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High glucose, unsaturated and saturated fatty acids differentially regulate expression of ATP-binding cassette transporters ABCA1 and ABCG1 in human macrophages

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Abbreviations: CVD, cardiovascular disease; FFA, free fatty acids; LXR, liver-X-receptor; RXR, retinoic acid receptor; TLDA, TaqMan low-density arrays

Abstract

The ATP-binding cassette transporters ABCA1 and ABCG1 are highly expressed in macrophage-derived foam cells and promote reverse cholesterol efflux via biogenesis of high-density lipoproteins. The aim of this study was to analyze the direct effects of bioactive factors related to the metabolic syndrome on macrophage transcript levels of all 47 human ABC transporters. Using *in vitro* M-CSF predifferentiated macrophages and TaqMan low density arrays we could show that linoleic acid, palmitic acid, and high glucose levels have a major impact on ABCA1 and ABCG1 expression but do not strongly affect most other human ABC transporters. In Western blot experiments we demonstrate that ABCA1 and ABCG1 protein levels are synchronously suppressed by high glucose levels and the ω6-unsaturated fatty acid linoleic acid. We conclude that metabolites associated with the metabolic syndrome enhance the formation of atherosclerotic lesions by diminishing the reverse cholesterol transport function of ABCA1 and ABCG1.

Keywords: ABCG1 protein, human; atherosclerosis; ATP binding cassette transporter 1; gene expression profiling; macrophage; metabolic syndrome X

Introduction

The metabolic syndrome is a concurrence of several metabolic risk factors (glucose intolerance, central obesity, elevated blood pressure and dyslipidemia) in one individual with increasing prevalence in Western populations. Importantly, patients with the metabolic syndrome have a significantly elevated risk of developing both type 2 diabetes and cardiovascular disease (CVD). One striking feature is atherogenic dyslipidemia, which is characterized by an elevation of triglycerides, free fatty acids (FFA) and proatherogenic small dense LDL particles, while anti-atherogenic HDL are decreased. In addition, this disordered lipid metabolism and high levels of FFA contribute to the development of insulin resistance, another important feature of the metabolic syndrome (Lee *et al*., 2006; Savage *et al*., 2007).

Atherosclerosis is histopathologically characterized by an accumulation of foam cells in the vessel wall. These foam cells are monocyte-derived macrophages accumulating large amounts of modified (e.g. oxidized) LDL. Under physiological conditions, macrophages have a high capacity for HDL-dependent reverse cholesterol transport. The formation of mature HDL particles mainly depends on the proper function of two cooperating ATP-binding cassette (ABC) transporters, ABCA1 and ABCG1 (Schmitz and Langmann, 2005). Thus, a high expression of these transporters counteracts the development of atherosclerotic plaques. Interestingly, diets enriched in polyunsaturated fatty acids, e.g. linoleic acid, have been shown to lower total cholesterol mainly at the expense of a decreased HDL-cholesterol concentration in monkeys and humans (Khosla and Hayes, 1992). In contrast, diets rich in saturated fatty acids, e.g. palmitic acid, had no such effect. Whether this is due to an altered ABC-transporter expression has not been explored yet.

The transcriptional regulation of ABCA1 and ABCG1 by modified lipoproteins and oxysterols via the nuclear receptors liver-X-receptor (LXR) and retinoic acid receptor (RXR) has been studied

Table 1. ATP-binding cassette transporter mRNA expression in monocyte-derived macrophages differentiated in the presence of M-CSF for 5 days (5d Mac) or 4 day predifferentiated cells which were stimulated with insulin (Ins), palmitic acid (PA), linoleic acid (LA), 9-cis-retinoic acid (RA), T0901317 (T0), and high-dose glucose (Glc) for further 24 h.

Gene	5d Mac	Ins	PA	LA	RA	T0	Glc
ABCA1	••	••	•••	••	•••	•••	•
ABCA2	••	••	••	••	•••	•••	••
ABCA3	••	•	•	•	•	•	•
ABCA4	-	-	-	-	-	-	-
ABCA5	••	••	••	••	••	••	••
ABCA6	••	••	••	••	••	••	••
ABCA7	•	•	•	•	••	••	•
ABCA8	-	-	-	-	-	-	-
ABCA9	•	•	•	•	•	•	•
ABCA10	-	-	-	-	-	-	-
ABCA12	-	-	-	-	-	-	-
ABCA13	-	-	-	-	-	-	-
ABCB1	-	-	-	•	••	••	-
ABCB2	••	••	••	••	••	••	••
ABCB3	••	••	••	••	••	••	••
ABCB4	•	-	•	•	•	•	•
ABCB5	-	-	-	-	-	-	
ABCB6	•	•	••	••	•	•	•
ABCB7	•	•	•	•	•	•	•
ABCB8	•	•	•	•	•	•	•
ABCB9	•	•	•	•	•	•	•
ABCB10	••	••	••	••	••	••	••
ABCB11	-	-	-	-	-	-	-
ABCC1	••	••	••	••	••	••	••
ABCC2	•	•	•	•	••	••	•
ABCC3	••	••	••	••	••	••	••
ABCC4	••	••	••	••	••	••	••
ABCC5	••	••	••	••	••	••	••
ABCC6	•	•	•	•	•	•	•
ABCC7	-	-	-	-	-	-	-
ABCC8	-	-	-	-	-	-	-
ABCC9	-	-	-	-	-	-	-
ABCC10	••	••	••	••	••	••	••
ABCC11	-	-	-	-	-	-	-
ABCD1	••	••	••	••	•••	•••	••
ABCD2	-	-	-	-	-	-	-
ABCD3	••	••	••	••	••	••	••
ABCD4	••	••	••	••	•••	•••	••
ABCE1	••	••	••	••	••	••	••
ABCF1	•	•	•	•	•	•	•
ABCF2	••	••	••	••	••	••	••
ABCF3	•	•	•	•	•	•	•
ABCG1	••	••	•••	••	•••	•••	•
ABCG2	•	•	•	•	•	•	•
ABCG4	-	-	-	-	-	-	-
ABCG5	-	-	-	-	-	-	-
ABCG8	-	-	-	-	-	-	-

high	•••	medium	••	low	•	absent	-

extensively (Langmann *et al*., 1999; Klucken *et al*., 2000; Venkateswaran *et al*., 2000; Fu *et al*., 2001; Kennedy *et al*., 2001). In contrast, little information is available on the influence of plasma metabolites prevalent in the metabolic syndrome on ABC-transporter gene expression. Therefore, our aim was to study the influence of insulin, glucose, and saturated as well as unsaturated fatty acids on ABC-transporter gene expression in human monocyte-derived macrophages. For comparison, cells were cultured in the presence of the synthetic LXR/RXR-ligands T0901317 and 9-cis retinoic acid as known ABCA1/G1 transcriptional regulators.

Results

Macrophage expression profile of all 47 human ATP-binding cassette transporter genes following stimulation with bioactive metabolic factors

TaqMan low-density arrays (TLDA) for real-time quantitative mRNA analysis were performed from three independent stimulation experiments with cells from independent blood donors. Monocytes were predifferentiated to macrophages for 4 days and were further incubated with different metabolic stimuli for 24 h. The effects of insulin, palmitic acid, linoleic acid, 9-cis-retinoic acid, the LXR-ligand

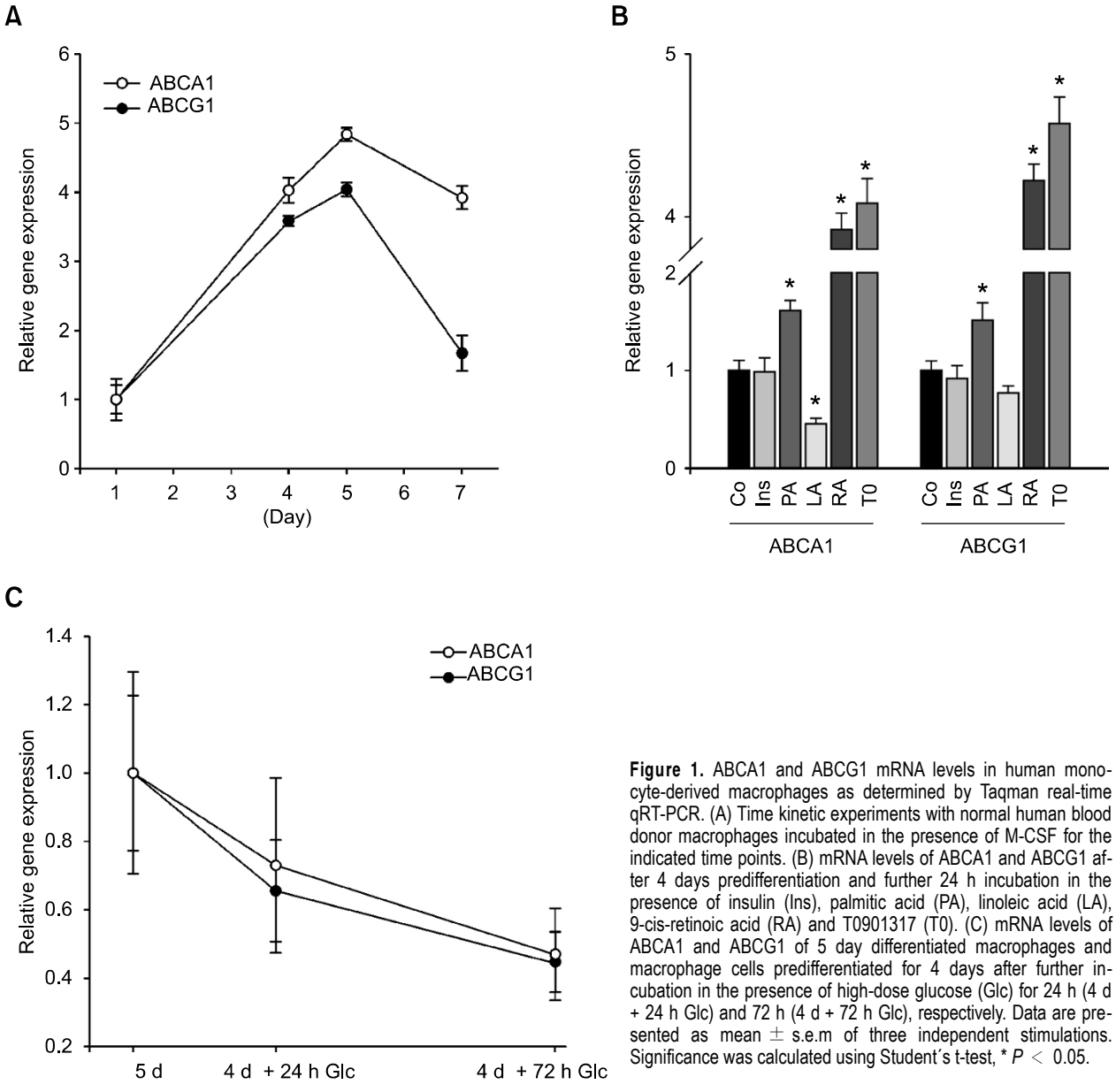

Figure 1. ABCA1 and ABCG1 mRNA levels in human monocyte-derived macrophages as determined by Taqman real-time qRT-PCR. (A) Time kinetic experiments with normal human blood donor macrophages incubated in the presence of M-CSF for the indicated time points. (B) mRNA levels of ABCA1 and ABCG1 after 4 days predifferentiation and further 24 h incubation in the presence of insulin (Ins), palmitic acid (PA), linoleic acid (LA), 9-cis-retinoic acid (RA) and T0901317 (T0). (C) mRNA levels of ABCA1 and ABCG1 of 5 day differentiated macrophages and macrophage cells predifferentiated for 4 days after further incubation in the presence of high-dose glucose (Glc) for 24 h (4 d + 24 h Glc) and 72 h (4 d + 72 h Glc), respectively. Data are presented as mean ± s.e.m of three independent stimulations. Significance was calculated using Student´s t-test, * $P < 0.05$.

T0901317, and glucose were analyzed in comparison to untreated, age-matched control samples. The TLDA results are displayed as a combination of dots and a color code for high (red), medium (yellow), low (blue) and very low (white) expression, which are based on individually calculated △Ct values [Ct (target gene)-Ct (18S rRNA)] (Table 1). For this analysis, the range between the lowest △Ct value and a △Ct of 25 was divided linearly into three groups for high, medium and low expressions. Genes with a △Ct above 25 cycles were defined as absent. For external calibration, ABC-transporter mRNA levels were also measured in a total RNA pool mixed from several human tissues (universal calibrator). When analyzing the relative expression in 5 day cultured macrophages, we detected transcripts for 31 ABC-transporters, with low to medium levels (Table 1). In concordance with earlier data from our group, 4 day M-CSF predifferentiation and further 24 h incubation with 9-cis-retinoic acid and T0901317 induced mRNA levels of ABCA1, ABCA2, ABCA7, ABCC2, ABCD1, ABCD4, and ABCG1. Interestingly, addition of the metabolic compounds insulin, palmitic acid, linoleic acid, and glucose to the medium showed a prominent effect on the expression of both ABCA1 and ABCG1. Thus, palmitic acid induced ABCA1/ABCG1 expression while glucose reduced both transcript levels. In addition, ABCA3 and ABCB6 were also differentially regulated with the above mentioned stimuli.

Time-dependent mRNA expression of ABCA1 and ABCG1 following stimulation with bioactive metabolic factors

Based on the TLDA data and their known role in the development of atherosclerosis, we focused on ABCA1 and ABCG1 for a detailed single sample Taqman analysis of macrophage differentiation and metabolic stimulation. In a time series experiment, monocyte-derived macrophages were cultured in M-CSF-containing medium for 1 day (1 d), 4 days (4 d), 5 days (5 d), and 7 days (7 d). ABCA1 and ABCG1 were not expressed in human monocytes (data not shown) and the quantification during macrophage differentiation showed a steep increase until day 5 with a decrease in expression until day 7 (Figure 1A). This indicated that our culture system with stimulation on day 4 resulted in matured macrophages with high ABCA1 and ABCG1 levels. Our single assay analysis fully confirmed the TLDA results and we were able to measure a significant increase of ABCA1 and ABCG1 by palmitic acid, whereas linoleic acid significantly suppressed ABCA1 transcript levels and showed a tendency for reduced expression of ABCG1. The positive controls 9-cis-retinoic acid and T0901317 strongly increased ABCA1 and ABCG1 mRNA levels (Figure 1B). Since our TLDA analysis indicated only a moderate effect of high glucose levels on both genes, a time kinetic experiment was performed. As depicted in Figure 1C, prolonged incubation in the presence of glucose caused only moderate expression changes but continously decreased ABCA1 and ABCG1 transcript levels.

Protein expression of ABCA1 and ABCG1 following stimulation with bioactive metabolic factors

To recapitulate our interesting mRNA findings on the protein level, we performed western blot analysis with independent macrophage cell extracts.

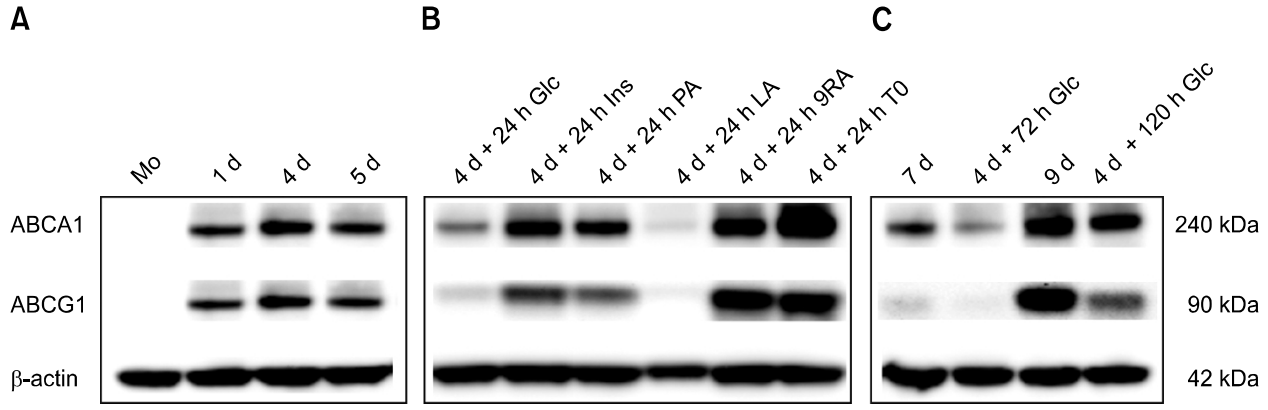

Figure 2. ABCA1 and ABCG1 protein levels in human monocyte-derived macrophages determined by Western blot analysis. (A) Time kinetic experiments with normal human blood donor macrophages incubated in the presence of M-CSF for the indicated time points. (B) Protein levels of ABCA1 and ABCG1 in 4 days predifferentiated macrophages after incubation in the presence of glucose (Glc), insulin (Ins), palmitic acid (PA), linoleic acid (LA), 9-cis-retinoic acid (RA) and T0901317 (T0) for 24 h. (C) Protein levels of ABCA1 and ABCG1 in 7 d and 9 d control incubated macrophages or cells after incubation in the presence of high-dose glucose for 72 (4 d + 72 h Glc) and 120 h (4 days + 120 h Glc), respectively.

ABCA1 and ABCG1 protein levels strongly increased during macrophage differentiation until day 5 (Figure 2A). Incubation of 4 d predifferentiated macrophages with insulin and palmitic acid increased the protein content, whereas linoleic acid markedly suppressed the amount of both proteins during a 24 h culture phase (Figure 2B). We also performed long term glucose culture experiments (Figure 2C), partially reflecting the mRNA data (Figure 1C). Macrophages treated with high glucose for 72 h and 120 h displayed a markedly decreased ABCA1 and ABCG1 expression profiles, when compared to control incubated cells (Figure 2C).

Discussion

In this study, we have shown that different metabolic factors elevated in the metabolic syndrome and type 2 diabetes affect the expression of ABCA1 and ABCG1 in primary human monocyte-derived macrophages and may thus contribute to the development of atherosclerotic lesions in these conditions. In contrast, most of the other ABC transporters were not influenced by metabolic stimuli and therefore may not be involved in the pathogenesis of CVD. We showed that linoleic acid, an ω6 unsaturated fatty acid, suppressed ABCA1 and ABCG1 transcripts and protein levels in human macrophages. In contrast, the saturated fatty acid palmitic acid had the opposite effect. Our data with primary human macrophages are in line with previous experiments employing murine and human macrophage cell lines (Uehara *et al*., 2002, 2007). Interestingly it has been reported, that in monkeys a diet rich in saturated palmitic acid leads to higher total cholesterol and HDL levels compared with a diet rich in polyunsaturated linoleic acid (Rudel *et al*., 1990; Khosla and Hayes, 1992). The reduction in total cholesterol was mainly due to a reduction of HDL cholesterol. This resulted in a higher total cholesterol/ HDL (TC/HDL) ratio, which has been shown to be highly correlated with ischemic heart disease (Lewington *et al*., 2007). As our findings show that palmitic acid has a stimulating effect on ABCA1 and ABCG1 expression it is tempting to speculate that dietary intake of palmitic acid could improve TC/HDL ratio. However, Hodson *et al*. (2001) even found a slightly decreased TC/HDL ratio in young adults eating self-selected diets either rich in saturated or polyunsaturated fatty acids. This may be due to the fact that the free-selected diet considerably differed in their fatty acid compositions compared to standardized diets under laboratory conditions, i.e. the PUFA diet contained almost as much palmitic acid as the SFA diet.

Importantly, our incubation experiments with high levels of glucose identified a clear reduction of both ABCA1 and ABCG1 mRNA and protein expression. These results are consistent with findings in experimental type 2 diabetic mice, which showed diminished ABCG1 expression in peritoneal macrophages (Mauldin *et al*., 2006). Supporting evidence for our data in humans comes from a recent study showing a negative correlation between blood glucose concentration and leukocyte ABCA1 levels (Albrecht *et al*., 2004). In addition to glucose, advanced glycated end products (AGEs), which are generated during persistent hyperglycemia, could be involved in destabilizing the ABCA1 protein (Passarelli *et al*., 2005).

Our data on the synchronous regulation of ABCA1 and ABCG1 by metabolic factors clearly point out a synergistic effect of dysregulated ABCA1 and ABCG1 in the pathophysiology of atheroslerosis. This hypothesis is corroborated by studies with $Abca1^{-/-}$ and $Abcg1^{-/-}$ knockout mice. Thus, LDL-receptor deficient mice transplanted with $Abca1^{-/-}$ $Abcg1^{-/-}$ double-knockout bone marrow displayed increased foam cell accumulation, inflammatory cell infiltration and apoptosis resulting in enhanced atherosclerosis (Yvan-Charvet *et al*., 2007). In addition, reverse cholesterol transport, the primary mechanism by which HDL protects against atherosclerosis (Cuchel and Rader, 2006), is more severely impaired in ABCA1 and ABCG1 double-knockdown macrophages, compared to single knockdown models (Wang *et al*., 2007). In summary, the studies described here provide a first mechanistic link between blood lipid and glucose levels and macrophage reverse cholesterol transport function.

Methods

Monocyte isolation and differentiation

Human peripheral blood monocytes were obtained from three healthy blood donors by leukapheresis and counterflow elutriation. Cells were cultured (5% CO_2 in air, 37°C) on plastic petridishes in macrophage SFM (Gibco, Graud Island, NY) containing 25 ng/ml of recombinant human macrophage colony stimulating factor (M-CSF) (R&D systems, Minneapolis, MN). The cells were allowed to differentiate into macrophages for different indicated time points (1 to 9 days). Then, the M-CSF containing medium was replenished and the stimuli were added as described below.

Macrophage stimulations

Four days predifferentiated macrophages were cultured in the presence of 25 mM glucose, 100 nM insulin, 100 μM

linoleic acid, 100 μM palmitic acid, 10 μM 9-cis retinoic acid, and 10 μM T0901317 or control medium for 24 to 120 h. For RNA isolation, cells were scraped in RLT buffer (Qiagen, Valencia, CA) and stored at -80°C until use. For protein isolation, cells were washed with PBS containing a protease inhibitor cocktail (Calbiochem, La Jolla, CA). After centrifugation, cell pellets were lysed in RIPA buffer (Pierce, Rockford, IL).

RNA isolation and reverse transcription

Total RNA was extracted using the RNeasy Protect Midi Kit (Qiagen). Purity and integrity of the RNA was assessed on the Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip reagent set (Agilent Technologies, Palo Alto, CA). The RNA was quantified spectrophotometrically and then stored at -80°C. cDNAs were generated using the Reverse Transcription System (Promega, Madison, WI).

Analysis of human ATP-binding cassette transporter gene expression by real-time quantitative RT-PCR

2 μl of single-stranded cDNA (equivalent to 100 ng of total RNA) were mixed with 48 μl of nuclease-free water and 50 μl of TaqMan Universal PCR Master Mix (Applied Biosystems, Fosfer City, CA). TaqMan low density arrays (TLDA, Applied Biosystems), which were previously developed by our group (Langmann *et al*., 2006), were loaded with 100 μl of a sample-specific PCR mixture, centrifuged twice for 1 min at 280 *g* and sealed. Real-time quantitative RT-PCR was performed with an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). The thermal cycling conditions were as follows: 2 min at 50°C hold, 10 min at 95°C hold, followed by 40 cycles of 15 s at 97°C melt and 1 min at 60°C anneal/extension. Measurements were carried out in duplicates. Results were analyzed with the ABI sequence detector software (version 2.2) using the ΔΔCt method for relative quantitation. Alle experiments were repeated three times.

Western blot analysis

The protein concentration of cell lysates was determined photometrically using the BCA Protein Assay kit (Pierce) and the concentration was adjusted to 1 μg/L by methanol/chloroform precipitation followed by resuspension in 1× sample buffer containing DTT. Proteins were heat-denatured and equal amounts (15 μg) were loaded and separated on NuPage SDS gels (Invitrogen, Gaithersburg, MD). After transfer, nitrocelluose membranes were blocked with milk powder (1%) for 1 h and were subsequently incubated with primary antibodies for 1 h. For the detection of ABCA1 and ABCG1, a monoclonal anti-human ABCA1 antibody (1:1000) (Abcam, Cambridge, MA) and a polyclonal anti-human ABCG1 antibody (1:1000) (Abcam) were used, respectively. A monoclonal antibody against β-actin (1:40000) (Abcam) served as a loading control. The primary antibodies were detected with a HRP-labled anti IgG secondary antibody (1:20000) (Invitrogen) in a chemoluminescence reaction. All immunoblots were repeated at least three times and representative experiments are shown.

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