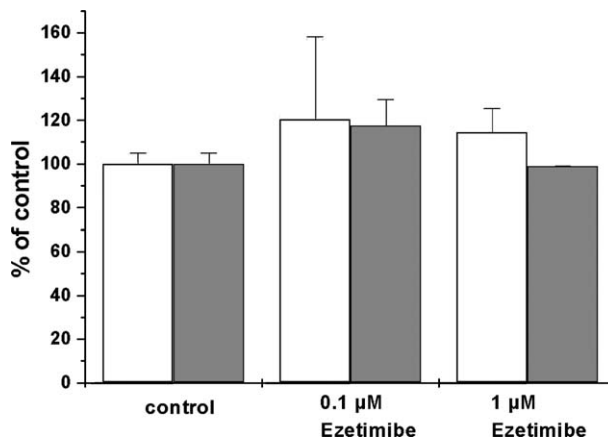


Fig. 3. Effect of SCH58235 on foam formation in human monocyte-derived macrophages loaded with Ac-LDL. Cells were incubated for 24h in medium supplemented with 80 μg acetylated LDL (Ac-LDL) per ml either in the absence (left bars) or presence of 0.1 or 1 μM SCH58235 as indicated in the figure. Data represent percentage of controls (cells incubated with 80 μg acetylated LDL (Ac-LDL) per milliliter in the absence of the drug). The bars shown in white indicate non-esterified cholesterol (FC), the grey-coloured bars represent cholesterol oleate.

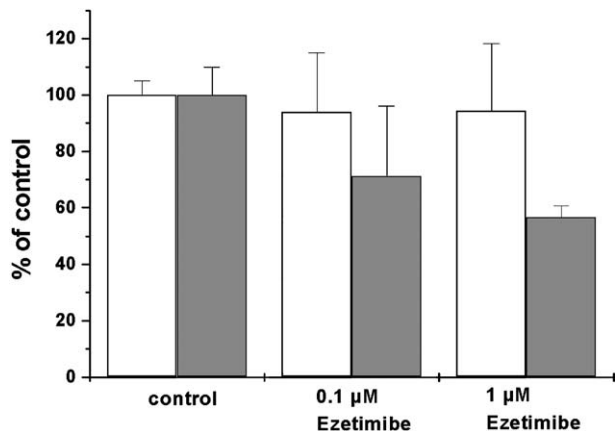


Fig. 4. Effect of SCH58235 on foam formation in human monocyte-derived macrophages loaded with Ox-LDL. Cells were incubated for 24h in medium supplemented with 80 μg oxidized LDL (Ox-LDL) per milliliter either in the absence (left bars) or presence of 0.1 or 1 μM SCH58235 as indicated in the figure. Data represent percentage of controls (cells incubated with 80 μg oxidized LDL (Ox-LDL) per ml in the absence of the drug). The bars shown in white indicate non-esterified cholesterol (FC), the grey-coloured bars represent cholesterol oleate.

macrophage lipid metabolism. The experiments were performed using the Taqman technique in order to quantify transcripts encoding ABCA1, ABCG1, HMG-CoA reductase, apo E, NPC1L1, LXR, and RXR. Expression of GAPDH served as internal control. As shown in Fig. 5, SCH58235 did not lead to repression of the lipid-induced induction of LXR/RXR target genes ABCA1 and ABCG1. Conversely, a very moderate dose-dependent repression occurred with respect to apo E expression if Ac-LDL loaded cells were treated with

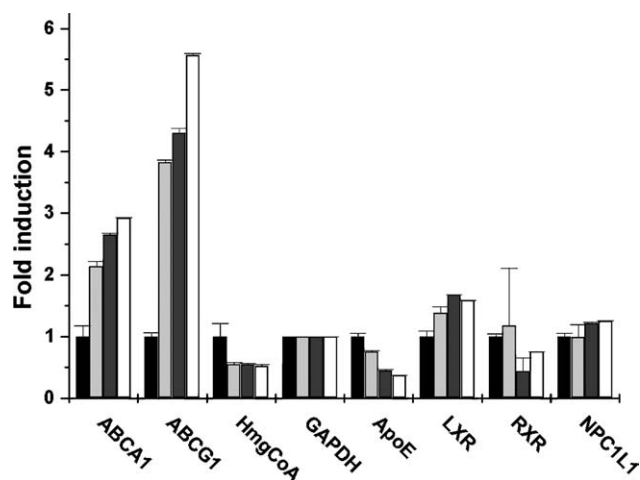


Fig. 5. Gene expression in Ac-LDL loaded human monocyte-derived macrophages treated with SCH58235. Data represent gene expression expressed as fold induction compared to non-treated cells. Bars from left to right: control (black); 80 μg/ml Ac-LDL for 24h (light grey); 80 μg/ml Ac-LDL plus 0.1 μM SCH58235 for 24h (dark grey); and 80 μg/ml Ac-LDL plus 1.0 μM SCH58235 for 24h (white).

SCH58235. In addition, neither the discrete differences of gene expression which were observed with respect to LXR and NPC1L1 nor the repression of RXR observed at a concentration of 0.1 μM of SCH58235 was statistically significant. Conversely, we observed a rather prominent, dose-dependent repression of LXR/RXR target gene induction (app. -40% at 0.1 μM for ABCA1, app. -50% at 0.1 μM of SCH58235 for ABCG1) in Ox-LDL loaded cells treated with SCH58235 (Fig. 6). A moderate repression was observed with respect to apo E and LXR expression. The ~2-fold induction of NPC1L1 expression observed in Ox-LDL loaded cells was only partly repressed in the presence of 1 μM SCH58235. The discrete differences of gene expression observed for HMG-CoA reductase and RXR were not statistically significant. Since it is known that LXR/RXR target gene expression is controlled in an important manner by the cholesterol loading state in macrophages [15], it seems likely that SCH58235 blocked the induction of these genes secondary to its effect on uptake and/or processing of Ox-LDL.

In summary, our results support that SCH354909 and SCH58235 inhibited uptake and/or processing of Ox-LDL but not of Ac-LDL in human monocyte-derived macrophages. Whereas data shown in Fig. 3 excluded involvement of SR-A, the results shown in Fig. 4 supported that SCH58235 affected the class B scavenger receptor-mediated uptake pathway known to be required for receptor-mediated endocytosis of Ox-LDL. According to previous studies, class B scavenger receptors CD36 and SR-BI are responsible for roughly 50% of ox-LDL uptake under the conditions used in our experiments [16]. The previous finding that Ezetimibe acts

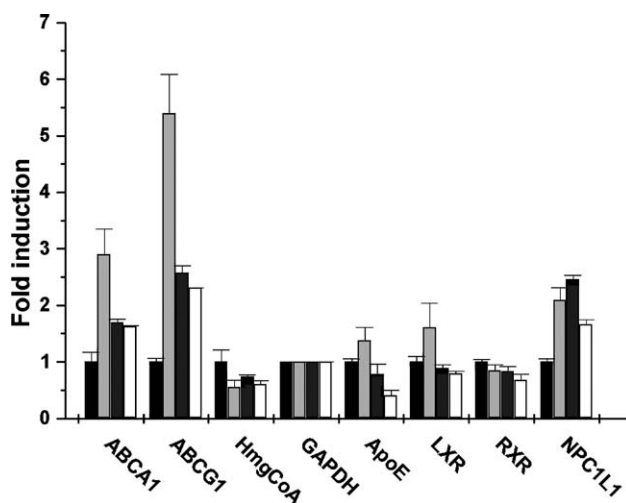


Fig. 6. Gene expression in Ox-LDL loaded human monocyte-derived macrophages treated with SCH58235. Data represent gene expression expressed as fold induction compared to non-treated cells. Bars from left to right: control (black); 80 μg/ml Ox-LDL for 24 hours (light grey); 80 μg/ml Ox-LDL plus 0.1 μM SCH58235 for 24h (dark grey); and 80 μg/ml Ox-LDL plus 1.0 μM of SCH58235 for 24h (white).

via caveolin-1/annexin-2/NPC1L1 implies that these proteins may not only be important for uptake of micellar cholesterol in enterocytes but also for class B scavenger receptor-mediated uptake of Ox-LDL in human macrophages.

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