

RESEARCH

Induction of the intestinal membrane transporters ABCA1 and ABCG8 by 27-hydroxycholesterol through a redox mechanism

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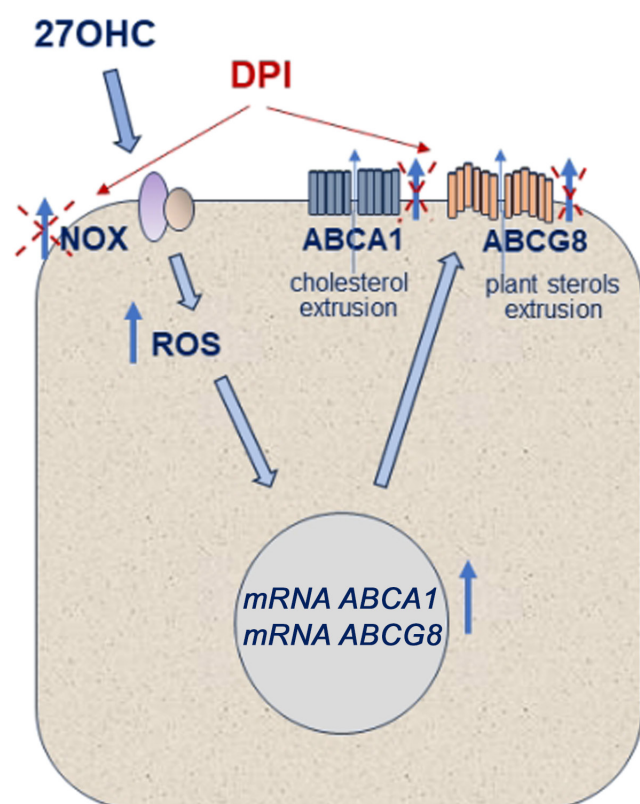
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Graphical abstract



Redox-induction of expression and synthesis of ATP binding cassette transporters in CaCo-2 cells by externally added 27-hydroxycholesterol.

27OHC: 27-hydroxycholesterol; NOX: NADPH oxidase; ROS: reactive oxygen species; ABCA1: ATP binding cassette A1; ABCG8: ATP binding cassette G8; DPI: diphenylene iodonium.

Abstract

Objective: We tested the effect of 27-hydroxycholesterol (27OHC) on the expression and synthesis of two membrane transporters involved in sterols extrusion from the intestinal epithelium into the gut lumen: ATP-binding cassette A1 (ABCA1) and G8 (ABCG8). Special attention was given to ABCG8, a key player in the intestinal cell discharge of plant sterols.

Methods: Differentiated CaCo-2 intestinal cells were supplemented with 27OHC, and added to the cell incubation medium at a final concentration of 1 or 5 μM . These 27OHC externally added amounts were proven to reach intracellular oxysterol levels within the range of those normally recovered in the human peripheral blood.

Results: An up-regulation of the *ABCA1* and *ABCG8* mRNAs was observed in the CaCo-2 cells supplemented with 27OHC. Moreover, both 1 μM and 5 μM 27OHC induced a net, and steady, statistically significant, increase of both ABCA1 and ABCG8 protein levels. Of interest, the cellular pre-treatment with diphenylene iodonium, a selective inhibitor of NADPH oxidase, i.e. a major intracellular source of reactive oxygen species, fully inhibited the 27OHC enhancement of both ABCA1 and ABCG8 protein synthesis.

Conclusion: This *in vitro* study shows for the first time that the addition of 27OHC to intestinal epithelial cells up-regulates ABCG8, the transporter discharging plant sterols into the gut lumen, besides confirming to induce ABCA1 as well. Importantly, the 27OHC-dependent up-regulation of the two transporters appears to involve a redox mechanism rather than the canonical liver-X-receptors-dependent pathway.

Significance statement

The 27OHC introduced with the diet might modulate the plant sterol extrusion in the gut, in parallel with that of cholesterol.

Keywords: 27-hydroxycholesterol; ABC transporters; ABCA1; ABCG8; CaCo-2 cells; oxysterols; plant sterols

Introduction

Of the broad family of oxysterols, cholesterol oxidation derivatives both of enzymatic and non-enzymatic origin (for a review see [Poli et al. 2022](#)), 27-hydroxycholesterol (27OHC) is by far the most represented one in human biology. In the peripheral blood of humans, this oxysterol is present in the range of 0.25–0.86 μM , i.e. 100–350 $\mu\text{g/L}$ ([Dzeletovic et al. 1995](#)), a blood concentration higher than that of vitamin D (30–100 $\mu\text{g/L}$) ([Holick 2009](#), [Sempos et al. 2018](#)) and of vitamin B12 (lower threshold 200 ng/L) ([Harrington 2019](#)). Notably, evolution has maintained relevant concentrations of 27OHC in mammalian milk, with an impressive peak of about 1 μM , i.e. 402 $\mu\text{g/L}$, in human colostrum ([Civra et al. 2019](#)).

27OHC can act as a potent agonist of liver X receptors (LXRs) ([Fu et al. 2001](#)), it modulates the expression of the estrogen receptors ([DuSell & McDonnell 2008](#)), peroxisome proliferator-activated receptors (PPARs), and toll-like receptors (TLRs) (for a review see [Vurusaner et al. 2016](#)), thereby being involved in diverse cell signaling pathways ([Vurusaner et al. 2016](#)). The LXRs orchestrate the up-regulation of genes pivotal for maintaining sterol equilibrium, including some genes coding for membrane transporters of the ATP-binding

cassette (ABC) family, in particular ABCA1 and ABCG1 ([Fu et al. 2001](#), [Edwards et al. 2002](#)), which are primarily involved in the ATP-dependent extrusion of cholesterol from the cells ([Ruiz et al. 2013](#)).

27OHC is well known to be a primary intermediate in the anti-atherogenic effect exerted by the synthetic sphingosine analog FTY720 in human primary monocyte-derived macrophages, through its up-regulation of the ABCA1 transporter ([Blom et al. 2010](#)). The up-regulation of ABCA1 expression by this oxysterol was also observed in the mouse brain cortex ([Wang et al. 2022](#)). Regarding the regulation of cholesterol efflux in the intestinal epithelium, rifampicin, a pregnane X receptor agonist, increased the level of 27OHC and the expression of both ABCA1 and ABCG1 in CaCo-2 and Ls174T intestinal cell lines ([Li et al. 2007](#)). The mechanism of action of 27OHC in inducing the two cholesterol-extruding pumps was always demonstrated or referred to as being dependent upon its activation of LXR-retinoid X receptor (RXR) heterodimers.

Beyond its function as an LXR ligand, 27OHC elicits responses that are indeed independent of LXR-RXR

activation, rather suggesting its involvement in redox-related pathways. These LXR-independent actions encompass pro-inflammatory effects, such as those observed in the CaCo-2 intestinal cell model, which is clearly mediated by an up-regulation of NADPH oxidase (NOX), a key source of intracellular generation of reactive oxygen species (ROS) (Mascia *et al.* 2010). The pro-fibrogenic effect of 27OHC on HSC-T6 hepatic stellate cells was also shown as well to be dependent on the higher ROS production induced by this oxysterol (Jiao *et al.* 2024).

In line with all the studies pointing to a pro-oxidant property of 27OHC, the up-regulation of ABCA1 and ABCG8 transporters exerted by this oxysterol in differentiated CaCo-2 epithelial cells seems to be redox-mediated. Moreover, while the increase of ABCA1 levels in CaCo-2 cells was previously described (Li *et al.* 2007), the up-regulation of ABCG8 by 27OHC has not been reported so far. Notably, while the ABCA1 and the ABCG1 extrude cholesterol out of cells, ABCG8 differs from other ABC family members because it facilitates the cellular extrusion of plant sterols back to the intestinal lumen (Ruiz *et al.* 2013), an action that underscores the physiological importance of this transporter. In fact, inactivating mutations of ABCG8 are the cause of sitosterolaemia, a rare genetic disease characterized by abnormal accumulation of plant sterols in the blood, increased LDL blood levels, and early atherosclerotic complications (Bydlowski & Levy 2024).

Materials and methods

Reagents

Unless otherwise indicated, all chemicals and reagents were from Sigma-Aldrich. DMEM with high glucose content, phenol red-free DMEM, fetal bovine serum (FBS), glutamine, and trypsin solution (5 g/L) were purchased from S.I.A.L. Srl (Rome, Italy). S.I.A.L. Srl also provided tissue culture flasks and six-well plates. 27OHC and deuterium-labeled 27OHC -25, 26, 26, 26, 27, 27-d₆ (d6-27OHC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

The protease inhibitors cocktail 'Complete ULTRA Tablets Mini EASYpacks' was from Roche SpA (Monza, Italy). The Bio-Rad protein assay dye reagent, the solution of 30% acrylamide/bis-acrylamide in mixture, 4–15% Mini-PROTEAN®TGX™ Precast Protein Gels (#4561086), and the enhanced chemiluminescence (ECL)® Western Blotting System were from Bio-Rad Srl. Santa Cruz Biotechnology (DBA Italia Srl, Segrate, Milan, Italy) provided mouse anti-ABCA1 (SC-58219) and rabbit anti-ABCG8 (SC-30111) polyclonal primary antibodies. Anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibody (7076S) and anti-rabbit IgG HRP-conjugated secondary antibody (7074S)

were purchased from Cell Signaling Technology (Euroclone SpA, Milan, Italy). Thermo Fisher Scientific (Life Technologies Italia, Monza, Italy) supplied the 10× dithiothreitol (DTT) reducing agent and the 4× lithium dodecyl sulfate (LDS) solution. Hybond ECL nitrocellulose membrane was obtained from GE Healthcare Srl.

A Milli-Q filter ultrapure water system Millipore (Milan, Italy) was used for dilutions. TRIzol reagent and SUPERase-In RNase inhibitor were from Invitrogen (S. Giuliano Milanese, Italy). Applied Biosystems provided the High-Capacity Complementary DNA (cDNA) reverse transcription kit, TaqMan gene expression assay kits for human ABCA1, ABCG8, and β-actin, TaqMan Fast Universal PCR master mix, and TaqMan Array 96-well plates.

Cell culture and treatments

The human colorectal adenocarcinoma CaCo-2 cell line was supplied by the Cell Bank Interlab Cell Line Collection (Genoa, Italy). Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS (v/v), 1% antibiotic/antimycotic solution (v/v) (100 U/mL penicillin, 0.1 mg/mL streptomycin, 250 ng/mL amphotericin B, and 0.04 mg/mL gentamicin), and 1% of glutamine (v/v) at 37 °C in a humidified atmosphere containing 5% CO₂. For treatments in the presence or absence of 27OHC, CaCo-2 cells were plated at 1 × 10⁶/mL density and cultured until reaching 100% confluence. Once confluent, cells were grown for an additional 18 days to allow their spontaneous differentiation into an enterocyte-like phenotype (Biasi *et al.* 2009). Before each experiment, differentiated CaCo-2 cells were incubated overnight in serum-free medium to make them quiescent and then placed in 1% FBS (v/v) phenol red-free DMEM, in six-well plates (3 × 10⁶ cells each well).

The supplementation with 27OHC was achieved by adding a single amount of this oxysterol to each well to reach a final concentration of 1 μM or 5 μM. Then, an appropriate number of cell aliquots were incubated at 37°C for various times, depending on the different analyses to be performed. Up to 12 h of incubation to quantify the amount of externally added 27OHC indeed recovered inside the cells. For 1, 2, 4, 6, and 8 h incubation, to measure the cellular levels of *ABCA1* and *ABCG8* mRNA were measured. For 24, 48, 72, and 96 h to measure the cellular levels of *ABCA1* and *ABCG8* protein. For 24 and 48 h in the experiments of pre-treatment with diphenylene iodonium (DPI), a selective inhibitor of NADPH oxidase was used. Aliquots of CaCo-2 to which an identical amount of ethanol was added to the incubation medium instead of 27OHC, underwent all experimental conditions, acting as an internal control. In the additional set of experiments with DPI, this drug was added to the cell cultures at the final concentration of 2 μM, 30 min

before the incubation in the presence or absence of 27OHC. DPI remained in the cell medium throughout the entire duration of the experiment.

Quantification of 27-hydroxycholesterol by isotope dilution GC-MS

At time 0 and after 12 h of incubation at 37°C, aliquots of 3×10^6 cells were added to vials filled with 500 ng 27OHC -25, 26, 26, 27, 27- d_6 (d6-27OHC) as internal standards, 50 μ L of butylated hydroxytoluene (BHT, 5g/L), 50 μ L of K3-EDTA (10 g/L) to prevent auto-oxidation, ethanol, and KOH 0.25 mol/L. The vials were then sealed with a Teflon septum. Each vial was flushed with argon for 10 min to remove air. Hydrolysis was carried out at room temperature, and then sterols and oxysterols were extracted twice with 5 mL of hexane. The two phases were evaporated under a nitrogen stream and derivatized with BSTFA+1% TCS at 70°C before being injected into GC-MS. Analysis was performed by GC-MS with an AB-XLB column (30 m $\times$ 0.25 mm i.d. $\times$ 0.25 μ m film thickness, J&W Scientific Alltech, Folsom, CA, USA) in an HP 6890N Network GC system (Agilent Technologies) connected with a direct capillary inlet system to a quadrupole mass selective detector HP5975B inert MSD (Agilent Technologies). The GC system was equipped with HP 7687 series autosamplers and HP 7683 series injectors (Agilent Technologies). The oven temperature program was as follows: an initial temperature of 180°C was held for 1 min, followed by a linear ramp of 20°C/min to 270°C, and then a linear ramp of 5°C/min to 290°C, which was held for 11 min. Helium was used as the carrier gas at a flow rate of 1 mL/min, and 1 μ L of sample was injected in splitless mode. Injection was carried out at 250°C with a flow rate of 20 mL/min. The transfer line temperature was 290°C. Filament temperature was set at 150°C, and quadrupole temperature at 220°C according to the manufacturer indication. Mass spectrometric data were acquired in selected ion monitoring mode for 27-hydroxycholesterol - d_6 and m/z =456 for 27OHC (Avanti Polar Lipids Inc. USA, SKU: 700021P). Peak integration was performed manually, and oxysterols were quantified from selected-ion monitoring analysis against internal standards using standard curves for the listed sterols. Recovery was 99% and precision 3% (Leoni et al. 2017, Staurengi et al. 2022).

Immunoblotting

At the end of each treatment, differentiated CaCo-2 cells (3×10^6 each plate well) were washed with ice-cold PBS 1 $\times$ and scraped. For protein extraction, 150 μ L of lysis buffer (PBS 1 $\times$ supplemented with 1% Triton X-100 (v/v), 1% sodium dodecyl sulfate (w/v) (final volume)) was added to each sample. Lysates were incubated for 30 min on ice and centrifuged at 12,052 g at 4°C for

15 min. Bio-Rad protein assay dye reagent was used for the evaluation of total cell extract protein concentration, following the protocol published by Bradford (1976). Samples containing 50 μ g of total proteins were boiled at 100°C for 5 min in the sample buffer (LDS Sample Buffer 4 $\times$ and DTT Sample Reducer 10 $\times$). Boiled samples were subjected to electrophoresis separation using 4–15% polyacrylamide gels, and subsequently, proteins were transferred to Hybond ECL nitrocellulose membranes. Saturation of nonspecific binding sites was performed at room temperature (RT) for 1 h with TTBS blocking buffer (TTBS: tris-buffered saline (TBS) supplemented with 0.05% (v/v) Tween 20) plus 5% (w/v) skimmed milk powder (final volume). At this time, the membranes were incubated overnight at 4°C with mouse anti-ABCA1 (1:250 dilution) or rabbit anti-ABCG8 (1:250 dilution) polyclonal primary antibodies in TBS containing 0.1% Tween 20 (v/v) and 5% skimmed milk powder (w/v). Three washes in TTBS were made and blots were incubated with anti-mouse IgG or anti-rabbit IgG HRP-conjugated secondary antibody (1:1000 dilutions) in TBS with 0.1% Tween 20 (v/v) and 5% skimmed milk powder (w/v) for 1 h at RT. In conclusion, blots were washed twice in TTBS for 10 min. Chemiluminescence was detected using the Clarity Western ECL kit and the ChemiDoc™ Touch Imaging System machine (Bio-Rad Laboratories). Image J Software (Bethesda, MD, USA) was used to quantify protein band densities.

Real-time quantitative RT-PCR

Total RNA was extracted from differentiated CaCo-2 cells (3×10^6 each plate well) with 1 mL TRIzol™ reagent following the manufacturer's instructions and then dissolved in RNase-free water RNase free with RNase inhibitors. RNA extract concentrations were detected by UV spectrophotometry measuring ribonucleic acid absorbance at 260 nm. cDNA was synthesized by the reverse transcription of 2 μ g of RNA using a commercial kit and random primers. Real-time quantitative RT-PCR (qRT-PCR) was performed on 30 ng cDNA with TaqMan gene expression probes for ABCA1, ABCG8, and β -actin, TaqMan Fast Universal PCR Master Mix, and a 7500 Fast Real-Time PCR System (Applied Biosystems, Thermo Fisher). The oligonucleotide sequences are not revealed by the manufacturer because of proprietary interests. PCR's cycling parameters were: 40 cycles of 3 s each at 95°C (melting) and 30 s at 60°C (annealing/extension). Results were normalized to the expression of β -actin, taken as the housekeeping gene. Target gene expression was quantified following Livak and Schmittgen protocol (Livak & Schmittgen 2001).

Statistical analysis

Results are reported as mean $\pm$ s.d. and data were analyzed with GraphPad InStat software. The statistical

differences among experimental data were evaluated by using the one-way ANOVA test associated with Bonferroni's multiple comparison post-test.

Results

Measured internalization of externally added 27OHC in differentiated CaCo-2 intestinal cells

In the first set of experiments, 27OHC was added to the incubation medium of CaCo-2 cells at final concentrations of 1 μ M or 5 μ M, and the amount of the oxysterol recovered within the cells was checked after 12 h of cell incubation at 37°C using GC-MS. The incubation time of 12 h to evaluate the actual amount of 27OHC internalized by the intestinal cells was selected because it is generally considered a convenient time to start checking protein synthesis in a cell line model system. After 12 h from the single addition to the cell medium of 1 μ M 27OHC (402 ng/mL), the internalized oxysterol amount was about 69 ng/mL, which is 1/6 of the added amount. In the case of 5 μ M 27OHC (2010 ng/mL) cell supplementation, the oxysterol's amount recovered inside the cells at 12 h was about 275 ng/mL, i.e. about 1/7 of the added one.

Effect of differentiated CaCo-2 cells supplementation with 1 μ M or 5 μ M 27OHC on *ABCA1* and *ABCG8* mRNA levels

As reported in Fig. 1A, the CaCo-2 cell supplementation with 1 μ M and 5 μ M of 27OHC appeared to increase the mRNA expression of *ABCA1*, checked up to 8 h of cell incubation, by about 1.5–2 times and 3–3.5 times, respectively. However, only the mRNA boost exerted by 5 μ M 27OHC was statistically significant.

As for *ABCG8*, while the mRNA expression was enhanced up to a maximum of 1.5 times in 1 μ M 27OHC-treated cells, it was amplified by four times in 5 μ M 27OHC-challenged cells (Fig. 1B). In this case as well, only the mRNA elevation provoked by 5 μ M 27OHC resulted in a statistically significant.

Increased levels of *ABCA1* and *ABCG8* in CaCo-2 cells supplemented with 1 μ M and 5 μ M 27OHC

The Western blotting quantification of cellular levels of the two membrane transporters was then carried out every 24 h up to 96 h from the 27OHC initial single supplementation. Both 1 μ M and 5 μ M 27OHC-supplemented CaCo-2 cells showed a steady and significant increase in both *ABCA1* and *ABCG8* protein levels throughout the whole entire analyzed time frame, namely up to 96 h of incubation (Fig. 2). At least in the set of experiments performed in this study, the

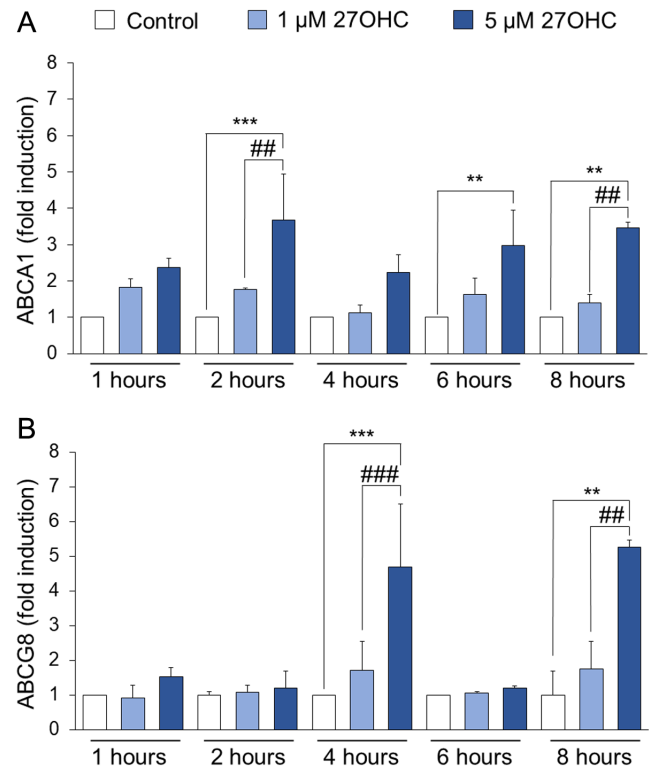


Figure 1

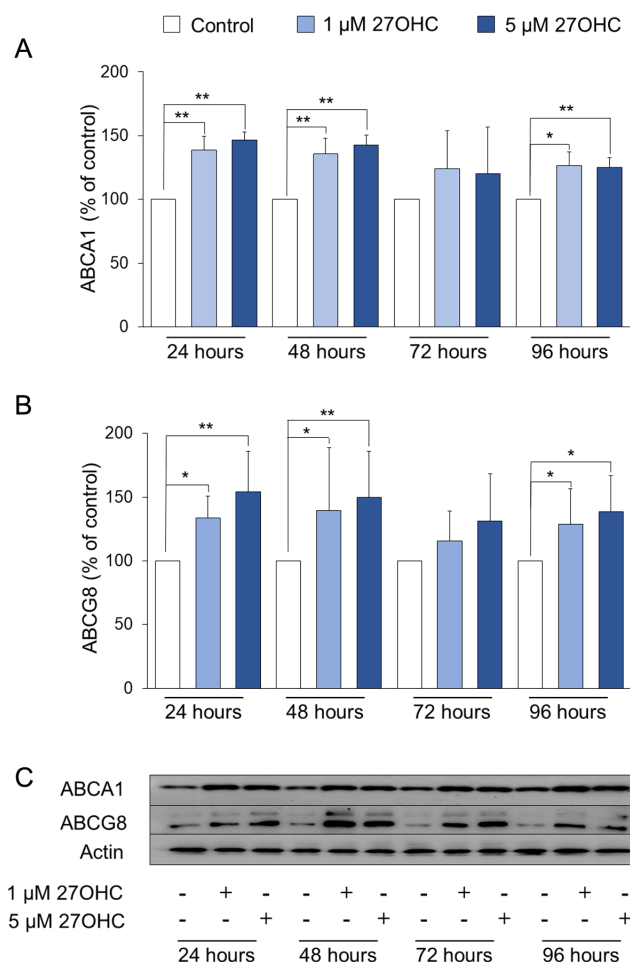
27OHC-induced *ABCA1* and *ABCG8* gene expression. *ABCA1* and *ABCG8* gene expression was evaluated through RT-PCR in differentiated CaCo-2 cells treated with 1 μ M 27OHC or 5 μ M 27OHC for 1, 2, 4, 6, and 8 h. Control: untreated cells. Data are indicated as means $\pm$ s.d. of three independent experiments. Significantly different vs control: ** P < 0.01, *** P < 0.001. Significantly different vs 1 μ M 27OHC: ## P < 0.01, ### P < 0.001.

increment of *ABCA1* and *ABCG8* protein levels exerted by the two 27OHC concentrations did not statistically differ from each other, i.e. a dose-dependent effect was not observed.

The inhibition of NADPH oxidase-dependent ROS generation prevents the enhancement of *ABCA1* and *ABCG8* cell protein levels due to 27OHC

The possible involvement of increased production of ROS in the 27OHC-induced rise of transporters *ABCA1* and *ABCG8* synthesis was tested. Prior to treatment with the oxysterol (1 and 5 μ M), differentiated CaCo-2 cells were incubated for 30 min in the presence of 2 μ M DPI, a selective inhibitor of NADPH oxidase (Buck et al. 2019), the latter being recognized as one of the two main intracellular sources of ROS up-regulated by 27OHC, the other one being the mitochondria (Vurusaner et al. 2014).

As reported in Fig. 3, pre-incubation of differentiated CaCo-2 cells with DPI completely prevented the 27OHC-provoked increase in *ABCA1* and *ABCG8* synthesis,

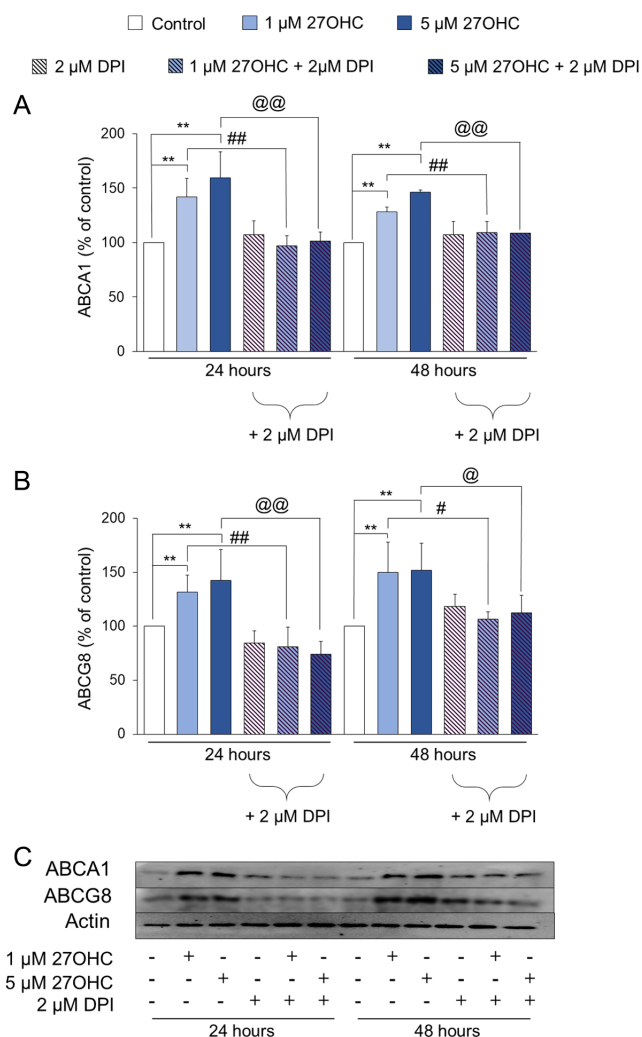
**Figure 2**

Increased ABCA1 and ABCG8 protein levels in differentiated CaCo-2 cells treated with 27OHC. Protein levels of ABCA1 and ABCG8 were detected by Western blotting in lysates from differentiated CaCo-2 cells treated with 1 μ M 27-OHC or 5 μ M 27OHC for 24, 48, 72, and 96 h. Control: untreated cells. Data are expressed as a percentage of control (taken as 100%). Values are indicated as means $\pm$ s.d. of three independent experiments. Significantly different vs control: * P < 0.05, ** P < 0.01.

observed both after 24 and 48 hours of challenge with the oxysterol. The prior addition of DPI to CaCo-2 cells, followed by 24 or 48 h of incubation in the absence of 27OHC, did not affect either cell viability (data not shown) or the normal concentration of the two membrane transporters under study (Fig. 3).

Discussion

The role of the two membrane transporters of the ABC family, ABCA1 and ABCG1, in the extrusion of cholesterol from the intestinal epithelial cell layer back to the intestinal lumen is fully recognized. Much less studied is another component of the same family of transporters, ABCG8, which is instead in charge of the

**Figure 3**

CaCo-2 cell pre-incubation with DPI inhibits the rise of ABCA1 and ABCG8 protein levels induced by 27OHC treatment. ABCA1 and ABCG8 cell protein levels were detected by Western blotting in lysates from differentiated CaCo-2 cells pre-treated for half an hour with 2 μ M DPI and then treated with 1 μ M 27OHC or 5 μ M 27OHC for 24 and 48 h. Control: untreated cells. Data are expressed as a percentage of control (taken as 100%). Values are indicated as means $\pm$ s.d. of three independent experiments. Significantly different vs control: ** P < 0.01. Significantly different vs 1 μ M 27OHC: # P < 0.05, ## P < 0.01. Significantly different vs 5 μ M 27OHC: @ P < 0.05, @@ P < 0.01.

extrusion of the plant sterols absorbed in the intestine back to the gut lumen (Ruiz et al. 2013). In fact, a defect of ABCG8 at the intestinal level would allow a dangerous increase of phytosterols in the peripheral blood. The latter event would exert a competitive interference with LDL for the receptor that mediates this lipoprotein removal from the bloodstream, with consequent rise in the hematic level of those lipoproteins (Bydlowski & Levy 2024).

While the 27OHC-mediated up-regulation of expression and levels of the ABCA1 and ABCG1 transporters has

been proven to occur in different cells, including *in vitro* cultivated human intestinal cells (Li et al. 2007), none have reported yet the enhancement of mRNA expression and synthesis of ABCG8 as induced in intestinal cells of human origin (differentiated CaCo-2 cells) by oxysterols. A reasonable explanation is that the cholesterol transport studies so far focused on those epithelial membrane pumps chiefly involved in the intestinal partial extrusion of the animal sterol. The remaining intracellular amount of absorbed cholesterol becomes included, with the dietary oxysterols, in the HDL and VLDL synthesized in the gut epithelial layer, then conveyed in the blood circulation (Poli et al. 2022).

Only a limited amount, about 1/6, of the two doses of 27OHC added to differentiated CaCo-2 cells in culture, 1 μ M (402 ng/mL) and 5 μ M (2010 ng/L), has been recovered within the cells, namely 69 and 275 ng/mL, respectively. Such an intracellular amount of 27OHC fits well within the physiological hematic concentration range of this oxysterol in humans, i.e. 100–350 μ g/L (Dzeletovic et al. 1995, Marcello et al. 2020). The limited 27OHC uptake by differentiated CaCo-2 cells was consistent with the modest bioavailability observed in previous unpublished *in vitro* measurements and the kinetics of this oxysterol recently afforded *in vivo* in a mouse model (Leoni et al. 2022).

The fact that the intracellular 27OHC concentration achieved by the performed treatment was mimicking the oxysterol's physiological plasma content should give more strength to the demonstrated up-regulation of expression and synthesis of ABCG8 together with that of ABCA1 in differentiated CaCo-2 cells supplemented with 27OHC (Figs. 2 and 3).

Besides the 27OHC-inducible enhancement of ABCG8, the plant sterol transporter essentially expressed in the intestinal epithelium, another original finding of the present study was the prevention of the 27OHC-exerted up-regulation of both ABCG8 and ABCA1 transporters by CaCo-2 cell pre-treatment with the NADPH oxidase selective inhibitor DPI (Fig. 3). This selective inhibitor was already shown by our group to markedly decrease the NADPH oxidase level in differentiated CaCo-2 cells challenged with a mixture of oxysterols, and consequently quench the rise of ROS intracellular levels provoked by the oxy-mixture itself (Biasi et al. 2009). Moreover, DPI was proved to strongly inhibit NOXs and, while recognizing more than one target, to display the great advantage to react and block only the activated flavoenzyme (Reis et al. 2020). On the other hand, being DPI a broad NOX inhibitor, it was not possible at this stage to understand which NOX isoform was activated by 27OHC.

Indeed, the preservation of ABCG8 and ABCA1 levels achieved by CaCo-2 cell pre-treatment with DPI seemed to be mediated by the drug-dependent quenching/inhibition of the pro-oxidant effect of 27OHC and not by its canonical activation of the LXR–RXR pathway.

In conclusion, a redox hypothesis is presented regarding the up-regulation of ABCG8, ABCA1, and possibly of other ABC family members inducible by 27-hydroxycholesterol through its pro-oxidant properties rather than by binding to the LXR–RXR receptor complex. The findings reported here, obtained with small-size experimental groups and in an *in vitro* model, certainly need to be supplemented by further experiments that focus on the redox signaling pathways triggered by 27OHC to up-regulate ABCA1 and ABCG8 levels, and possibly consider other oxysterols, like 7 β -hydroxycholesterol, 7-ketocholesterol, and 25-hydroxycholesterol. Not least of all, the interesting enhancement of the plant sterol transport observed as exerted by relatively low amounts of 27OHC in the CaCo-2 cell line should be further examined by means of tissue-engineered cell-based *in vitro* models.

Declaration of interest

FB, VL, and GP are members of the Editorial Board of Redox Experimental Medicine and were not involved in the review or editorial process for this paper, on which they are listed as authors. The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the study reported.

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Author contribution statement

FB and GP conceived and supervised the overall study; GP and NI wrote the manuscript; NI performed all the molecular biology experiments; VL performed the 27OHC quantitative measurements and provided, together with NI, significant contribution to the discussion of the results.

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