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Short Communication

ABCA1 and *ABCG1* expressions are regulated by statins and ezetimibe in Caco-2 cells

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Abstract

Background: Enterocytes play a crucial role in high-density lipoprotein (HDL) biogenesis. Statins and ezetimibe are potent lowering-cholesterol drugs, which can also influence HDL plasma concentrations. We hypothesized that these drugs could modulate the expression of intestinal *ABCA1* and *ABCG1*, two genes involved in HDL metabolism.

Methods: Caco-2 cells were used as a model of the human intestinal cells and were treated with statins (0.01–1 μmol/L) and/or ezetimibe (0.5–5.0 μmol/L) for 12 h or 24 h. Gene expression was examined using real-time PCR.

Results: *ABCA1* level was more abundant than *ABCG1* in Caco-2 cells. *ABCA1* was downregulated after 12-h and 24-h treatment with atorvastatin (0.1 and 1.0 μmol/L) or simvastatin (0.01, 0.1 and 1 μmol/L) ($p<0.05$). In statin-treated cells, *ABCG1* levels remained unaltered. Ezetimibe alone did not induce change of *ABCA1* or *ABCG1* mRNA levels ($p>0.05$) but 24-h ezetimibe (2.5 or 5.0 μmol/L) plus simvastatin (1 μmol/L) treatment decreased the transcription of *ABCA1* and *ABCG1* ($p<0.05$).

Conclusions: Our findings reveal that, at the concentrations studied, statins isolated or combined with ezetimibe, but not ezetimibe alone, downregulate *ABCA1* mRNA expression in Caco-2 cells. Moreover, simvastatin combined with ezetimibe treatment also decrease the *ABCG1* levels in these cells.

Keywords: *ABCA1*; *ABCG1*; Caco-2 cells; lipid-lowering drugs; mRNA expression.

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Plasma cholesterol is derived from two main sources in the body: cholesterol biosynthesis (peripheral and hepatic) and intestinal cholesterol absorption; the relative contribution of cholesterol from these sources depends on various factors, including genetic predisposition, diet and drug therapies [reviewed in (1)]. Lipid-lowering drugs, such as statins and ezetimibe, are used to normalize serum lipids and to reduce risk of cardiovascular disease. Statins are widely prescribed for reduction of serum levels of low-density lipoprotein cholesterol (LDL-C) in both the primary and secondary prevention settings [reviewed in (2)]. They act by inhibiting of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and, thereby, suppressing the cholesterol biosynthesis. Ezetimibe is an effective agent for reducing LDL-C, either used alone or in combination with statins. Ezetimibe potently inhibits the absorption of biliary and dietary cholesterol from the small intestine by binding to the Niemann-Pick C1-like 1 (NPC1L1) transporter, which keeps cholesterol in the intestinal lumen for excretion [reviewed in (2)].

In addition to their LDL-C lowering action, statins and ezetimibe can also influence the metabolism and/or plasma concentrations of other lipoproteins, one of which being the high-density lipoprotein (HDL). The effects of these drugs on plasma HDL-cholesterol (HDL-C) have been assessed in several large clinical trials with variable outcomes: there was no change or modest increase or decrease in HDL-C serum level (3), [reviewed in (1) and (4)].

We have previously shown that lipid-lowering drugs can influence the expression of two important genes involved in the HDL metabolism, *ABCA1* and *ABCG1*, in a cell-specific manner (5). According to our findings, atorvastatin, simvastatin or ezetimibe treatment downregulated *ABCA1* and *ABCG1* mRNA expression in peripheral blood mononuclear cells of hypercholesterolemic individuals, even in the absence of changes in serum HDL-C or apolipoprotein AI (apoAI), the major protein of HDL. On the contrary, in *in vitro* study with human hepatocellular carcinoma (HepG2) cells, different concentrations of statin or ezetimibe treatments did not alter the *ABCA1* transcription but caused upregulation in the expression of *ABCG1*.

Here, we have examined the effects of these two common statins (atorvastatin and simvastatin) and the ezetimibe on *ABCA1* and *ABCG1* mRNA expression in enterocyte (human colorectal adenocarcinoma/Caco-2) cell line. The findings of the present study are relevant because the intestine is an important tissue to study HDL metabolism.

Briefly, Caco-2 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 44 mmol/L sodium bicarbonate, 10,000 µg/mL streptomycin and 10,000 UI/mL penicillin. Cells were grown at 37°C in a humidified atmosphere, containing 5% CO_2. Culture medium was replaced twice a week and cells were trypsinized and subcultured every 7 days.

Caco-2 cells were treated with atorvastatin (kindly provided by Pfizer Pharmaceuticals Ltd., Guarulhos, SP, Brazil), simvastatin (Sigma, St. Louis, MO, USA) or ezetimibe (kindly provided by Merck/Schering-Plough, NJ, USA) as previously reported (5, 6). For mRNA expression measurements, Caco-2 cells were seeded at a density of 1.0×10^6 cells per 75 cm^2, cultured for 3 days, and then treated with atorvastatin (0–1 µmol/L), simvastatin (0–1 µmol/L) and/or ezetimibe (0–5 µmol/L) for 12 h or 24 h. For atorvastatin and simvastatin, none of the concentrations tested (0–1.0 µmol/L) in Caco-2 cells induced a loss of cell viability or DNA fragmentation after 24-h treatment (data not shown). Cytotoxicity of ezetimibe was based on the results described elsewhere (7). *ABCA1* and *ABCG1* mRNA levels were measured by TaqMan® quantitative PCR (qPCR) assay using *HMBS* as the reference gene. Details about cytotoxicity assays, cell treatments, primer and probe sequences used for *ABCA1*, *ABCG1* and *HMBS* mRNA detection, qPCR assay and analyses of a relative amount of the transcript in the sample were previously described (5, 6).

In Caco-2 cells, baseline *ABCA1* level was higher than *ABCG1* (data not shown), and corroborating with this result, the same baseline expression profile was found in hamster enterocytes (8).

Regarding the lipid-lowering drug effects, as shown in Figure 1, we have observed that statins decreased *ABCA1* mRNA expression in a dose-independent manner ($p<0.05$), but ezetimibe alone did not induce a change of *ABCA1* mRNA levels in Caco-2 cells ($p>0.05$). Atorvastatin decreased *ABCA1* transcript levels after 12 h at 0.1 (0.55±0.06) and 1.0 µmol/L (0.58±0.04) compared with control (1.00±0.02, $p<0.01$). After 24-h treatment, *ABCA1* mRNA levels were significantly decreased also at 0.1 (0.64±0.08 vs. 1.00±0.01, $p<0.05$) and 1.0 µmol/L (0.58±0.10 vs. 1.00±0.01, $p<0.05$). Likewise, after simvastatin treatment, there was also a reduction in *ABCA1* level. This reduction was significant after 12-h and 24-h treatments at 0.01 (12 h: 0.59±0.02; 24 h: 0.62±0.04), 0.1 (12 h: 0.50±0.01; 24 h: 0.59±0.14) and 1.0 µmol/L (12 h: 0.54±0.06; 24 h: 0.55±0.15) compared with controls (12 h: 1.00±0.11; 24 h: 1.00±0.15, $p<0.05$) (Figure 1). Moreover, compared with the vehicle control, Caco-2 cells treated with 1.0 µmol/L of simvastatin plus 2.5 or 5.0 µmol/L of ezetimibe for 24 h also resulted in a decrease in the *ABCA1* transcription level ($p<0.05$, Figure 2).

In a study with hamsters treated with cholestyramine (bile acid sequestering resin) and lovastatin, intestinal *ABCA1* mRNA levels remained similar to those in non-treated controls (8). On the contrary, consistent with our finding, treatment with a mixture of pravastatin and colestimide (also a bile acid binding resin) lowered Abca1 mRNA and protein levels in the intestine of rats (9). This effect in rat intestinal tissue seems to

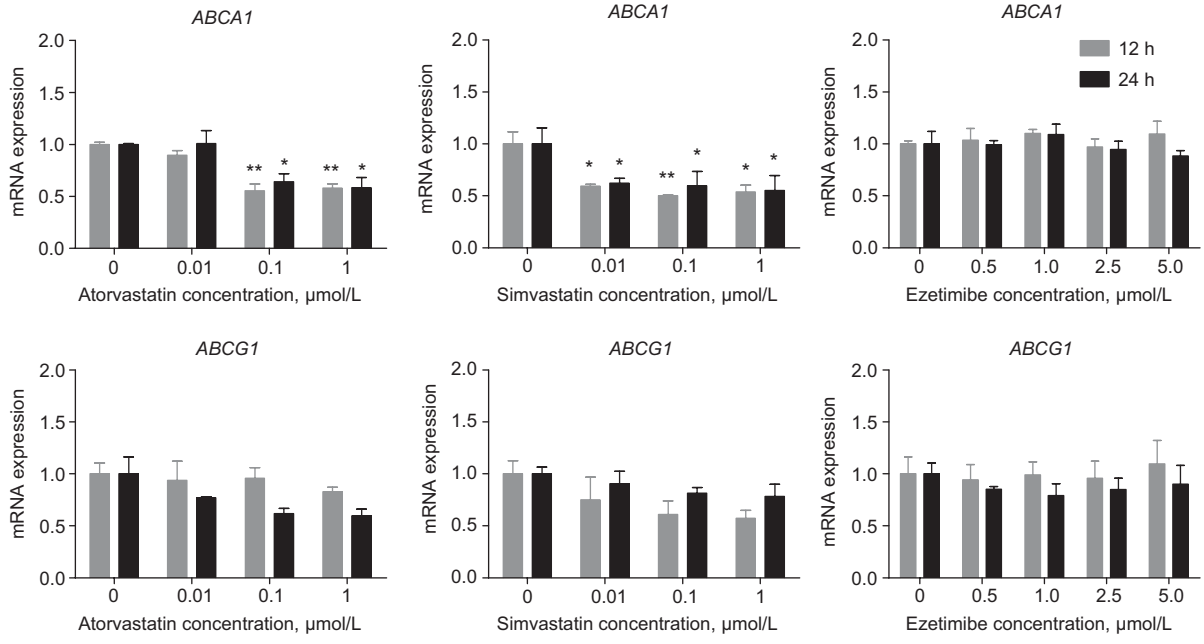

Figure 1 Effects of lipid-lowering drugs on *ABCA1* and *ABCG1* mRNA expression in Caco-2 cells.
Real-time quantitative PCR was performed using total RNA extracted from 12 h and 24 h atorvastatin, simvastatin or ezetimibe-treated and vehicle control (0 µmol/L) cells. The mRNA levels were normalized with *HMBS*. Values are reported as mean±SEM up to four independent experiments and expressed as relative value to control ($2^{-\Delta\Delta CT}$ formula was used). *$p<0.05$ and **$p<0.01$ as compared to 0 µmol/L statin as indicated by two-way analysis of variance (ANOVA) followed by Bonferroni post-tests.

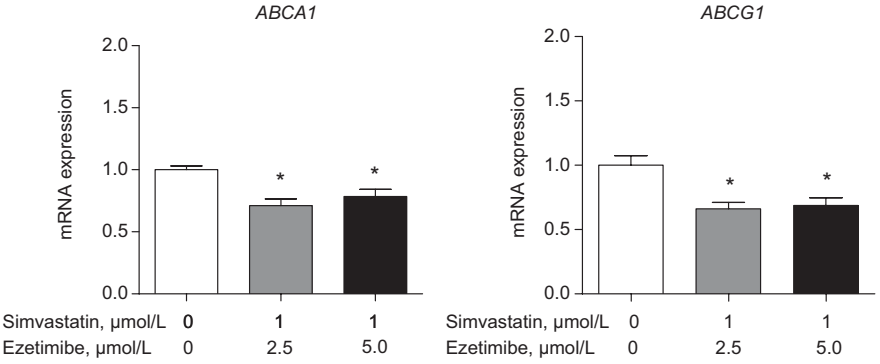

Figure 2 Effects of simvastatin and ezetimibe treatments on *ABCA1* and *ABCG1* mRNA expression in Caco-2 cells.
Real-time quantitative PCR was performed using total RNA extracted from 24 h simvastatin and ezetimibe-treated and vehicle control (0 µmol/L) cells. The mRNA levels were normalized with *HMBS*. Values are reported as mean±SEM up to four independent experiments and expressed as relative value to control ($2^{-\Delta\Delta CT}$ formula was used). *p<0.05 as compared to 0 µmol/L lipid-lowering drugs as indicated by one-way ANOVA followed by the Tukey test for multiple comparisons.

result from the modulation of a single major *Abca1* transcript, which is liver X receptor response element (LXRE)-driven (9). Because statins inhibit the cholesterol synthesis, they also limit the availability of oxysterols, the true ligand for LXR. Although we have not evaluated ABCA1 protein expression or activity, Field et al. (10) demonstrated that lovastatin did not alter ABCA1-mediated cholesterol efflux in 14-day differentiated Caco-2 cells incubated with LXR agonist, which maximizes the ABCA1 expression. These same authors also reported that ABCA1-mediated cholesterol efflux is independent of normal NPC1L1 function (10). Moreover, like us, they found that ezetimibe did not alter *ABCA1* transcription (10). Interestingly, in differentiated Caco-2 cells, disruption of the *NPC1L1* resulted in unchanged *ABCA1* mRNA levels (11).

On the contrary, During et al. (12) showed that the mRNA levels for *ABCA1* were significantly lower in 3-week-differentiated Caco-2 cells treated with ezetimibe than in control cells. There are some potential explanations for these different findings: the differentiation status of Caco-2 cells, the ezetimibe addition to the culture medium [During et al. (12) used the Zetia® tablet crushed and not the pure drug] and the cholesterol content into cell culture medium during the ezetimibe treatments. Whereas in Field et al. (10) and in our studies Caco-2 cells were maintained in DMEM supplemented with 10% fetal bovine serum, the same cells were maintained in the serum-free DMEM culture medium by During et al. (12). This last hypothesis is in agreement with previous observations showing that the treatment with SCH58053, an analog of ezetimibe, did not change *Abca1* mRNA level in enterocytes (jejunal mucosa) of mice fed with a high-cholesterol diet (1.0% w/w cholesterol), although SCH58053 treatment downregulated *Abca1* expression when mice are treated with a basal diet (0.02% w/w cholesterol) (13). Another important explanation for the differences observed between our results and those of During et al. (12) is the reference gene used in qPCR assay. In our experiments, *HMBS* was selected as stable for Caco-2 cells, according to the analysis by GeNorm software® (http://medgen.ugent.be/~jvdesomp/genorm/) [see details in (6)]. Nevertheless, During et al. (12) found that in cells treated with ezetimibe, the mRNA level for *HMBS* tended to be lower than in control cells (approx. 54% reduction).

It is important to note that the maximum concentration of ezetimibe used in our study (5 µmol/L) was lower than that of others, but it was a condition established by the pharmaceutical company that provided the pure drug (Merck/Schering-Plough) and it is close to the calculated pharmacologically active concentration (2.5 mg/L or approx. 6 µmol/L) that would be found in the small intestine of a human adult after the intake of the recommended dose (10 mg ezetimibe/day) (12). Likewise, the doses of statins used herein also closely relate to blood levels in patients treated with these drugs. Assuming complete absorption of the drugs, 10 mg of atorvastatin or simvastatin is estimated to result in blood levels of approximately 1.8 µmol/L (in a 70-kg man with a blood volume of 5 L) (14, 15).

It is known that ABCA1-mediated assembly of phospholipid and free cholesterol with apoAI plays an important role in HDL biogenesis, mainly in the liver and to a lesser extent in the small intestine (16). Actually, intestinal ABCA1 seems to contribute to approximately 30% of the circulating HDL-C (16). Although further studies are needed to confirm it, together, these results support human data showing that statins or ezetimibe either do not significantly alter or modestly change HDL-C or apoAI levels (3), [reviewed in (1) and (4)].

Unlike the *ABCA1* transcription, in Caco-2 cells, *ABCG1* was not modulated by atorvastatin (p=0.15), simvastatin (p=0.07) or ezetimibe (p=0.90) treatments (Figure 1). Although simvastatin treatment showed a trend to reduce *ABCG1* levels, this reduction, shown in Figure 1, was not significant. Nevertheless, interestingly, *ABCG1* mRNA expression was significantly lower in Caco-2 cells incubated with simvastatin (1.0 µmol/L) plus ezetimibe (2.5 or 5.0 µmol/L) for 24 h than in control cells (p<0.05, Figure 2). In three intestinal segments (duodenum, jejunum and ileum) from hamsters ingesting cholestyramine and lovastatin, expression of *ABCG1* was markedly decreased compared with control animals (8).

At present, despite increasing interest and extensive study, relatively little is known about the role of ABCG1. Several transcripts are known for human *ABCG1* and lipids have been characterized as the most effective transcriptional modulators of this gene (mainly by LXR activation) [reviewed in (17)]. Nevertheless, we do not know the regulation and the biological function of ABCG1 in enterocytes. A previous study suggested different functions of intestinal ABCG1 and ABCA1 (8) and it seems that ABCG1 has no role in lipid absorption (18). Therefore, further studies are needed to elucidate our findings in statin or ezetimibe-treated Caco-2 cells.

In summary, our findings reveal that, at the concentrations studied, statins isolated or combined with ezetimibe, but not ezetimibe alone, downregulate *ABCA1* mRNA expression in Caco-2 cells. Moreover, simvastatin combined with ezetimibe treatment also decrease the *ABCG1* levels in these cells.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.
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