

## Pitavastatin increases ABCA1-mediated lipid efflux from Fu5AH rat hepatoma cells

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### Abstract

ATP binding cassette A1 (ABCA1) is responsible in vivo for the formation of HDL by promoting the lipidation of apoprotein A-I (apoA-I) via cholesterol and phospholipid efflux from the liver. Treatment of patients with statins produces an increase in HDL plasma level, but the underlying mechanism is not completely understood. In this work we investigated the ability of pitavastatin to modulate ABCA1-mediated efflux from Fu5AH rat hepatoma cells, that here we demonstrate to express functional ABCA1 upon treatment with 22OH/cRA. In both basal and ABCA1 expressing cells pitavastatin 0.1–50 μM induced a dose-dependent increase in cholesterol efflux to apoA-I; this effect was reversed by mevalonate or geranyl geraniol. A stimulatory effect was also observed on phospholipid efflux. Similar results were obtained with compactin, suggesting a class-related effect of statins. These results indicate a potential mechanism for the improvement in HDL plasma profile observed in patients treated with statins.

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ATP binding cassette A1 (ABCA1) is a transporter that mediates the unidirectional efflux of lipids from cell membranes to extracellular acceptors [1]. A major physiological role of this protein involves HDL formation in the liver by the promotion of cholesterol and phospholipid release from hepatocytes to the apolipoprotein A-I (apoA-I) and the consequent generation of pre-beta lipidated particles, the precursors of mature HDL [2]; this function is confirmed by clinical observations in human Tangier disease, where mutations of ABCA1 are correlated to a severe hypoalphalipoproteinemia [3]. Since epidemiological studies have consistently shown an inverse relation between plasma levels of HDL and risk of cardiovascular disease [4], ABCA1-mediated

hepatic synthesis of HDL is likely to explain the atheroprotective role of this receptor. The importance of ABCA1 in regulating HDL cholesterol level is evidenced also by its abundant expression in the liver, where both mRNA and protein are found at high levels; in particular, hepatocytes are found to be ABCA1-enriched, as shown by in situ hybridization and immunohistochemistry experiments [5]. In addition, a recent work reveals ABCA1 specific localization on the basolateral (vascular) plasma membrane domain, consistent with its role in HDL cholesterol metabolism and reverse cholesterol transport [6]. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) cause an improvement of clinical events in patients with atherosclerosis related to the classical effect of reducing LDL cholesterol and possibly to non lipidic mechanisms, the so-called “pleiotropic effects” [7,8]. Moreover, the

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reported ability of statins to increase HDL levels may contribute to their protective activity against cardiovascular diseases [9–12]; to date, the mechanisms of improvement of HDL by statins have not been fully elucidated. In this paper we investigated if statins may potentially act by stimulating ABCA1-mediated lipid efflux in hepatocytes. To this end we studied the effect of the HMG-CoA reductase inhibitors pitavastatin and compactin on ABCA1 function in hepatic cells using Fu5AH rat hepatoma cells as experimental model. These cells have been extensively used to investigate intracellular cholesterol processing and lipid exchange with lipoproteins for many years, as documented by several works where they have been used for both physiological and pharmacological studies [13–16]. A recent work aimed to correlate ABCA1 expression and function in several cell types indicates no expression of ABCA1 in Fu5AH in basal conditions [17]; however, these results do not exclude the possibility to induce ABCA1 in these cells. In the present paper we found that a functional ABCA1 may be induced in Fu5AH and we considered them an appropriate model to investigate the pharmacological effect of statins.

## Materials and methods

**Materials.** Fetal calf serum (FCS), bovine serum albumin (BSA), 3',5'-cyclic monophosphate (cpt-cAMP), 9-*cis* retinoic acid (cRA), 22-hydroxy-cholesterol (22OH), compactin, mevalonate, and geranyl geraniol (GGOH) were purchased from Sigma (St. Louis, MO, USA). Pitavastatin was gently provided by Kowa (Japan).

ApoA-I was kindly donated by Dr. Laura Calabresi (University of Milan). Organic solvents were purchased from Merck (Darmstadt, Germany). [1,2-<sup>3</sup>H]Cholesterol and [methyl-<sup>3</sup>H]choline chloride were from Amersham Biosciences (Uppsala, Sweden). Tissue culture flasks and plates were from Corning (Corning, NY, USA) and Falcon (Lincoln, NY, USA). Dulbecco's minimum essential medium (DMEM) and phosphate-buffered saline (PBS) were purchased from BioWhittaker (Walkersville, MD). Western blot buffers and supplies were purchased from Invitrogen (Carlsbad, CA). Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit antibody and ECL-Plus chemiluminescent kits were purchased from Amersham-Pharmacia (Little Chalfont, Buckinghamshire, England). Rabbit-anti-mouse ABCA1 antibody was purchased from Novus Biological (Cambridge, UK).

**Cell culture.** Fu5AH rat hepatoma cells were cultured in DMEM supplemented with 10% FCS and seeded in 24-well plates for the experiments. Cells were incubated at 37°C, 5% CO<sub>2</sub>.

**Western blotting analysis.** Cell monolayers were lysed with a 1% Triton X-100, 0.5% NP-40, and 10mM Tris buffer and homogenized through a 27-gauge needle. Equal amounts of protein (20µg) were separated on 3–8% Tris-acetate gels and transferred to PVDF membrane. ABC1 was detected with a rabbit primary antibody. Membranes were then incubated with a donkey anti-rabbit IgG conjugated to horseradish peroxidase with visualization by enhanced chemiluminescence ECL Plus according to the manufacturer's conditions.

**Cholesterol efflux.** Cholesterol release from Fu5AH was measured as previously described [18]. Briefly, 80–90% confluent cells were radiolabeled with 2µCi/ml [1,2-<sup>3</sup>H]cholesterol for 24h; in the next 18h ABCA1 was up-regulated by the treatment with 22OH (5µg/ml) and cRA (10µM) in presence or absence of pitavastatin. Pitavastatin was

previously dissolved in DMSO (Merck) and added to the culture media as 0.5% of the volume. Monolayers were then incubated for 4h in the presence of 20µg/ml apoA-I. Cholesterol efflux was quantified by removing media from the cell monolayers and centrifuging them to remove floating cells. The radioactivity present in the incubation medium was determined by liquid scintillation counting.

**Measurement of phospholipid efflux.** The phospholipid efflux was performed as previously described [19]. Fu5AH were incubated for 48h in DMEM containing 1% FCS, and 4µCi/ml [methyl-<sup>3</sup>H]choline chloride and in presence or absence of 22OH (5µg/ml) and cRA (10µM) and pitavastatin in the next 18h. Lipid efflux was promoted to 20µg/ml apoA-I for 4h. At the time point media were collected and centrifuged. The supernatant was separated and lipids were extracted using the method of Bligh and Dyer [20]. The aqueous phase was aspirated, and to remove any remaining free [<sup>3</sup>H]choline, the chloroform phase was washed three times with 10:9 (v/v) methanol:water. The chloroform phase was dried under a stream of nitrogen (N<sub>2</sub>) and redissolved in 1ml toluene. A 200µl aliquot of each sample was quantitated by scintillation counting. To analyze cellular lipids, the cell monolayers were washed 3 times with PBS, and lipids were extracted by the addition of 1ml of 2-propanol. The 2-propanol extracts were dried under a stream of N<sub>2</sub>, resuspended in water, and extracted with organic solvents as described before.

**Statistical analysis.** Results are reported as means of triplicates ± SD. Statistical significance was determined by two-tailed Student's *t* test.

## Results and discussion

### Expression of functional ABCA1 in Fu5AH cells

Western blotting analysis demonstrates that stimulation with the LXR/RXR agonists 22OH/cRA induces a significant up-regulation of ABCA1 protein in Fu5AH hepatoma cells (Fig. 1). Concomitantly, cells treated with 22OH/cRA exhibited a 2.4-fold increase in cholesterol efflux to apoA-I compared to unstimulated cells (Fig. 2A). Similarly, ABCA1 up-regulation promoted a more efficient efflux of phospholipids (Fig. 2B). Altogether these results demonstrate for the first time that Fu5AH hepatoma cells may express an LXR-dependent functional ABCA1. This conclusion is entirely consistent with the concept that cholesterol and phospholipid efflux to apoA-I are specific processes mediated by ABCA1 [21]. A recent study has shown that ABCA1 basal expression is virtually absent in Fu5AH and not sensitive to cAMP-mediated stimulation [17]. The lack of effect by cAMP stimulation is not surprising since

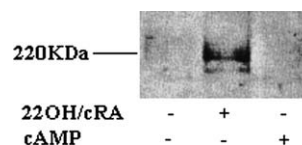


Fig. 1. ABCA1 protein content in Fu5AH. Cells were treated with DMEM + 0.2% BSA, cAMP 0.3mM or 22OH (5µg/ml)/cRA (10µM) overnight. Protein was isolated as described in Materials and methods; ABCA1 was detected with a rabbit primary antibody to ABCA1 and a secondary donkey antibody conjugated to horseradish peroxidase.

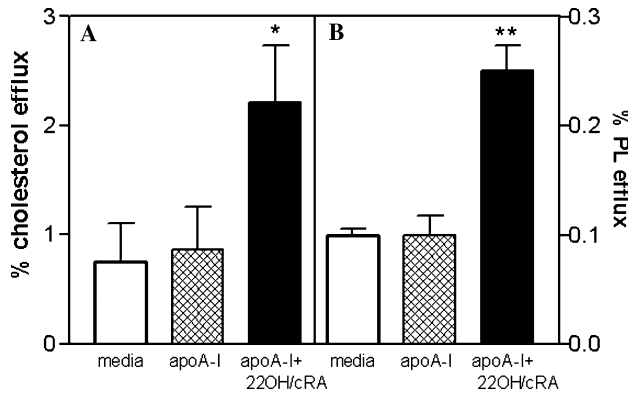


Fig. 2. (A) Cholesterol efflux to apoA-I from Fu5AH. Cells were radiolabeled and incubated in the presence or absence of 22OH (5  $\mu$ g/ml)/cRA (10  $\mu$ M) as described under Materials and methods. Efflux was promoted to apoA-I 20  $\mu$ g/ml for 4h and expressed as: cpm in medium/(cpm in the monolayer + cpm in medium)  $\times$  100. Data are expressed as means  $\pm$  SD ( $n = 3$ ). (B) Phospholipid efflux to apoA-I from Fu5AH. Cells were radiolabeled with 4  $\mu$ Ci/ml [methyl- $^3$ H]choline chloride for 48h and successively incubated in the presence or absence of 22OH 5  $\mu$ g/ml-cRA 10  $\mu$ M and apoA-I 20  $\mu$ g/ml. Phospholipid content in cell medium and monolayer was assessed after lipid extraction as described under Materials and methods. Efflux was calculated as: cpm in medium/(cpm in the monolayer + cpm in medium)  $\times$  100. Data are expressed as means  $\pm$  SD ( $n = 3$ ). \*\* $p < 0.01$ ; \* $p < 0.05$  compared to cells treated with apoA-I alone.

several studies have reported that ABCA1 upregulation is induced by cAMP only in murine macrophages; this suggests that second messenger pathways activated by cAMP are not operative in many cells [17,22]. The expression of functional ABCA1 and the ability to extensively modulate the level of protein expression make Fu5AH an appropriate cell model to investigate drug effects on hepatic ABCA1-mediated lipid efflux.

#### Effect of pitavastatin on lipid efflux from Fu5AH cells

Pitavastatin induces a dose-dependent increase in cholesterol efflux from Fu5AH cells to apoA-I, reaching the maximum effect at 10  $\mu$ M (Fig. 3, left panel). A more pronounced effect was observed in the presence of 22OH/cRA, where pitavastatin 50  $\mu$ M produced a 1.6-fold increase of cholesterol release compared to untreated cells (Fig. 3, right panel). Pitavastatin 10  $\mu$ M promoted also phospholipid efflux to apoA-I (1.5-fold increase;  $p < 0.05$ ). In Fu5AH cells exposed to compactin 50  $\mu$ M, cholesterol release in the extracellular medium increased about 1.5-fold compared to control, suggesting a class-related effect of statins.

Simultaneous incubation of pitavastatin 10  $\mu$ M with mevalonate 100  $\mu$ M or GGOH 10  $\mu$ M abolished the stimulatory effect of the statin both in the absence or in the presence of 22OH/cRA (Fig. 4). Our present results indicate for the first time that statins may induce lipid efflux in hepatic cells. A line of evidence indicates

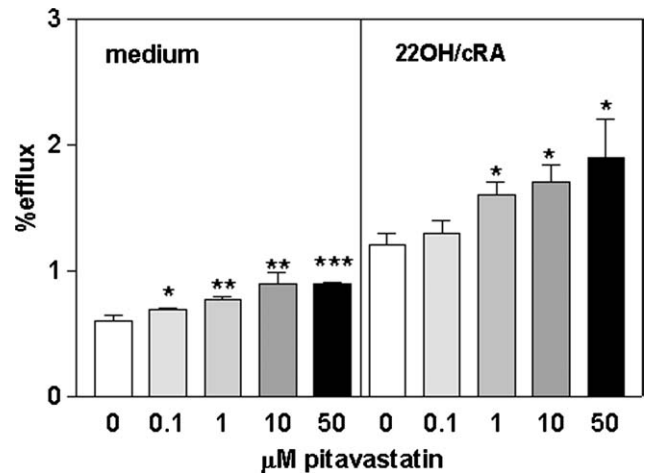


Fig. 3. Effect of pitavastatin on cholesterol efflux from Fu5AH. Cells were radiolabeled and treated with 22OH (5  $\mu$ g/ml)/cRA (10  $\mu$ M) or medium alone in presence of increasing concentrations of pitavastatin. Efflux was promoted to apoA-I 20  $\mu$ g/ml for 4h and expressed as: cpm in medium/(cpm in medium + cpm in monolayers)  $\times$  100. Data are expressed as means  $\pm$  SD ( $n = 3$ ). \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; and \* $p < 0.05$  compared to cells treated with apoA-I alone.

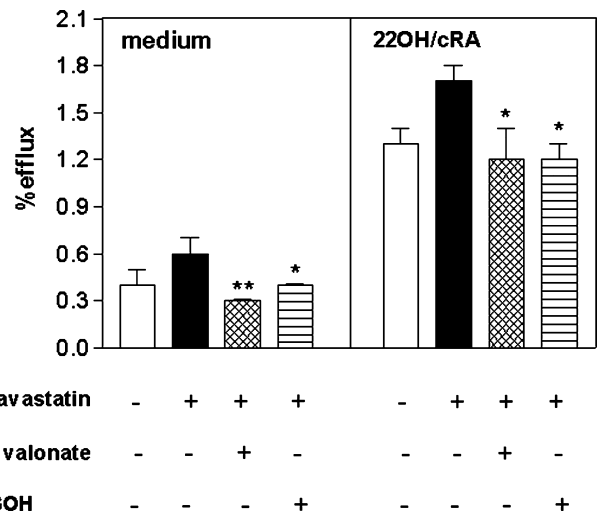


Fig. 4. Effect of mevalonate and geranyl geraniol on pitavastatin-induced increase in cholesterol efflux. Fu5AH were radiolabeled and treated with 22OH (5  $\mu$ g/ml)/cRA (10  $\mu$ M) or medium alone in presence or absence of pitavastatin 10  $\mu$ M, mevalonate 100  $\mu$ M, and GGOH 10  $\mu$ M. Efflux was promoted to apoA-I 20  $\mu$ g/ml for 4h and expressed as: cpm in medium/(cpm in monolayers + cpm in medium)  $\times$  100. Data are expressed as means  $\pm$  SD ( $n = 3$ ). \*\* $p < 0.01$ ; \* $p < 0.05$  compared to cells treated with pitavastatin alone.

that this effect is mediated by stimulation of the ABCA1 pathway: the increase of cholesterol efflux is observed in the presence of apoA-I, the specific ABCA1 ligand [17]. The stimulatory effect is observed also on phospholipid efflux, a process specifically mediated by this pathway [21]. Consistent with our conclusion, fluvastatin has been reported to increase the level of RNA for ABCA1 in HepG2 hepatoma cells [23]. As suggested by the

antagonism of mevalonate and geranyl geraniol, the mechanism of action of pitavastatin-induced lipid efflux involves an inhibitory effect of statins on HMG-CoA reductase and the isoprenoid pathway. It has been reported that geranylgeranyl pyrophosphate may reduce ABCA1 expression acting as an LXR inhibitor through two different mechanisms: directly as an LXR antagonist or indirectly by activation of the Rho GTP-binding proteins that in turn may change the activation status of LXR [24]. Moreover, statins may inhibit the Rho-signaling pathway and activate PPAR $\alpha$  [25]. Interestingly, PPAR $\alpha$  activation may stimulate the ABCA1 pathway via LXR [26]. Altogether these observations suggest that statins may stimulate ABCA1 expression and activity in hepatic cells by counteracting the inhibitory effect of the isoprenoid pathway on LXR activity. This conclusion is entirely consistent with our observation that Fu5AH cells may express a functional ABCA1 through LXR activation. Whatever the mechanism involved, our results indicate that the inhibitory effect of statins on HMG-CoA reductase is not only responsible for the increased expression of the LDL receptor in the liver and reduction of plasma LDL [27], but might also induce an increase of plasma HDL by stimulating the ABCA1-mediated cholesterol efflux.

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