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ABCA1 and ABCG1 Synergize to Mediate Cholesterol Export to ApoA-I

Ingrid C. Gelissen, Matthew Harris, Kerry-Anne Rye, Carmel Quinn, Andrew J. Brown, Maaïke Kockx, Sian Cartland, Mathana Packianathan, Leonard Kritharides, Wendy Jessup

Objective—To study the acceptor specificity for human ABCG1 (hABCG1)-mediated cholesterol efflux.

Methods and Results—Cells overexpressing hABCG1 were created in Chinese Hamster Ovary (CHO-K1) cells and characterized in terms of lipid composition. hABCG1 expressed in these cells formed homodimers and was mostly present intracellularly. Cholesterol efflux from hABCG1 cells to HDL₂ and HDL₃ was increased but not to lipid-free apolipoproteins. A range of phospholipid containing acceptors apart from high-density lipoprotein (HDL) subclasses were also efficient in mediating ABCG1-dependent export of cholesterol. Importantly, a buoyant phospholipid-containing fraction generated from incubation of lipid-free apoA-I with macrophages was nearly as efficient as HDL₂. The capacity of acceptors to induce ABCG1-mediated efflux was strongly correlated with their total phospholipid content, suggesting that acceptor phospholipids drive ABCG1-mediated efflux. Most importantly, acceptors for ABCG1-mediated cholesterol export could be generated from incubation of cells with lipid-free apoA-I through the action of ABCA1 alone.

Conclusions—These results indicate a synergistic relationship between ABCA1 and ABCG1 in peripheral tissues, where ABCA1 lipidates any lipid-poor/free apoA-I to generate nascent or pre- β -HDL. These particles in turn may serve as substrates for ABCG1-mediated cholesterol export. (*Arterioscler Thromb Vasc Biol.* 2006;26:534-540.)

Key Words: cholesterol efflux ■ ApoA-I ■ phospholipids

In atherosclerosis, cholesteryl ester deposition in macrophage foam cells occurs because cholesterol accumulation exceeds the capacity of the cells to export excess cholesterol to extracellular acceptors. The inverse relationship between plasma high-density lipoprotein (HDL) levels and atherosclerosis risk is thought to reflect in part the role of HDL and its apolipoproteins (mainly apoA-I and apoA-II) to act as cholesterol acceptors.¹ This is highlighted by Tangier disease, in which genetic deficiency in the ATP-binding cassette (ABC) transporter ABCA1 leads to low HDL levels and macrophage foam cell formation.^{2,3,4} ABCA1 is a key regulator of cholesterol and phospholipid export to lipid-free apolipoproteins, forming nascent HDL, but it is unlikely to be involved in cholesterol export to mature HDL.⁵ Recently, ABCG1 was identified as a more likely mediator of cholesterol transport to HDL. ABCG1 belongs to the ABCG subfamily, which comprises a number of ABC half transporters that are implicated in sterol metabolism and drug resistance in various tissues.⁶ ABCG1 is thought to require another half transporter, either by homodimerization or heterodimerization, to form a functional complex.⁷ Expression of this ABC trans-

porter is highly upregulated during macrophage differentiation and cholesterol loading and downregulated by cholesterol depletion and statins.^{8,9} Several recent studies have indicated that mouse¹⁰ and human^{8,11} ABCG1 stimulate cholesterol export to HDL but not to lipid-free apoA-I. Targeted disruption of ABCG1 in mice on a high-fat diet led to extensive lipid accumulation in tissue macrophages, whereas overexpression of ABCG1 protected against diet-induced lipid deposition.¹² No change in total plasma HDL was found in these mice, suggesting that the most important action of ABCG1 may be in tissue macrophages.

In the present study we have used a cell line stably overexpressing hABCG1 to characterize the transporter and its acceptor specificity. We confirm that ABCG1 stimulates export of cell cholesterol to HDL but not to lipid-free apoA-I or apoA-II. We furthermore identify that ABCA1-mediated lipid efflux transforms apoA-I into an efficient substrate for ABCG1-dependent cholesterol efflux, in proportion to the phospholipid content of lipidated apoA-I. ABCA1 and ABCG1 may therefore act in series to mediate lipid efflux from macrophages to apoA-I. This suggests that macrophage

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ABCG1 may be a major contributor to cholesterol efflux to apoA-I as well as to mature HDL.

Methods

For a full description of the methods used, please see the online supplementary methods at <http://atvb.ahajournals.org>.

Plasmid Constructs

Full-length human ABCG1 (hABCG1) cDNA was generated from THP-1 macrophages, incubated overnight with 22(R)-hydroxycholesterol (10 μ mol/L) to upregulate ABCG1 expression. Primers for a nested polymerase chain reaction (PCR) strategy were designed using mRNA sequence NM_016818 (Table I, available online at <http://atvb.ahajournals.org>), which encodes for a 674 AA protein. Constructs were generated with either a V5 or myc tag at the C-terminus.

Cell Cultures

CHO-K1 cells (ATCC) were used for transient and stable transfection of hABCG1. THP-1 monocytes (ATCC) were differentiated to macrophages using phorbol myristate acetate. Baby hamster kidney (BHK) cells expressing human ABCA1 under the mifepristone inducible GeneSwitch system¹³ were a generous gift from Dr Ashley Vaughan (Department of Medicine, University of Washington, Seattle, Wash).

RNA Isolation and Gene Expression Analysis by Quantitative Reverse-Transcription PCR

Semi-quantitative reverse-transcription PCR and quantitative (real-time) PCR were performed as described in supplementary methods.

Lipoproteins and Acceptor Particles

Lipoproteins and apolipoproteins were prepared from human serum or plasma and phospholipid-apoA1 discs and PC small unilamellar vesicles (PC-SUV) generated as previously described.^{14,15,16}

Cholesterol Efflux Assays

Parent and hABCG1-expressing CHO-K1 cells were labeled for 24 hours with [³H]cholesterol (Amersham), washed, and equilibrated for 90 minutes in serum-free medium, then incubated in efflux medium containing bovine serum albumin (1 mg/mL) \pm acceptors for up to 6 hours. Cells and media were assayed for radioactivity and cholesterol mass. Efflux is cholesterol present in medium as a percentage of total cholesterol in the culture.

Homodimerization

CHO-K1 cells were transiently transfected with hABCG1-myc alone, or hABCG1-myc plus hABCG1-V5. Nontransfected cells were a control. Cell lysates were immunoprecipitated with anti-V5, and the pellet was separated by SDS-PAGE and blotted with anti-myc.

Western Blotting and Immunofluorescence Microscopy

Details of Western blotting, cell staining, and microscopy are in supplementary methods.

Results

Generation and Characterization of a Cell Line Stably Overexpressing hABCG1

To determine the role of ABCG1 in cholesterol transport, we generated CHO-K1 cells stably expressing hABCG1. Figure 1A and 1B show that these cells express hABCG1 RNA and protein, whereas no ABCG1 was detected in the parent cells. Cells expressing hABCG1 were viable and proliferated at the same rate as the parental line (data not shown). The expres-

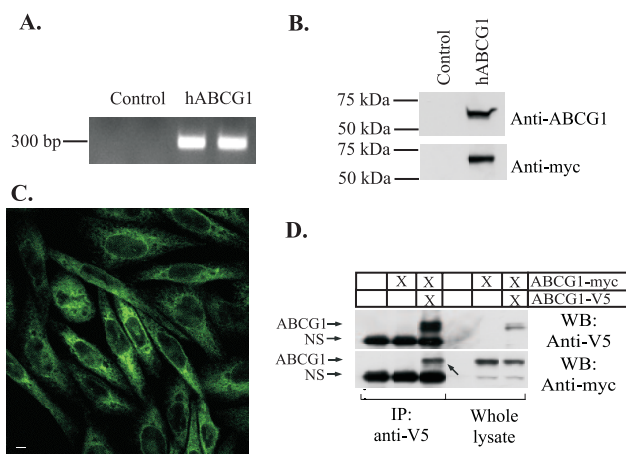


Figure 1. Expression of hABCG1 in control and stably transfected CHO-K1 cells. A, Reverse-transcription PCR for hABCG1. B, Western blot of protein expression, using either sheep anti-human ABCG1 or mouse anti-myc antibodies (15 μ g protein/lane). C, Cellular distribution of hABCG1, visualized by immunofluorescence microscopy. Bar=50 μ m. D, Cells were transiently transfected with hABCG1-myc \pm hABCG1-V5, as shown by Western blotting using anti-myc or anti-V5 antibodies (RHS) (15 μ g protein per lane). Nontransfected cells were also included as controls. Immunoprecipitation using anti-V5 antibodies coprecipitated hABCG1-myc, only in the V5-expressing cells (LHS, homodimer indicated with arrow). The nonspecific (NS) band below ABCG1 originates from the protein A/G beads and is \approx 50 kDa (Pierce).

sion of hABCG1 also had no effect on cellular free (control versus hABCG1; 54.8 ± 4.6 versus 47.7 ± 7.2 nmol/mg cell protein; $n=6$ experiments) and esterified cholesterol (5.2 ± 2.8 versus 7.1 ± 1.9 nmol/mg cell protein). The percentage of cholesterol esterified was slightly higher in hABCG1-expressing cells ($12.6 \pm 3.6\%$) compared with controls ($8.6 \pm 3.1\%$), as described,¹¹ but this difference was not significant (paired *t* test, $P < 0.05$ for significance). Introduction of hABCG1 also had no effect on the level of expression of endogenous hamster genes ABCG1 (which was very low), ABCA1, low-density lipoprotein receptor (LDL-R), or HMGCoA-reductase (Figure I, available online at <http://atvb.ahajournals.org>).

hABCG1 protein displayed a largely perinuclear distribution (Figure 1C), similar to that previously reported in human macrophages¹⁷ and in fibroblasts expressing gfp-ABCG1.¹⁸ However, some protein could be detected on the cell periphery, presumably at the plasma membrane. This was not investigated in detail.

ABCG1 Can Form Homodimers

Earlier studies^{10,11} have shown that overexpression of ABCG1 alone induces transport activity, consistent with its function as a homodimer, although it does not exclude the possibility that transfected hABCG1 may dimerise with other endogenous ABC half-transporters to form a functional complex. To more directly determine whether hABCG1 can homodimerize, cells were transfected with hABCG1-myc alone or a combination of V5 and myc-tagged hABCG1 and cell lysates subjected to immunoprecipitation (IP) with anti-V5 antibodies. Nontransfected cells were included as a control for detection of nonspecific IP products, whereas cells

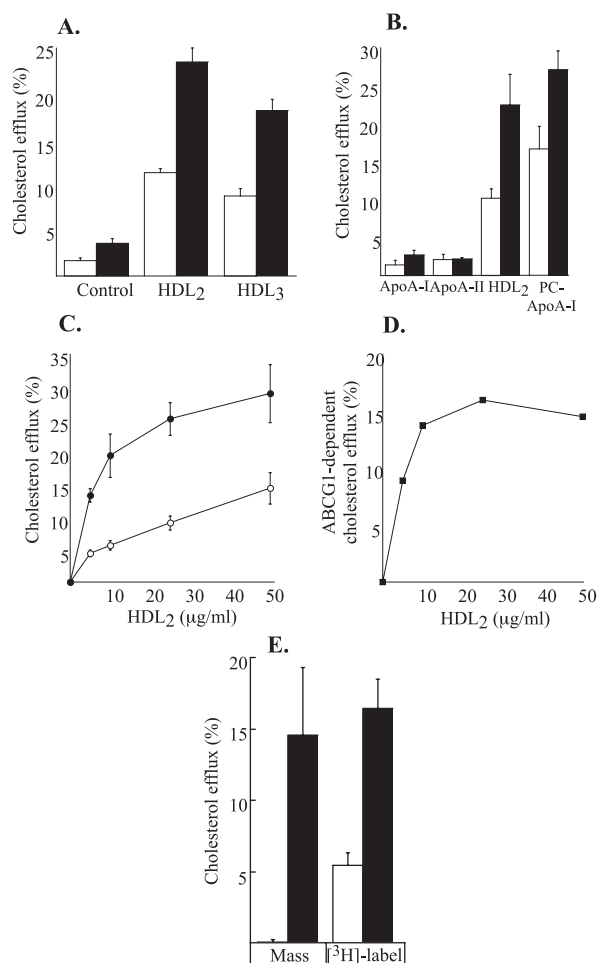


Figure 2. Accepter specificity for ABCG1-mediated cholesterol efflux. Cholesterol efflux from hABCG1-expressing (filled bars and symbols) or control cells (open bars and symbols) for 6 hours to various acceptors. All data are means \pm SD of 3 to 7 separate experiments, except for apoA-II, which is mean \pm range of 2 experiments. A, [3 H]-cholesterol efflux to HDL₂ or HDL₃ (25 μ g HDL protein/mL) or bovine serum albumin alone (control). B, Acceptor specific (less control) [3 H]-cholesterol efflux to apoA-I or apoA-II (25 μ g/mL), HDL₂ (25 μ g protein/mL), and PC/apoA-I discs (25 μ g/mL apoA-I, 76 μ g/mL PC). C, HDL₂-specific efflux of [3 H]-cholesterol at the indicated concentrations to ABCG1-expressing cells (black circles) or control cells (white circles). D, "ABCG1-dependent" cholesterol efflux, determined as the difference in HDL₂-specific efflux between control and hABCG1-expressing cells, using the data shown in (C). E, HDL₂-specific efflux (10 μ g/mL), assessed by mass analysis (high-performance liquid chromatography [HPLC]) and [3 H]-label release from hABCG1-expressing (filled bars) or control cells (open bars). Specific efflux (less efflux to bovine serum albumin and subtraction of cholesterol supplied by HDL₂ itself) was calculated for both methods.

expressing hABCG1-myc only were used to control for nonspecific immunoprecipitation of hABCG1-myc. Figure 1D shows that hABCG1-myc coprecipitated with hABCG1-V5 only in cotransfected cells, consistent with the formation of V5/myc hABCG1 homodimers.

Accepter Specificity for ABCG1-Mediated Efflux

Figure 2A shows that hABCG1 expression increased cholesterol efflux to both HDL₂ and HDL₃ as previously shown.^{10,11} Incubation of control cells with HDL also caused efflux of

[3 H]-cholesterol, but this was much less than in hABCG1-expressing cells. Similar to mouse ABCG1,¹⁰ efflux was slightly greater to HDL₂ than HDL₃ when matched for protein concentrations. Interestingly, and like a previous study,¹⁰ ABCG1 expression consistently initiated more [3 H]-cholesterol release under basal (ie, no added acceptor) conditions (Figure 2A, control). Consistent with others,^{10,11} we found lipid-free apoA-I did not stimulate cholesterol efflux, nor did apoA-II (Figure 2B). However, lipidation of apoA-I with only PC to form PC/apoA-I discs was sufficient to generate an acceptor as efficient as HDL₂ at 25 μ g protein/mL.

Figure 2C shows the concentration dependence of [3 H]-cholesterol efflux from ABCG1 expressing and control cells to HDL₂. The rate of efflux was greater from ABCG1-expressing cells at all HDL concentrations. However, the increment in efflux as HDL concentrations increased at higher levels was similar for both cell lines. This suggests that there are at least 2 components of [3 H]-cholesterol efflux to HDL₂; an ABCG1-dependent process that is saturated at relatively low HDL₂ concentrations, and an ABCG1-independent process (possibly exchange) that predominates at higher HDL₂ concentrations. This is shown more clearly in Figure 2D, in which "ABCG1-specific" efflux is shown as the difference between the rates of efflux of cholesterol from hABCG1-expressing and control cells. The ABCG1-specific contribution to cholesterol export reached a maximum at \approx 10 μ g HDL₂ protein/mL. This concentration of HDL₂ was used in most subsequent experiments.

As efflux of [3 H]-cholesterol can represent mass export and/or exchange of label, we also directly measured changes in cholesterol mass between cells and medium after addition of HDL (Figure 2E). This confirmed that expression of hABCG1 stimulates net mass cholesterol export from cells to HDL₂. In contrast, no detectable export of cholesterol mass could be measured from control cells to HDL, suggesting that the export of [3 H]-cholesterol (Figure 2) in this case reflects only exchange of cholesterol between cells and HDL₂.

Human Macrophages Incubated With Lipid-Free ApoA-I Generate an Accepter for ABCG1-Mediated Cholesterol Efflux

The experiments with natural and reconstituted HDLs indicate that a variety of lipidated particles are capable of acting as acceptors for lipid efflux from hABCG1-expressing cells. It is known that macrophages transfer cellular cholesterol and phospholipid to apoA-I to form "nascent HDL" particles.^{19,20} To determine whether these particles are sufficiently lipidated to stimulate ABCG1-dependent lipid efflux, we conditioned apoA-I by incubation with THP-1 macrophages for 24 hours. Figure 3A shows that THP-1-conditioned apoA-I stimulated [3 H]-cholesterol efflux significantly relative to lipid-free apoA-I, and this stimulation was greater for ABCG1-expressing cells than controls. In the same experiment, mass cholesterol export from hABCG1-expressing cells to "conditioned apoA-I" was similar to label ($8.7 \pm 1.5\%$ after 6 hours) but for control cells mass efflux was the same as efflux to lipid-free apoA-I ($2.7 \pm 0.2\%$ per 6 hours), suggesting again that cholesterol export from control cells was merely caused by exchange of label and not by active cholesterol transport.

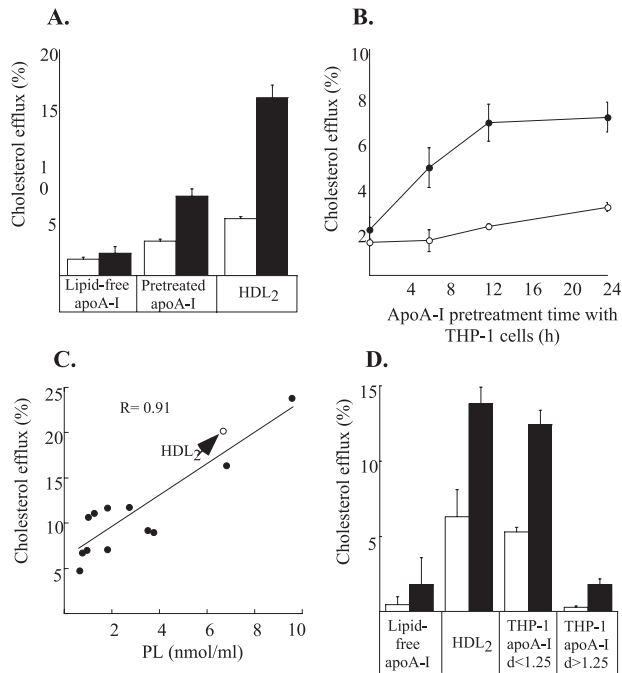


Figure 3. Human macrophages incubated with lipid-free apoA-I generate an acceptor for ABCG1-mediated cholesterol efflux. A, [³H]-cholesterol-labeled control (open bars) or hABCG1-expressing (black bars) cells were incubated for 6 hours with THP-1-“conditioned” apoA-I (see Methods for preparation). ApoA-I and HDL₂ (10 μg protein/mL) were included as negative and positive controls. Data are means ± SD of triplicates from a single representative experiment. B, ApoA-I-containing media (10 μg protein/mL) were pre-incubated with THP-1 macrophages (2 mL/35 mm well) for the times indicated, then harvested and used to stimulate cholesterol efflux from control (white circles) or hABCG1-expressing (black circles) cells. C, ApoA-I (10 μg/mL) was incubated with THP-1 macrophages (35 mm wells or 175 cm² flasks, see supplementary Methods (<http://atvb.ahajournals.org>)) (black circles), then analyzed for their phospholipid (PL) concentration and ability to stimulate [³H]-cholesterol efflux from hABCG1-expressing cells. For comparison, efflux to HDL₂ (10 μg/mL) is also shown (white circle). Line of best fit (excluding HDL₂) is shown. D, [³H]-cholesterol efflux as from control (open bars) and hABCG1-expressing cells to THP-1-conditioned medium, subjected to ultracentrifugation to separate d<1.25 and d>1.25 fractions. All data are expressed as specific efflux and are means ± SD of triplicates.

The degree to which lipidation of apoA-I by THP-1 macrophages converted the apoA-I to an efficient acceptor for ABCG1-mediated cholesterol efflux was time-dependent (Figure 3B) and increased if the THP-1 cells were first cholesterol loaded and/or the cell number-to-medium volume ratio increased (data not shown). These are conditions that increase lipidation of apoA-I.^{19,20} Therefore, we measured the cholesterol and phospholipid composition of all THP-1-conditioned apoA-I media and compared this with their ability to stimulate ABCG1-dependent cholesterol efflux. THP-1-conditioned media contained predominantly unesterified cholesterol, phosphatidylcholine (PC), and sphingomyelin (SM). There were approximately equimolar amounts of PC and sphingomyelin in these conditioned media (PC/SM=1.14±0.30; n=7). We found a strong association between the total phospholipid content of conditioned media and their ABCG1-dependent efflux activity (Figure 3C). Of

the lipids, the strongest correlation was with PC content ($R=0.92$), whereas cholesterol content was less strongly associated ($R=0.77$)

Under the same experimental conditions as used here, it was previously shown that only a minor proportion of apoA-I becomes lipidated by THP-1 macrophages.¹⁹ We anticipated that it was this “nascent HDL” population of lipidated apoA-I that was active in inducing ABCG1-mediated cholesterol efflux. Therefore, THP-1-conditioned apoA-I was collected and separated by ultracentrifugation into d<1.25 (“nascent” HDL) and d>1.25 (lipid-free/poor apoA-I) fractions. Consistent with previous work,¹⁹ only 7% to 8% of the added apoA-I was recovered in the lipidated (d<1.25) fraction. This fraction was a much more efficient acceptor for ABCG1-mediated cholesterol efflux compared with the lipid-poor/free apoA-I (both tested at 10 μg apoA-I/mL; Figure 3D). The results clearly indicate that the buoyant, lipid-containing fraction that contains the “nascent HDL” is most active in stimulating cholesterol efflux from hABCG1-expressing cells.

ABCA1 Activity Alone Is Sufficient to Generate an ABCG1 Acceptor

The previous experiments showed that lipidation of apoA-I by THP-1 macrophages, which was most likely dependent on their endogenous ABCA1 activity, was sufficient to convert apoA-I into an acceptor for ABCG1-mediated cholesterol efflux. However other possible mechanisms also exist; for example, apoA-I also stimulates secretion of apoE from human macrophages,^{21,22} which also contributes to the formation of “nascent” HDL by these cells.¹⁹

To determine whether ABCA1 activity alone was sufficient to generate an acceptor for ABCG1-dependent efflux, we moved to a simpler system for apoA-I lipidation, using BHK cells stably transfected with human ABCA1 under a mifepristone-inducible expression system.¹³ In these cells, ABCA1 is essentially undetectable in basal conditions but is rapidly and highly expressed after exposure to 10 nmol/L mifepristone (Figure 4A). This is associated with a marked increase in apoA-I-mediated cholesterol and phospholipid efflux from these cells.¹³ ABCA1 expression in the mifepristone-treated cells remained high throughout the experiment (Figure 4A). Figure 4B shows that apoA-I that was preincubated with BHK cells without mifepristone had little effect on cholesterol export from control cells and was indistinguishable from lipid-free apoA-I not exposed to cells. In contrast, apoA-I conditioned by exposure to ABCA1-expressing BHK cells stimulated a large increase in cholesterol export from hABCG1-expressing cells but not from control cells. This indicates that ABCA1 activity alone is sufficient to convert lipid-free apoA-I into an efficient acceptor to stimulate ABCG1-dependent cholesterol efflux. Under the same conditions, bovine serum albumin-only control media incubated with BHK cells did not stimulate cholesterol efflux from control or ABCG1-expressing cells above basal levels (data not shown). Thus, the generation of an ABCG1 acceptor was dependent on apoA-I and did not reflect nonspecific acceptor release by the BHK cells. The phospholipid content of the apoA-I containing media exposed to BHK

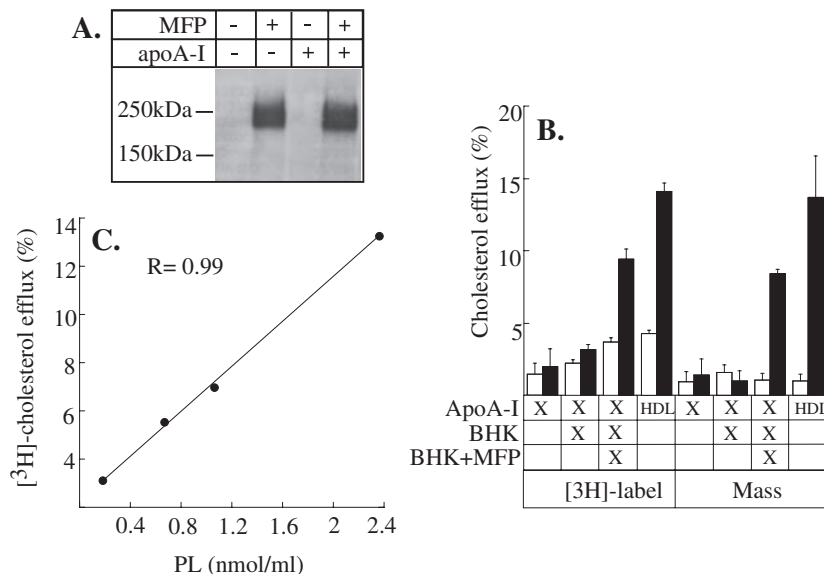


Figure 4. BHK cells overexpressing ABCA1 transform apoA-I containing media into acceptors for ABCG1-mediated cholesterol efflux. **A**, ABCA1 protein levels in stably transfected BHK cells¹³ after incubation \pm 10 nmol/L mifepristone for 12 hours, \pm further incubation for 24 hours with apoA-I (10 μ g/mL). Protein loading was 10 μ g per lane. **B**, Cholesterol efflux from control (white bars) and hABCG1-expressing (black bars) cells after 6 hours with apoA-I, HDL₂ (10 μ g protein/mL) or with apoA-I preincubated with BHK cells with or without induction of ABCA1 expression using 10 nmol mifepristone (MFP). Data are specific efflux (bovine serum albumin alone subtracted) and are means \pm SD of triplicate measurements. Efflux was determined both by released [³H]-cholesterol and cholesterol mass. **C**, "BHK-conditioned" media after induction with MFP were assayed for phospholipid (PL) content by HPLC and for their ability to stimulate cholesterol efflux from hABCG1-expressing cells. Line of best fit is shown.

cells was strongly correlated with the cholesterol efflux capacity (Figure 4C), again suggesting that ABCG1-mediated cholesterol efflux is driven by acceptor phospholipids. Stimulation of ABCG1-mediated cholesterol efflux was achieved when the total phospholipid (PL) concentration in the medium was as low as \approx 1 nmol/mL. In comparison, HDL₂ as used in these experiments contributed $>$ 6 nmol phospholipid/mL (Figure 3C) (627 nmol phospholipid/mg HDL protein; added to medium at 10 μ g protein/mL). These results indicate that relatively lipid-poor apoA-I, produced from lipidation of lipid-free apoA-I via the action of ABCA1 alone, is capable of inducing ABCG1-mediated cholesterol efflux from cells.

Phospholipid Alone Stimulates Cholesterol Efflux From ABCG1-Expressing Cells

Because previous experiments showed a strong correlation between hABCG1-mediated cholesterol efflux and the phospholipid content of the acceptors, we examined the possibility that phospholipid-only-containing acceptors would be sufficient to induce ABCG1-mediated cholesterol efflux. Figure 5A shows that cholesterol efflux to PC-containing small unilamellar vesicles (PC-SUV) was similar to that stimulated by PC/apoA-I discs when matched for phospholipid concentration. As was previously found for HDL₂, the rate of

[³H]-cholesterol efflux to PC-SUV was dose-dependent and greater from hABCG1-overexpressing than control cells (Figure 5B). The ABCG1-specific component of efflux (Figure 5C) reached saturation at 30 to 40 nmol/mL. In comparison, HDL₂ reached saturation at a much lower phospholipid concentration (10 μ g/mL HDL₂ protein, which corresponded to 6.3 nmol/mL total phospholipid) (Figure 3C). Therefore, the presence of phospholipid alone, although a requirement for inducing ABCG1-mediated cholesterol efflux, was less efficient compared with acceptor particles that contain apoA-I in combination with phospholipids.

Discussion

It is clear from recent studies that ABCG1 is emerging as a major contributor to cholesterol export from macrophages. Its expression is highly responsive to cholesterol status.⁸ ABCG1 overexpression protects tissues from cholesterol accumulation, whereas its ablation leads to accumulation of high levels of neutral sterols in tissue macrophages without any changes to plasma HDL levels,¹² suggesting that the main mode of action may be predominantly in tissue macrophages.

Until now, it was thought that whole HDL and/or its subfractions were the most likely acceptors for ABCG1-mediated cholesterol export. We show here that a range of

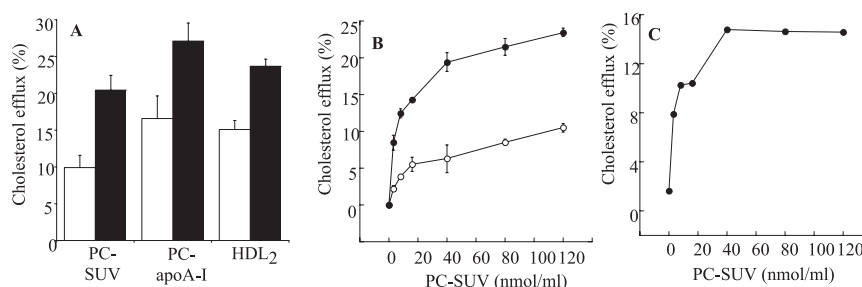


Figure 5. Phospholipid-only containing acceptors stimulate ABCG1-dependent cholesterol efflux. [³H]-cholesterol efflux from control (white bars) and hABCG1-expressing (black bars) cells was measured after 6 hours of incubation. **A**, With PC-SUV (76 μ g/mL), PC/apoA-I discs (76 μ g/mL PC; 25 μ g/mL protein), or HDL₂ (25 μ g protein/mL). **B**, With the indicated concentrations of PC-SUV. **C**, "ABCG1-dependent" cholesterol efflux, determined by subtracting the rate of efflux to PC-SUV by control cells from that to hABCG1-expressing cells, using the data shown in (B). Data are means \pm SD of triplicates of a representative experiment.

phospholipid-containing acceptors other than HDL subclasses are efficient in mediating export of cholesterol via ABCG1. Furthermore, a buoyant phospholipid-containing fraction generated from incubation of lipid-free apoA-I with macrophages was nearly as efficient as HDL₂. Most importantly, acceptors for ABCG1-mediated cholesterol export can be generated from incubation of cells with lipid-free apoA-I through the action of ABCA1 alone. This implies a potential synergistic relationship between ABCA1 and ABCG1 in peripheral cholesterol export, where ABCA1 lipidates lipid-poor/free apoA-I to generate nascent or pre- β -HDL, and these particles in turn serve as substrates for ABCG1-mediated cholesterol export. It should be emphasized that efflux to apoA-I via ABCG1 requires previous conversion of apoA-I into a phospholipid-containing acceptor, and that ABCA1 can, but ABCG1 cannot, perform this conversion.

In macrophages, expression of ABCA1 and ABCG1 are regulated via similar mechanisms, consistent with their coordinated activity in cholesterol export.^{9,23} Recently, another example was presented of potential coordination between ABCG-transporters and an ABC full transporter in mice. The heterodimers ABCG5 and ABCG8, 2 half transporters from the same subfamily as ABCG1, were shown to be dependent on activity of Mdr2, a phospholipid transporter necessary for secretion of cholesterol into bile.²⁴ Further studies will elucidate whether there is any overlap in the modes of action of these ABC transporter pairs.

The mechanism by which ABCG1 mediates cholesterol efflux is not known. We do not yet know whether direct interaction between ABCG1 and acceptor particles is necessary for efflux, as has been suggested for ABCA1. The fact that the transporter distributes partially to the cell surface, similar to ABCA1, suggests that it might function there, acting either to facilitate aqueous diffusion-controlled efflux by binding acceptor particles or by directly altering cholesterol distribution in the plasma membrane. Vaughan et al¹¹ showed that inducible overexpression of ABCG1 in BHK cells increases a cholesterol-oxidase sensitive cholesterol pool that can be accessed by HDL. Their inducible overexpression of ABCA1 using the same cell system also increased a cholesterol oxidase sensitive cholesterol pool accessible by apoA-I, but not by HDL phospholipids. This might suggest that different membrane domains, albeit both accessible to cholesterol oxidase, may be involved in ABCA1 versus ABCG1-mediated cholesterol efflux. We have shown that in macrophages apoA-I first interacts with lipid raft domains, depleting this rapidly accessible pool of cholesterol.²⁵ Subsequent efflux from a slower pool of cholesterol, possibly of nonraft origin, may be involved in the further lipidation of the nascent HDL particles via ABCG1. These concepts are currently under investigation.

It was previously reported that ABCA1 facilitates cholesterol efflux to lipid-free/poor apoAI, whereas ABCG1 functions with spherical HDL. Previous studies as well as our results showed that HDL₂ was more efficient than HDL₃ as a cholesterol acceptor, which may be explained by the relatively higher phospholipid-to-protein ratio of HDL₂. Phospholipid-only containing acceptors were also able to induce ABCG1-mediated cholesterol efflux, although less

efficiently than apoA-I containing acceptors matched for PL content. Significantly, the acceptor phospholipid content was shown to be highly correlated with its capacity to induce ABCG1-mediated cholesterol efflux. These results may help to explain, in part, the inverse correlation found in several studies between HDL phospholipid content and risk of atherosclerosis.^{26,27} Importantly, ABCG1 was effective in cholesterol export even at much lower PL-to-apoA-I ratios than found in mature HDL. Thus, ABCG1 may play an important role in cholesterol export from peripheral tissues where poorly lipidated "pre- β " apoA-I may predominate over HDL as an acceptor. The importance of acceptor phospholipid for efflux via ABCG1 has parallels with previous studies of cholesterol efflux via scavenger receptor BI (SR-BI).^{28,29} Because ABCG1 is induced by the same transcriptional processes as ABCA1 and, like ABCA1 but unlike SR-BI,³⁰ is upregulated in response to cellular cholesterol accumulation in macrophages, we propose that ABCG1 is most likely to cooperatively interact with ABCA1 in peripheral tissues. Whether ABCG1 and SR-BI act as coexistent parallel pathways of efflux to all or some phospholipid-containing acceptors requires further investigation.

The current studies present the first direct evidence to our knowledge that ABCG1 can indeed form homodimers, using ABCG1 constructs with 2 different tags, an approach that has been used successfully for other ABC half-transporters.^{31,32} Homodimerization is supported by the fact that overexpression of ABCG1 alone has a functional outcome. Furthermore, Vaughan et al showed that cross-linking of ABCG1 in overexpressing cells produced a product that had twice the molecular weight of ABCG1.¹¹ Nonetheless, it is possible that other potential partners for ABCG1 may exist, depending on tissue expression of other ABCG transporters. One such transporter may be ABCG4, although in macrophages its expression level is low compared with ABCG1, even after liver x receptor (LXR) activation.¹⁰

In summary, we have shown that acceptors for ABCG1-mediated cholesterol export can be generated from incubation of cells with lipid-free apoA-I through the action of ABCA1. Moreover, we showed that the phospholipid content of the acceptor was strongly correlated with its cholesterol efflux inducing capacity. These results imply a possible synergistic relationship between ABCA1 and ABCG1 in tissue macrophages. It also suggests that ABCG1 may make a major contribution, in concert with ABCA1, to cholesterol export to lipid-free/poor apoA-I.

Acknowledgments

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SUPPLEMENTARY METHODS

Plasmid constructs

Full-length human ABCG1 (hABCG1) cDNA was generated from THP-1 macrophages, incubated overnight with 22(R)-hydroxycholesterol (10 μ M) to upregulate ABCG1 expression, using Platinum Taq high fidelity DNA polymerase (Invitrogen). Primers for a nested PCR strategy were designed using mRNA sequence NM_016818 (**Supplementary Table I**), which encodes for a 674 AA protein. The stop codon was omitted to allow for addition of a V5 tag to the C-terminus by cloning the cDNA into pcDNA3.1 (V5/his)(Invitrogen). The plasmid was cut with BamHI and NotI (Fermentas) and ligated into pcDNA4.0 (myc/his) using T4 DNA ligase (Invitrogen). All vectors were sequence verified (SUPAMAC, University of Sydney).

Cell culture conditions

CHO-K1 cells (ATCC) were routinely cultured in Ham's F-12 medium (Gibco) containing 10% (v/v) heat-inactivated fetal calf serum (HIFCS), 2 mM L-glutamine (Trace Biosciences), penicillin (100U/ml, Sigma) and streptomycin (100 μ g/ml, Sigma) at 37°C in 5% CO₂.

THP-1 monocytes (ATCC) were maintained in RPMI medium containing 10% (v/v) HIFCS, L-glutamine and penicillin/streptomycin (see CHO-K1), at 37°C in 5% CO₂. The cells were seeded at 2 x 10⁶/ml into 35mm culture wells (2ml/well) or at 60 x 10⁶ cells in 175cm² flasks (60ml/flask) in medium supplemented with 50 ng/ml phorbol 12-myristate 13-acetate (Sigma) for 3 days to differentiate into macrophages. In some cases THP-1 macrophages were cholesterol-loaded by incubation for 48h with acetylated low density lipoprotein (AcLDL; 100 μ g/ml) in RPMI containing 10% (v/v) lipoprotein deficient serum (LPDS).

BHK cells expressing human ABCA1 under the mifepristone inducible GeneSwitch system¹ were a generous gift from Dr Ashley Vaughan (Department of Medicine, University of Washington). The cells were grown in DMEM containing 10% (v/v) HIFCS plus 2mM L-glutamine, penicillin(100U/ml) and streptomycin (100 μ g/ml) until experimental treatment.

ABCA1 expression was induced by incubating cells for 12h in DMEM with 1mg/ml bovine serum albumin (BSA; fatty acid free; Sigma) and 10nM mifepristone (Invitrogen).

Transfection and generation of stable expressors

CHO-K1 cells were seeded at 2×10^5 /ml into 35mm culture wells (2ml/well) and transfected using lipofectamine (Invitrogen) according to the manufacturers instructions. For generation of stable expressors, the myc-tagged hABCG1 construct was transfected into CHO-K1 cells as above. After 24 hours, cells were transferred to selection medium containing zeocin (400 μ g/ml, Invitrogen). Stable colonies were selected and screened for ABCG1-myc expression by Western blotting. Positive clones were subjected to a further round of dilution cloning. Cells were maintained routinely in 200 μ g/ml zeocin.

RNA Isolation and Gene Expression Analysis by Quantitative Reverse Transcription-PCR

Cells were harvested for total RNA using Tri Reagent according to the manufacturer's instructions (Sigma). Concentrations of total RNA were measured by spectrophotometry (Nanodrop ND-100 Spectrophotometer, Biolab). Reverse transcription-PCR was performed according to the manufacturer's protocol for SuperScript III First Strand cDNA Synthesis (Invitrogen).

Semi-quantitative RT-PCR for hABCG1 was performed using Red Hot DNA polymerase (ABGene) according to the manufacturer's instructions. Quantitative ('real-time') Reverse Transcriptase-PCR (QRT-PCR) was performed using iQ SYBR Green Supermix" (Biorad) and an ABI 7700 Sequence Detector followed by analysis of data using ABI Prism Sequence Detector Soft-ware v1.6.3 (PE Biosystems). Primer pairs (synthesised by Sigma-Genosys) used for the amplification reaction of various genes from cDNAs are listed in the **Supplementary Table I**. PCR products were verified by sequencing. The change in gene expression levels was determined by normalizing mRNA levels of the gene of interest to the mRNA level of the house-keeping gene, porphobilinogen deaminase (PBGD). Melting curve analysis was performed to confirm production of a single product in each reaction.

Lipoproteins and acceptor particles.

Low-density lipoprotein (LDL), acetylated LDL (AcLDL), lipoprotein-deficient serum (LPDS) and lipid-free apoA-I and apoA-II were prepared from human serum as described

previously^{2,3}. HDL₂ and HDL₃ were prepared from human plasma according to Sattler *et al*⁴. Discs containing 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC; Avanti Polar Lipids) and apoA-I (PC/apoA-I; molar ratio 114:1) were prepared using the cholate dialysis method⁵ and PC small unilamellar vesicles (PC-SUV) by sonication³.

THP-1 'conditioned' apoA-I was generated by incubation of THP-1 macrophages in flasks or 35mm wells with apoA-I (10µg/ml, 20ml or 2ml respectively) in RPMI medium for up to 24 h. The medium was then harvested and centrifuged to remove any detached cells. In some experiments, the preconditioned apoA-I was concentrated (Amicon Centriplus YM-10) and subjected to ultracentrifugation to separate $d < 1.25$ and $d > 1.25$ fractions⁶. After dialysis against 4 changes of PBS, each fraction was diluted in fresh medium to 10µg protein/ml.

Cholesterol efflux assays

Parent and hABCG1-expressing CHO-K1 cells were plated at 0.9×10^5 /22mm well (Costar) and left overnight. The cells were then labelled for 24 h with 1 µCi/ml [$1\alpha,2\alpha(n)$ -³H]cholesterol (Amersham), washed twice with phosphate buffered saline (PBS) followed by 90 min equilibration in serum-free Ham's F-12 medium containing 1mg/ml BSA (fatty acid-free). The cells were washed twice with PBS and incubated in 1 ml/well serum-free efflux medium containing BSA (1mg/ml) and acceptors as indicated for up to 6 h. At the end of efflux, incubation media were collected, centrifuged to remove any detached cells and an aliquot counted for radioactivity. Cells were washed in PBS, lysed in 0.2M NaOH and samples taken for measurement of radioactivity, cholesterol mass and protein content (BCA assay; Pierce). Cell and media samples were extracted and cholesterol, cholesteryl esters and individual phospholipids determined by HPLC as previously described^{7,8}.

Homodimerisation assays

CHO-K1 cells were transiently transfected as described above in 6-well dishes with either hABCG1-myc alone, or hABCG1-myc plus hABCG1-V5. Non-transfected cells were also included as a control. A transient transfection system was used to co-express both myc- and V5-tagged hABCG1. The total amount of DNA was kept constant between conditions at 1 µg/well (5 µl lipofectamine/well). After 24 hours, the cells were washed twice with PBS and transferred to lysis buffer (10 mM Tris, 1% sodium cholate, 0.1% Triton, 0.1 % SDS, pH

7.5, 0.4 ml/well). Cell lysates (1.5 ml total from 4 wells each) were pre-cleared with 50 μ l protein A/G beads (Pierce) for 45 minutes at 4° C. After pre-clearing, cell lysates were incubated with 1 μ l anti-V5 monoclonal antibody (Invitrogen) for 1 hr at 4° C. The cell lysate/antibody solution was further incubated with 50 μ l of protein A/G beads to immunoprecipitate the V5-hABCG1. The pellet was washed 6 times with lysis buffer and incubated with SDS/PAGE loading buffer (see below) and separated on a 4-12% SDS-PAGE gel (Nupage, Invitrogen). An aliquot of whole cell lysate pre-IP was also run to check for plasmid expression. The IP products were blotted with anti-myc antibody (Invitrogen) to detect the presence of non-specific IP products (non-transfected or myc-only transfected cells) or the V5/myc homodimer (co-transfection).

hABCG1 Antibody generation

A polyclonal antiserum against a 15 amino acid peptide (AA 616-630; DLHCDIDETCHFQKS; Mimotopes) of hABCG1 was generated in sheep (Chemicon, Australia). The antibody was affinity purified using the 15 AA peptide conjugated to Sepharose.

Western blotting.

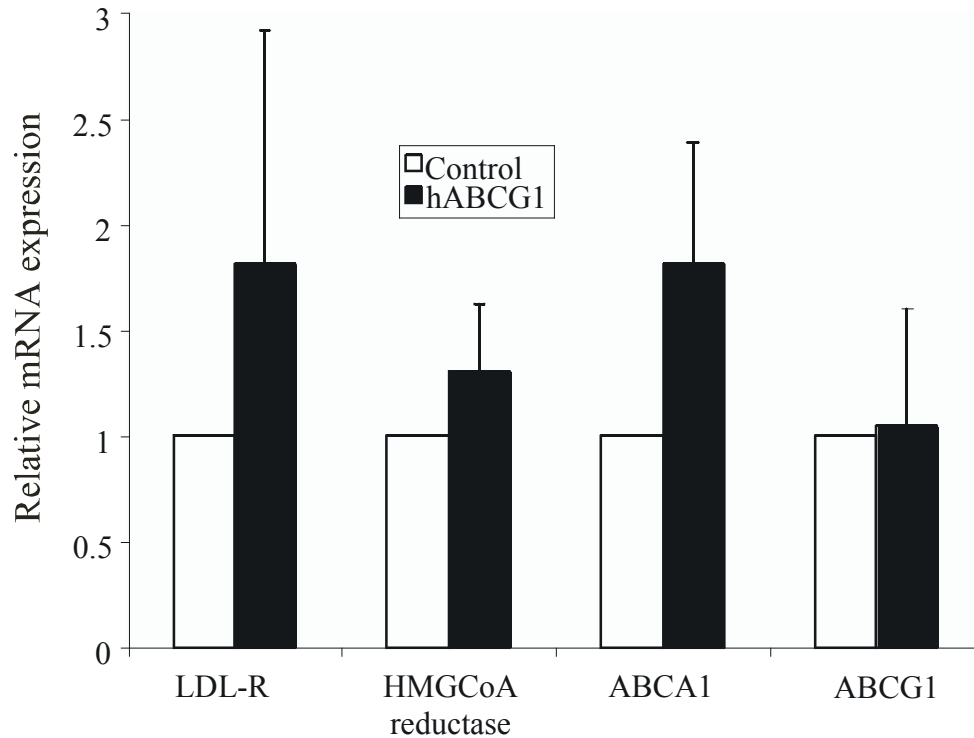
Cells were washed twice with PBS and lysed (10 mM Tris, 0.1 % SDS, 1% sodium cholate, 0.1% Triton, pH 7.5). Equal amounts of cell protein were mixed with 5 x SDS/PAGE loading buffer (150 mM Tris, 50 mM EDTA, 20% glycerol, 5% SDS, 50 mM DTT, 0.025% bromoblu) and separated using precast 4-12% gels (Nupage, Invitrogen). After transfer onto nitrocellulose (Amersham), membranes were blotted using monoclonal anti-myc or anti-V5 antibodies (1:1000; Invitrogen) followed by HRP-conjugated anti-mouse secondary (1:5000; Jackson Laboratories). For native ABCG1, the purified polyclonal anti-hABCG1 antibody was used (1:300; details in supplementary methods), followed by an anti-sheep secondary antibody (1:5000; Jackson Laboratories). ABCA1 was blotted using a polyclonal anti-ABCA1 (Novus; 1:1000) followed by anti-rabbit secondary antibody (1:5000; Jackson Laboratories). Bands were visualised using ECL® (Amersham).

Immunofluorescence microscopy.

Parent and hABCG1-expressing cells were grown on coverslips, fixed in 4% paraformaldehyde and permeabilized with 0.1% saponin and incubated in 1% (w/v) BSA to block nonspecific binding, overnight at 4 °C with anti-myc (1:200; Invitrogen) followed by anti-mouse-FITC (Sigma; 1:100), both in 1% BSA. Cells were mounted in mounting media containing anti-fade agents (Biomedica). Fluorescent images were collected using a Leica TCS SP confocal DMIRB inverted microscope.

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Supplementary Figure I

mRNA expression of LDL-receptor, HMGC0A reductase, ABCA1 and ABCG1 (native hamster) in control and hABCG1 expressing CHO cells. Results are mean + standard deviation of four separate experiments. No significant differences, using paired t-tests (significance $p < 0.05$) were observed between control and hABCG1 expressing cells.

Supplementary Table I.

Primer sequences used for plasmid construction, RT-PCR and QRT-PCR.

Gene	Accession Number	Direction	Primer Sequence	Predicted PCR product	Reference
hABCG1 plasmids (nested PCR)	NM_016818	Outside Forward Outside Reverse Inside Forward Inside Reverse	5' AGCCAGCGCAGCCTCGGC 3' 5' CTCGATTCTAGACGTGCCC 3' 5' ATGGCCGCTTCTCGGTCG 3' 5' CCTCTCTGCCCCGATTTTG 3'	2 kB 2 kB	Present study
ABCA1* (hamster)	NM_013454	Forward Reverse	5' ATAGCAGGCTCCAACCCTGAC 3' 5' GGTACTGAAGCATGTTTCGATGTT 3'	103 bp	Field <i>et al.</i>
ABCG1* (hamster)	AF323659	Forward Reverse	5' GGGATCAGAACAGTCGCCTG 3' 5' CGAGGTCTCTCTTATAGTCAGCGTC 3'	72 bp	Field <i>et al.</i>
LDL receptor (hamster)	M94387.1	Forward Reverse	5' AAGGAGAAGGACACTGTTCC 3' 5' ATGCTGGAGATAGAGTGGAG 3'	246 bp	Present study
HMG-CoA Reductase (hamster)	X00494.1	Forward Reverse	5' CTGGTGATGGGAGCTTGCTGTG 3' 5' AATCACAAGCACGAGGAAGACG 3'	244 bp	Present study
PBGD* (hamster)	NM_013551	Forward Reverse	5' AGATTCTTGATACTGCACTC 3' 5' TGAAAGACAACAGCATCACA 3'	192 bp	Present study
hABCG1	NM_207630	Forward Reverse	5' ACGCAGTTCTGCATCCTCTTC 3' 5' TGTCAGAACAGTAGGCATGAG 3'	222 bp	Wong <i>et al.</i>

* Published mouse primers were used for hamster PBGD as no hamster mRNA sequence available. Primers for ABCA1 and ABCG1 were also derived from mouse and used by others (Field *et al.*).

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Wong, J., Quinn, C.M. & Brown, A.J. (2004) *Arterioscler Thromb Vasc Biol* **24**;2365-71.