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Expression of ATP binding cassette-transporter ABCG1 prevents cell death by transporting cytotoxic 7β-hydroxycholesterol

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Abstract Oxysterols result from cholesterol by enzymatic or oxidative processes. Some exert cytotoxic effects leading to necrosis or apoptosis. Detoxification of these compounds mainly occurs in the liver and requires transport from peripheral tissues towards it. Some ATP-binding cassette transporters are involved in export of cytotoxic compounds. In the current study, we investigated whether ABC transporter family member G1 (ABCG1) may be involved in oxysterol transport, since its gene expression is highly responsive to oxysterol loading. TetOff HeLa cells stably expressing ABCG1 showed decreased mass uptake of 7β-hydroxycholesterol (7β-HC) whereas that of other physiologically relevant oxysterols was unaffected. Application of 7β-HC to ABCG1 expressing cells induced hyperpolarization of mitochondrial membrane potential and production of reactive oxygen species, indicating energy consumption by the ATP-binding cassette transporter when it is activated by its correct substrate. Our study points to detoxification as one of potential cellular functions of ABCG1. We assume that ABCG1 protects against 7β-HC-induced cell death, an important role in prevention of neurodegenerative and cardiovascular disease.

Keywords: ATP-binding cassette transporter; ABCG1; 7β-Hydroxycholesterol; Cholesterol; Substrate

1. Introduction

Oxysterols are biologically active products of cholesterol and are present at high levels in macrophages of the atherosclerotic plaque or in the brain of patients with neurodegenerative diseases [1–4]. Various oxysterols repress the pacemaker enzyme of cholesterol biosynthesis, HMGCoA-reductase [5–7]. In addition, hydroxycholesterol (HC) derivatives oxidized in the side chain activate the transcription factor liver-X-receptor (LXR) [8]. Oxysterol formation occurs specifically by enzymatic conversion of cholesterol during bile acid and steroid hormone synthesis (7α-HC) or when excess cholesterol in the brain (24-HC) or in macrophages (27-HC) is targeted for export and subsequent degradation [1,2]. Contact with peroxides, reactive nitric oxide species, or superoxide-radicals results in non-specific oxidation of cholesterol and produces 7β-HC, 7-ketocholesterol, 25-HC, and 5,6-epoxides as the major products [1,4]. HC derivatives are produced when mitochondria hyperpolarize or peroxisomes are highly active under conditions of cell stress [9,10] and show cytotoxic effects. For instance, 7β-HC was shown to efficiently induce cell apoptosis and necrosis in U937 monocytes [11] by destabilizing membranes leading to lysis of mitochondria and lysosomes [9,12,13]. For this reason, oxysterols have to be effectively removed from cells, but mechanisms underlying their transport are poorly understood.

ATP-binding cassette transporters are involved in energy dependent translocation of various substrates including sugars, lipids, peptides and xenobiotics across membranes [14]. Family member 1 of the G-subgroup of mammalian ABC transporters (ABCG1) is thought to be involved in cellular export of cholesterol and sphingomyelin [15,16]. However, in the ABCG1 deficient mouse model no changes in plasma cholesterol levels were observed [15], and a study using ABCG1-deficient mice in the homogenous genetic background of LDL-receptor deficiency attributed a moderately anti-atherogenic role to ABCG1 already at relatively low plasma cholesterol levels [17]. Another study using an independently generated ABCG1-deficient mouse model suggested involvement of ABCG1 in the development of obesity [18].

Since ABCG1's gene expression is highly responsive to oxysterol treatment, we argued that the transporter may be involved in cellular export of its inducers. In this respect, it may have a function comparable to its family member ABCG2 which is involved in export of cytotoxic xenobiotics, e.g. used in chemotherapy against breast cancer [19]. Other ABC transporters such as multidrug resistance protein (MDR, ABCB1), MDR-related protein 1 (MRP1, ABCC1), and the obligate heterodimeric ABCG5/ABCG8 complex are also involved in clearance of toxic compounds from cells [15,20,21]. Bile acids can counteract the activation of ABCG1 gene expression induced by oxysterols [22].

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Abbreviations: ABC, ATP-binding cassette; ABCA1, ABC transporter family member A1; ABCG1, ABC transporter family member G1; DHE, dihydroethidium; HA, heamagglutinin; HC, hydroxycholesterol; LXR, liver-X-receptor; RXR, retinoid-X-receptor; TMRE, tetramethylrhodamine ethyl ester

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In the current study, we used our stably expressing ABCG1 cell lines [16] to analyze involvement of ABCG1 in transport of specific oxysterols. We identified a ring-substituted oxysterol as its substrate which indicates that ABCG1 is involved in cellular detoxification rather than in cholesterol export. Our results may give new insights into prevention of pathologic conditions involving oxysterols, namely neurodegenerative diseases and atherosclerosis.

2. Materials and methods

2.1. Reagents

Anti-α-tubulin antibody was obtained from Sigma–Aldrich (Deisenhofen, Germany), anti-heamagglutinin (HA) antibody, as well as LXR and retinoid-X-receptor (RXR) agonists, as previously described [16]. Avasimibe was a kind gift of Parke–Davis. Tetramethylrhodamine ethyl ester (TMRE) and dihydroethidium (DHE) were purchased from Molecular Probes (Eugene, OR, USA). Reagents for lipid analysis were of analytical grade and were supplied by Sigma–Aldrich except water and organic solvents (HPLC-grade; Mallinckrodt Baker, Griesheim, Germany).

2.2. Cells

TetOff-HeLa cell lines stably cotransfected with pTK-Hyg and pTRE expression plasmids (Clontech) containing wt (clone 94) or mutant (clone 47) $(HA)_3$-ABCG1(666) [16] were maintained and used in experiments, as previously described [16].

2.3. Western blot analysis

Samples were analyzed as previously described [16]. Filters were cut and developed with the anti-α-tubulin antibody or the anti-HA antibody. α-Tubulin migrated at 50 kDa, $(HA)_3$-ABCG1 at ~70 kDa.

2.4. FACS measurement

In case of the TetOff HeLa cell lines 1×10^5 cells were seeded per well of a 24-well plate with 1 μg or 100 pg doxycycline per ml medium. Thirty hours later medium was replaced with fresh medium supplemented with identical concentration of doxycycline plus oxysterols, as indicated in the figure legends. Twelve hours later cells were incubated with 20 nM TMRE for 20 min at room temperature, or 10 μM DHE at 37 °C for 15 min [11]. Cells were detached from the plate, pelleted, resuspended in PBS, and analyzed employing a FACScan flow cytometer equipped with Lysis II software (Becton-Dickinson, Heidelberg, FRG). Subconfluent RAW264.7 macrophages were seeded at a density of 1.5×10^5 cells per well in complete medium with or without 1 μM LXR and 0.1 μM RXR agonists. After 24 h medium was replaced with DMEM supplemented with antibiotics, with or without LXR and RXR agonists, respectively, 0.2% bovine serum albumin, 5 μM acyl-CoA acyl transferase (ACAT) inhibitor avasimibe and increasing concentration of 7β-HC. After 24 h cells were analyzed for TMRE fluorescence, as describe above for the HeLa cells. Cells within the TMRE negative region were regarded as dead, those in the TMRE-positive region as live. Triplicate samples were analyzed.

2.5. Quantitative RT-PCR measurement

HeLa cells (3×10^6) were seeded in 6 cm petri dishes in complete medium supplemented with the respective concentration of doxycycline. Twenty-four hours later medium was replaced with fresh one plus the respective concentration of doxycycline and cells were incubated for further 16 h. Cells were harvested, RNA was extracted, reverse transcribed, and quantitative PCR was performed as previously described [23] except SuperScriptIII (Invitrogen, Karlsruhe, FRG) was used. Primers for detection of endogenous and exogenous ABCG1 at the same time were: 5′-CTG GCT GGA TAC AGG GAG AC-3′; 5′-CCT GCA TGA TGT AGC AGG AC-3′; those for mouse ABCG1 transcript: 5′-CAC GCA AGT CTG ACG ATG AC-3′; 5′-CCA ACC GTC CTA CAT CTT CC-3′.

2.6. Preparation of lipid emulsion

Fifty milligrams of egg yolk phosphatidylcholine (PC) in chloroform (Sigma) and 50 μg of individual oxysterol, respectively, in methanol were mixed together in a conical glass tube. Solvent was evaporated under a stream of nitrogen gas and 25 ml of DMEM was added. Mixture was vigorously vortexed and subsequently sonicated with a Sonopuls GM70 (Bandelin electronic, Berlin, FRG) until a transluscent solution was obtained. Emulsion was centrifuged for 5 min at $580 \times g$ and supernatant fraction supplemented with 0.1% bovine serum albumin was used in the experiment.

2.7. Lipid analysis

Cells (3×10^6) were seeded in 6 cm petri dishes in complete medium supplemented with the respective concentration of doxycycline. After 24 h complete medium was replaced and supplemented again with fresh doxycycline. Sixteen hours later cells were washed twice with DMEM/PSG and were incubated in DMEM/PSG for 30 min at 37 °C. Medium was replaced with 5 ml lipid emulsion plus doxycycline and cells were incubated for 4 h. Cells were washed twice with DMEM/PSG and incubated for 30 min in DMEM/PSG. Cells were washed and scraped off the plate in PBS/0.1% EDTA and harvested by centrifugation. Cell pellet was resuspended in 1.3 ml distilled water and sonicated for 10 s (Sonopuls GM70, as above). Protein content was measured using the BCA protein assay (Pierce, Rockford, IL; USA). Cholesterol determination was performed as described previously employing a 100 μl aliquot of the sample [23].

For oxysterol measurement as an internal standard and anti-oxidant reagent a methanolic solution of 1 ng/μl 22*S*-HC with 20 ng/μl 3,5-di-*tert*-butyl-4-hydroxytoluol (BHT) was used. Four hundred microliters of this solution were added to 1 ml of cell homogenate and subjected to saponification with 2 ml of 20% tetramethyl-ammoniumhydroxide/2-propanol (w/v, 30 min, 80 °C). Three milliliters of water were added to the cold sample followed by twice extraction with 4 ml of *n*-hexane. The upper layers were combined in a silanized glass tube and the organic solvent was removed under a steam of nitrogen. The residue was resolved in 100 μl of HPLC eluent (solvent system 2-propanol/*n*-heptane/acetonitrile, 35:12:52, v/v/v). To remove cholesterol the sample was subjected to HPLC-fractionation (conditions described previously but with a 100 μl loop injector) [24]. Oxysterols were collected in silanized glass tubes within the 3rd and 6th minute after HPLC-injection (fractionating apparatus: Advantec SF2120), derivatized [24], and subjected to further analysis by gas chromatography–mass spectrometry (GC–MS).

GC–MS analysis of sterols was performed on a GCQ system (Thermo-Finnigan, Bremen, Germany) equipped with an ion trap mass analyzer and a HT-5 fused silica capillary column (SGE, Darmstadt, Germany; 25 m, inner diameter 0.22 mm, film thickness 0.1 μm). Twenty microliters of the sample were injected in large volume solvent split mode at 55 °C and vaporized splitless at 280 °C. Helium was used as carrier gas at a constant velocity of 30 cm/s. The temperature of the column oven was maintained at 50 °C for 4 min, increased with 25 °C/min to 200 °C and finally increased with 10 °C/min to 280 °C. The parameters for the ion trap mass analyzer were as follows: transfer line at 280 °C, positive electron ionization mode (70 eV), ion source at 180 °C, full scan on masses 50–650 Da. Oxysterol compound concentrations were quantified by comparing the ion current response of the sum of characteristic quantitation masses with the peak area response obtained for the known amount of the added internal standard. Amounts of oxysterol were normalized to the protein content. Duplicate samples were analyzed.

3. Results

In order to identify whether ABCG1 is able to transport oxysterols we used our previously characterized TetOff HeLa cell lines which allow regulatable expression of ABCG1(666) or the ABCG1(666,K124M) defective mutant [16] over four orders of magnitude. Oxysterols were applied as an emulsion of 0.1% (w/w) oxysterol/phosphatidylcholine for 4 h, which protected the cells from destructive unspecific effects of oxysterols and allowed to analyze their cellular uptake and export

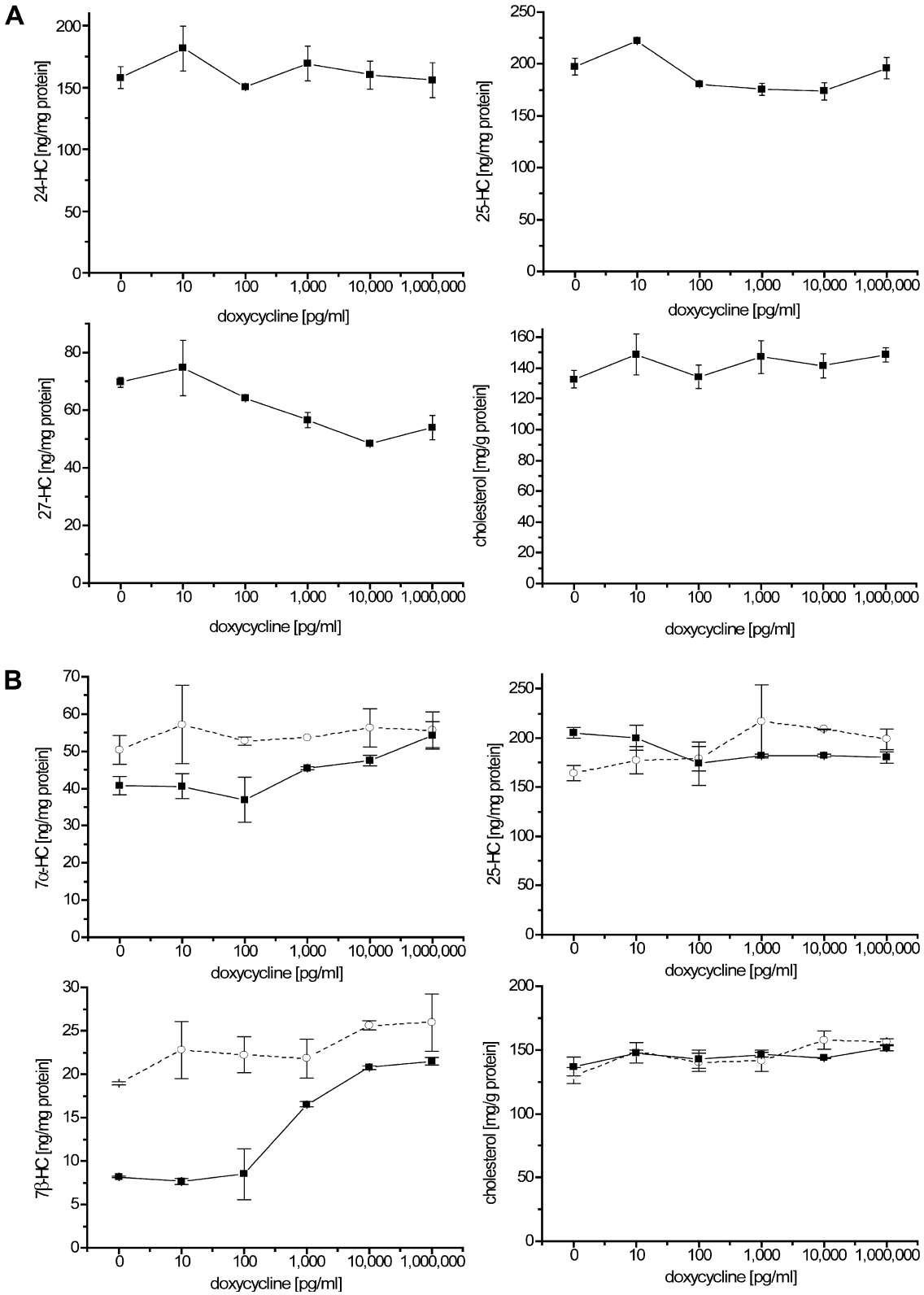

Fig. 1. ABCG1 expression does not interfere with uptake of side chain oxidized HC derivatives, inducers of endogenous ABCG1 gene expression, but prevents uptake of 7β-HC. (A) TetOff HeLa cells expressing variable amounts of human ABCG1 by application of increasing doxycycline concentrations were incubated with a PC emulsion supplemented with 24-HC (upper left panel), 25-HC (upper right panel), and 27-HC (lower left panel) for 4 h. Subsequently the cells were analyzed for cholesterol (lower right panel) and oxysterol content, as described in Section 2. (B) TetOff HeLa cells expressing variable amounts of wt (closed boxes, straight lines) or mutant (open circles, dotted lines) form of ABCG1 by application of increasing doxycycline concentrations were incubated with a PC emulsion supplemented with 7α-HC (upper left panel), 7β-HC (lower left panel), and 25-HC (upper right panel) for 4 h. Subsequently the cells were analyzed for cholesterol (lower right panel) and oxysterol content, as described in Section 2.

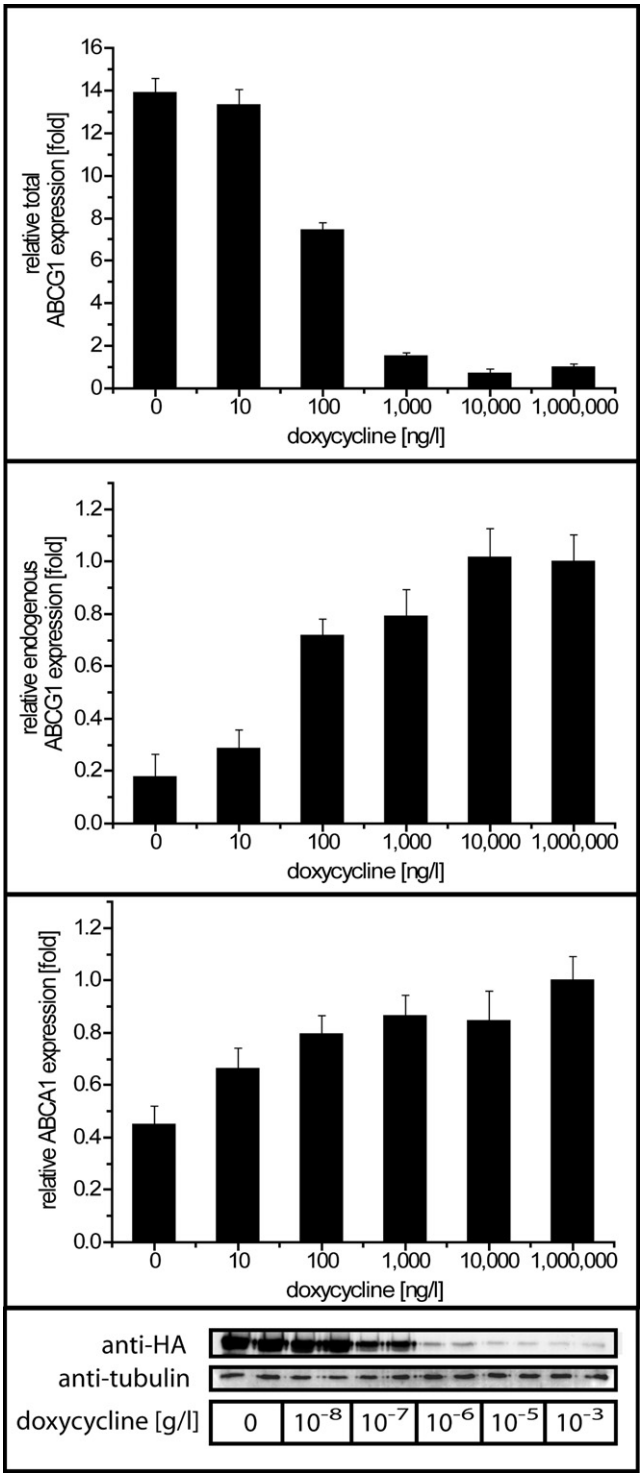

Fig. 2. Doxycycline dependent gene regulation in ABCG1 expressing TetOff HeLa cells. Expression of different genes was measured by reverse transcriptase quantitative PCR in TetOff HeLa cells expressing variable amounts of wt ABCG1 by application of increasing amounts of doxycycline, as described in Section 2. Results are normalized to the expression level at 1 μg/ml doxycycline. Relative expression levels for total ABCG1 transcripts (endogenous and expressed; upper panel), endogenous ABCG1 transcripts (2nd panel), and ABCA1 transcripts (3rd panel) are shown. Recombinant ABCG1 protein levels within the same cells detected with an anti-HA antibody are shown in the lower panel, α-tubulin serves as a loading control. Duplicate samples were analyzed.

at steady state. If the ABC transporter recognizes its substrate, the substrate should be actively expelled from the cells as soon as it enters them and, consequently, cellular uptake of the lipid should be diminished in response to increased expression of the transporter at low doses of doxycycline. As shown in Fig. 1A, uptake of none of the classical LXR-agonists, the side chain oxidized 24-HC, 25-HC, and 27-HC, was reduced. In a marked contrast, uptake of ring oxidized 7β-HC but not its stereoisomer 7α-HC was inhibited, as shown in Fig. 1B. Under the used experimental conditions no change in cellular cholesterol content was observed (Fig. 1A and B). Uptake inhibition of 7β-HC occurred already at an ABCG1 transcription level approx. 10-fold lower (Fig. 2) than required for radioactive cholesterol transport measured previously in this cell line [16]. The dose response curve for 7β-HC uptake was sigmoidal, a characteristic feature of saturable processes (Fig. 1B).

Quantitative RT-PCR showed that the decrease of 7β-HC uptake was inversely related to the expression of total ABCG1 transcripts, as shown in Fig. 2. As soon as the expression level of the exogenous ABCG1 transcript exceeds that of the endogenous one, below a doxycycline concentration of 1 ng/ml, 7β-HC mass uptake is decreased (compare with Fig. 1B). ABC transporter family member A1 (ABCA1) and endogenous ABCG1 expression levels decreased significantly below 100 pg/ml doxycycline. The maximally achieved expression level of total ABCG1 within the wt ABCG1 expressing cell line was 5.6(±0.4)-fold above the level of endogenous gene expression after maximal stimulation with synthetic agonists specific for transcription factors LXR and RXR (Fig. 3).

In order to evaluate whether decreased uptake of 7β-HC depended on the interaction of the oxysterol with ABCG1, we incubated wt and mutant ABCG1 expressing cell lines with individual oxysterols, respectively, and measured ATP consumption by detecting mitochondrial membrane potential with

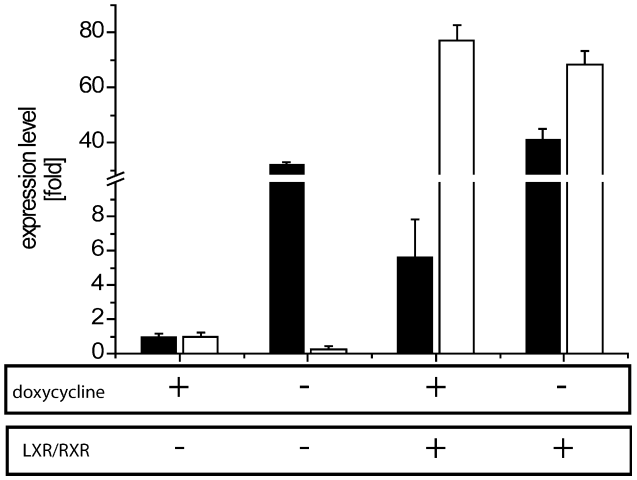

Fig. 3. Liver-X-receptor and retinoid-X-receptor dependent gene regulation in ABCG1 expressing TetOff HeLa cells. Expression of ABCA1 (white bars) and total ABCG1 (black bars) transcripts was measured by reverse transcriptase quantitative PCR in TetOff HeLa cells expressing wt ABCG1, as described in Section 2. ABCG1 expression was turned off by application of 1 μg/ml doxycycline (+ doxycycline) or induced by omitting doxycycline (− doxycycline). Before harvesting the cells synthetic agonists specific for liver-X-receptor and retinoid-X-receptor (1 μM and 0.1 μM, respectively) were added for 24 h simultaneously to doxycycline, as indicated. Duplicate samples were analyzed.

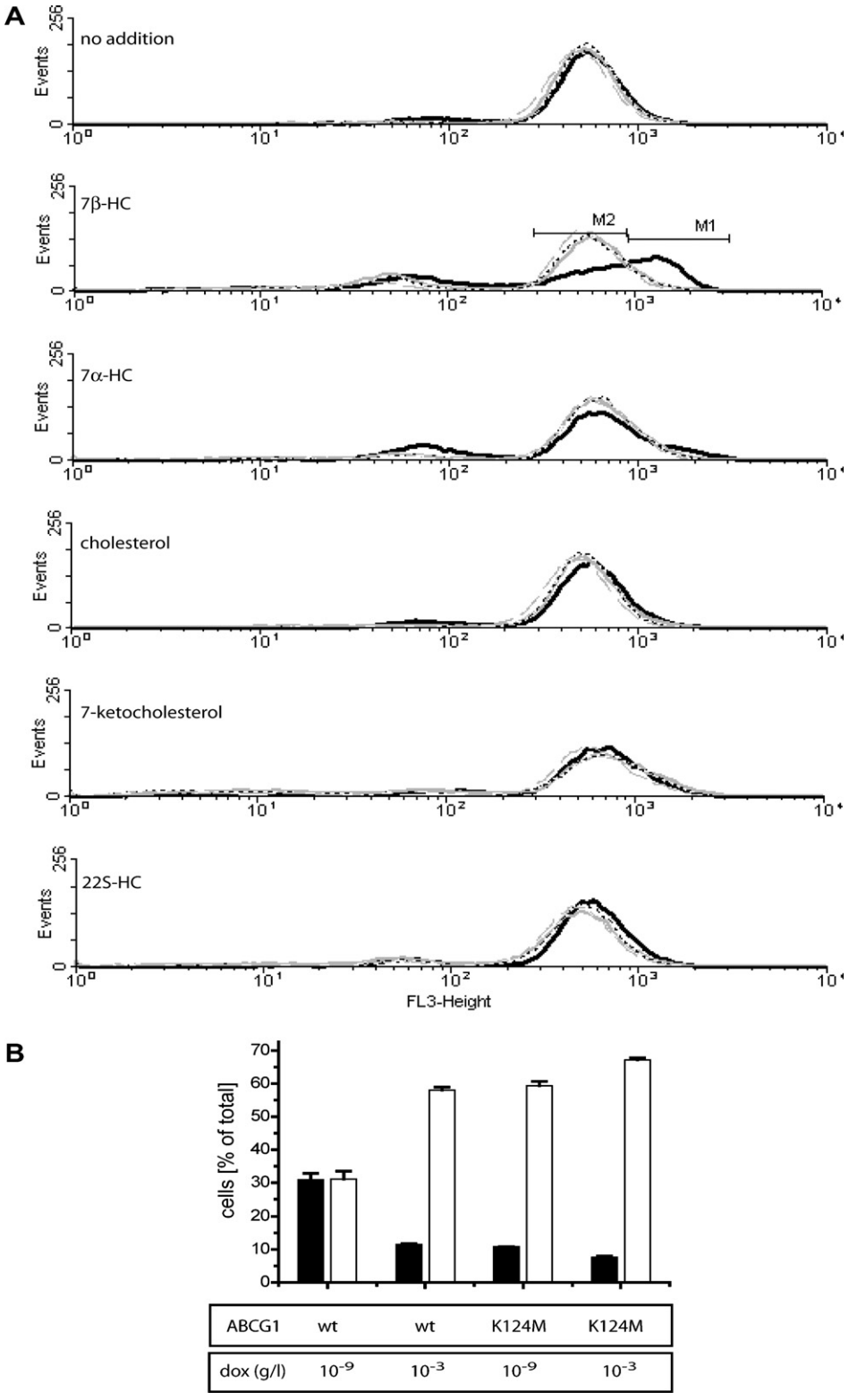

Fig. 4. ABCG1 expression triggers mitochondrial hyperpolarization in the presence of 7β-HC. (A) TetOff HeLa cell lines expressing (black) or not expressing (gray) wt (straight) or mutant (dotted/dashed) form of ABCG1, were incubated with 20 μg of either 7β-HC (2nd panel), 7α-HC (3rd panel), cholesterol (4th panel), 7-ketocholesterol (5th panel), or 22S-HC (last panel), or no sterol (1st panel) for 12 h. TMRE fluorescence was recorded, as described in Section 2. (B) Amounts of cells after treatment with 7β-HC present within the region M1 (black bars) and M2 (white bars), as indicated in the 2nd panel of (A), are shown. Triplicate samples were analyzed.

TMRE. As shown in Fig. 4, only 7β-HC was able to cause hyperpolarization of the mitochondrial membrane potential, indicative of pronounced energy consumption when the ABC transporter exports its correct substrate out of the cell by

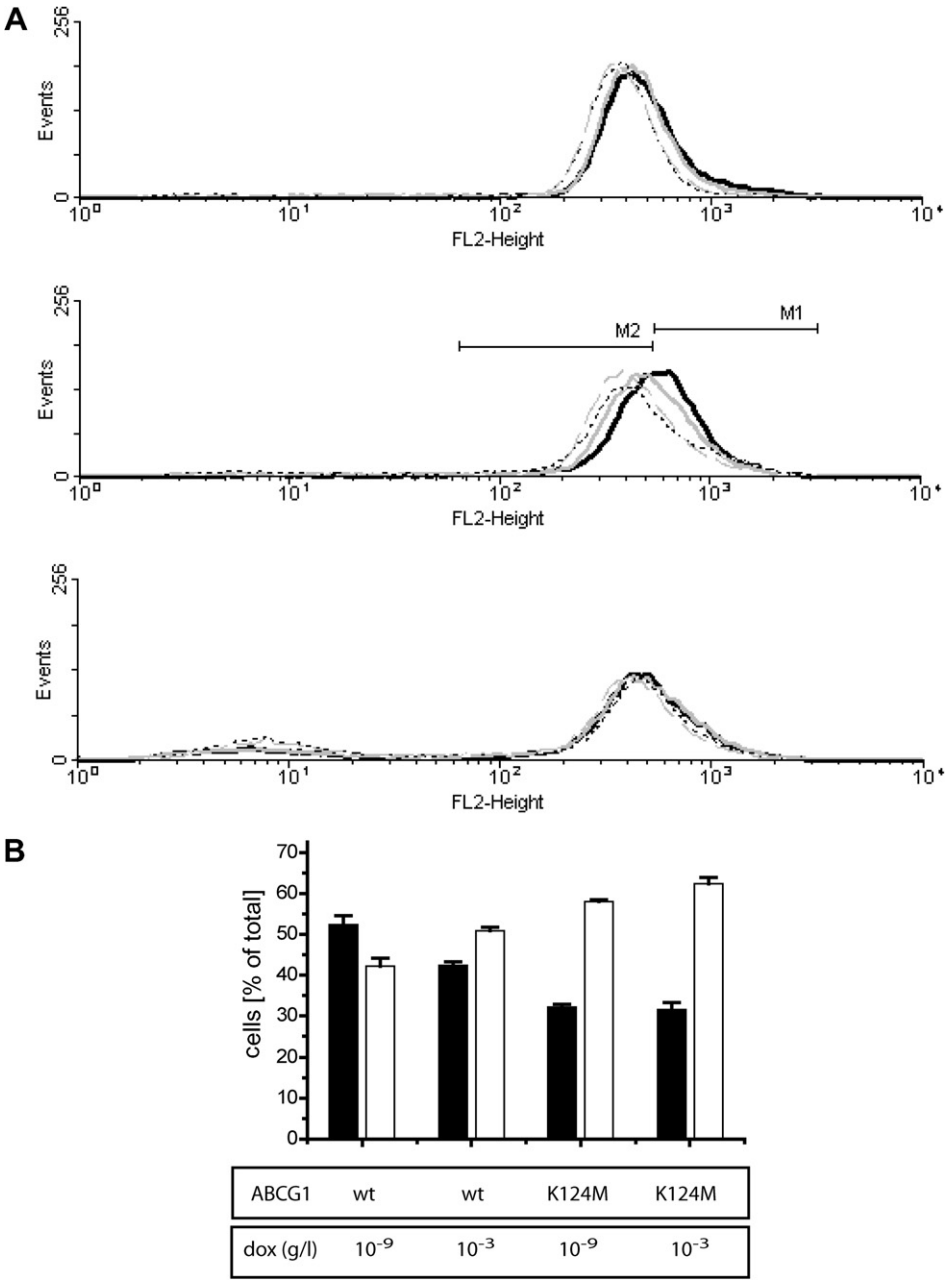

Fig. 5. ABCG1 expression induces oxidative stress in the presence of 7β-HC. (A) TetOff HeLa cell lines expressing (black) or not expressing (gray) wt (straight) or mutant (dotted/dashed) form of ABCG1 were incubated with 20 μg 7β-HC (middle panel), with 20 μg 7-ketocholesterol (lower panel), or without oxysterol (upper panel) for 12 h. Fluorescence of oxidized DHE was recorded, as described in Section 2. (B) Amounts of cells after treatment with 7β-HC present within the region M1 (black bars) and M2 (white bars), as indicated in the middle panel of (A), are shown. Triplicate samples were analyzed.

means of its ATPase. Cholesterol was ineffective in this respect. As shown in Fig. 5, mitochondrial hyperpolarization was accompanied by generation of reactive oxygen species in a specific manner only if 7β-HC was applied and expression of wt ABCG1 was induced.

Next, we analyzed whether ABCG1 expression protects against 7β-HC-induced cell death. As due to low p53 tumor suppressor content as a consequence of interference with papilloma virus E6 protein HeLa cells may respond incorrectly to induction of cell death, and as ABCG1 seems to be very important for macrophages, since it is highly expressed in these cells [15], we used a macrophage cell line. Macrophages are involved in clearance of dead and apoptotic cells and recycling of lipids. As a consequence they are exposed to huge amounts of oxysterols and may require an efficient export of these compounds. ABCG1 expression can be induced approx. 10-fold in RAW264.7 macrophages by application of synthetic LXR and RXR agonists (Fig. 6). This treatment protects the cells against cell death induced by 7β-HC up to a concentration of 500 ng oxysterol/ml (Fig. 6). By contrast, untreated macrophages die proportionally to increasing 7β-HC concentration.

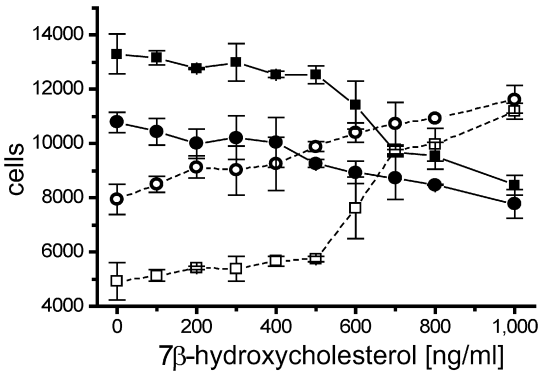

Fig. 6. Induction of ABCG1 expression prevents 7β-HC mediated macrophage cell death. RAW264.7 macrophages were treated with (squares) or without (circles) LXR and RXR agonists and increasing concentration of 7β-HC for 24 h and TMRE fluorescence was recorded, as described in Section 2. Live cells, filled symbols; dead cells, open symbols. 20 000 cells were counted. Inset shows macrophage ABCG1 transcript level with or without LXR and RXR agonist treatment for 24 h relative to untreated cells.

4. Discussion

Our presented results show that ABCG1(666) is a functional ABC transporter which is involved in transport of 7β-HC. We could demonstrate by direct mass analysis in the absence of isotopic labeling, which may give false results due to conversion/oxidation of the radiolabeled compounds during the time scale of the experiment, that uptake of this oxysterol is inhibited by ABCG1 expression. In addition, we have shown that combination of ABCG1 and its substrate, 7β-HC, but not cholesterol, cause massive ATP consumption, implying activation of the transporter in the presence of this oxysterol. Similar burst of energy expenditure has been observed in cells overexpressing ABC-transporter MDR1 following to stimulation with chemotherapeutic drugs [25–27]. We assume that both molecules, ABCG1 and 7β-HC, fit together like key and lock, which is contradictory to the previous assumption that cholesterol is the substrate for ABCG1 [15,16]. In our study, the cellular cholesterol content did not decrease, as would be expected when ABCG1 is overexpressed and cholesterol is exported. It cannot be excluded that the transporter's affinity for cholesterol is too low to result in efficient cholesterol export at the relatively low ABCG1 expression levels employed in our study. Another plausible explanation for increased cholesterol export by very high level ABCG1 expression is that cholesterol homeostasis is affected by cellular depletion of 7β-HC. For instance, 7β-HC already at very low doses inhibits HMGCoA-reductase [7]. Removal of an inhibitor would result in overproduction of cholesterol biosynthesis and subsequently cholesterol export and cholesterol ester storage should be observed, as was reported in BHK cells overexpressing ABCG1 [15]. Side chain oxidized sterols have the same inhibitory effect on HMGCoA-reductase [6], but unfortunately no data exist comparing the efficiency of inhibition of 7β-HC versus side chain oxidized sterols side by side. It is possible that 7β-HC may act as ligand for transcription factors or repressors but such a role has not yet been clarified.

Cholesterol and 7β-HC both have a planar constitution of the cholestane ring structure but have different polarity. The 7β-hydroxyl group causes a shift in orientation within the membranes which may be the major cause for increased membrane permeability and subsequent cytotoxicity associated with 7β-HC [28,29]. Consistent with our results, no activation of the ABCG1 ATPase activity was observed by non-oxidized sterols in a study performed by Cserepes and colleagues [30].

In conventional tissue culture samples the amounts of oxysterols present are relatively low, requiring enrichment of oxysterols from exogenous sources for successful quantification by the methodology used within this study. However, high oxysterol levels may occur under chronic pathologic conditions like those observed in the atherosclerotic plaque or in neurodegenerative diseases [3,31]. For instance β-amyloid and amyloid precursor protein are highly expressed in Alzheimer's disease and can produce 7β-HC [32]. Our uptake scenario is well tolerated by the cells because we deliver exogenously supplied oxysterols as a dilute mixture within a large excess of lecithin emulsion which differs extensively from conditions which have been used to study the influence of oxysterols on phase transitions in biological membranes in vitro [29]. Incubation with the lipid emulsion enables both uptake and intracellular delivery of oxysterols and at the same time allows analysis of the steady state level influenced by the amount of expressed active transporter. Consistent with our results on oxysterol uptake, it was previously shown that even phospholipid vesicles can take up radiolabeled cholesterol from ABCG1 overexpressing cells [33].

The moderate accumulation of 27-HC levels at high level ABCG1 expression observed in Fig. 1A likely reflects the repression of the expression of ABCA1 (Fig. 2) which was attributed a role in the export of the side chain substituted HC derivatives 27-HC, 24-HC, and 25-HC [34].

Application of LXR and RXR agonists to macrophages induces gene expression of ABCG1 and other genes. In the context of all results of our study we assume that the protective effect of agonist treatment against 7β-HC cytotoxicity, as shown in Fig. 6, is due to the enhanced ABCG1 expression. Consistent with our assumption, an enhanced rate of macrophage apoptosis in the ABCG1 deficient mouse model was discussed to explain the phenotype of enhanced early onset atherosclerotic lesion development and deposition of lipid filled alveolar macrophages in the lung of these animals, where lipid oxidation likely occurs at an enhanced rate due to extensive exposure to atmospheric oxygen [15,17].

As ABCG1-deficiency may be expected to decrease 7β-HC export capacity, 7β-HC levels should increase over time in ABCG1-deficient pneumocytes and alveolar macrophages leading to apoptosis and subsequent lipid deposition in the subpleural region of the lung, a process that resembles parallels to the formation of the lipid-filled core of the atherosclerotic plaque. Actually, increased amount of apoptotic macrophages was observed in the vessel wall of LDL-receptor-deficient mice transplanted with ABCG1-deficient bone marrow fed a high fat/high cholesterol diet [35], which corroborates well with results of the present study.

We assume that ABCG1 is a protective factor against 7β-HC-induced cell death. Whether it protects against apoptosis or necrosis needs to be evaluated in a separate study. Once the oxysterol is exported from the cell it is probably taken up by lipoproteins where it in a first step is detoxified by esterification mediated by LCAT [36]. Lipoproteins are then cleared from the circulation by the liver and after lysosomal liberation of the oxysterol cytochrome-P450 containing oxidases form 7β-substituted bile acids which may leave the body via the

gut [37]. ABCG1 may thus be involved in 'reverse transport' of 7β-HC. This newly defined activity of ABCG1 could play an important role in prevention of oxysterol-induced cell death in the atherosclerotic plaque, in Alzheimer's diseased brain cells, and in alveolar macrophages.

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