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Unsaturated fatty acids suppress the expression of the ATP-binding cassette transporter G1 (ABCG1) and ABCA1 genes via an LXR/RXR responsive element

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Abstract

ATP-binding cassette transporters (ABC) G1 and ABCA1 are membrane cholesterol transporters and have been implicated to mediate cholesterol efflux from cells in the presence of high density lipoproteins and its major protein constituent apolipoprotein A-I, respectively. We previously demonstrated that unsaturated fatty acids suppress the stimulatory effects of oxysterols and retinoids on ABCA1 gene transcription. We here demonstrate that unsaturated fatty acids significantly suppress the stimulatory effects of oxysterols and retinoids on the expression of ABCG1 mRNA and protein and the activity of the wild-type human ABCG1 promoter as well as ABCA1. Mutation or deletion of the DR4 element within the ABCG1 or ABCA1 promoters, to which the transcriptional inducers LXR and RXR bind, abolished the suppressive effects of unsaturated fatty acids. Our observations provide the first evidence that unsaturated fatty acids suppress ABCG1 gene expression by a mechanism which involves the binding of LXR/RXR to the promoters. Suppression of both the ABCA1 and ABCG1 genes may indicate that unsaturated fatty acids suppress not only cholesterol efflux to apoA-I and thereby nascent HDL formation but also HDL-dependent cholesterol efflux from vascular cells.

Keywords: ATP-binding cassette transporter; Cholesterol transport; Diabetes; Unsaturated fatty acid; Highdensity lipoprotein; LXR; RXR; Direct repeat 4

1. Introduction

Dyslipidemia, notably hypertriglyceridemia and low plasma HDL cholesterolemia, is a common phenomenon in diabetes mellitus type 2 and insulin resistance and increases the risk of cardiovascular disease in these patients [1–3]. HDL exerts various potentially anti-atherogenic effects. For example, HDL particles transport cholesterol from cells in the arterial wall to the liver and to steroidogenic organs, in which cholesterol is used for the synthesis of bile acids, lipoproteins, vitamin D, and steroid hormones [2]. The details and the pathogenesis of low HDL cholesterol and the

Abbreviations: ABC, ATP-binding cassette transporter; apo, apolipoprotein; ATP, adenosine triphosphate; BSA, bovine serum albumin; CETP, cholesterol ester transfer protein; CYP7A, 7α-hydroxylase; DR4, direct repeat 4; EPA, eicosapentaenoic acid; LA, linoleic acid; AraA, arachidonic acid; OA, oleic acid; PA, palmitic acid; SA, stearic acid; HDL, high density lipoprotein; LXRα, liver-X-receptor alpha; LXRβ, liver-X-receptor beta; RXRα, retinoid-X-receptor alpha; USFA, unsaturated fatty acid; TG, triglyceride; 9-cis-RA, 9-cis retinoic acid; 22R-HC, 22(R)-hydroxycholesterol

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increased risk for atherosclerosis in insulin resistance are not well understood. Another typical abnormality in type II diabetic patients is elevated plasma levels of free fatty acids [4]. Free fatty acids play an important physiological role in many tissues, for example by inhibiting insulin-mediated glucose uptake and glycogen synthesis. Furthermore, it is well known that the dietary intake of polyunsaturated fatty acids exerts lipid-lowering effects on all lipoprotein classes including LDL, VLDL (i.e. triglycerides) and HDL [5,6].

The ATP-binding cassette transporter (ABC) A1 has previously been identified as a pivotal gene in the regulation of both plasma HDL cholesterol levels and cellular cholesterol homeostasis, which is defective in patients with Tangier disease [2,7–9]. In these patients and their heterozygous relatives, mutations in the ABCA1 gene cause gene-dosage-dependent decreases in plasma levels of HDL cholesterol and in the capacity of skin fibroblasts and monocyte-derived macrophages to release cholesterol in the extracellular presence of apolipoprotein (apo) A-I or apoE [2,7–9]. Clinically, homozygous patients with Tangier disease accumulate macrophage-derived foam cells in various tissues. We previously demonstrated that unsaturated fatty acids downregulate ABCA1 gene expression and inhibit apoA-I-induced cellular cholesterol efflux in cultivated macrophages and hepatocytes [10].

ABCG1 (formerly termed ABC8) is another member of the ATP-binding cassette transporter superfamily which has been mapped to chromosome 21q22.3 [11–16]. By contrast to ABCA1, which is a full ABC transporter with two ATP binding cassettes and transmembrane domains, ABCG1 is a half transporter containing only one ATP binding cassette and one transmembrane domain [14,15]. It is therefore believed to need a dimeric partner for being active. Wang et al. recently reported ABCG1 contributes to HDL2- and HDL3-dependent cellular cholesterol efflux [17].

Several ABC lipid transporters including ABCA1 and ABCG1 have been shown to be strongly regulated by cholesterol-derived oxysterols. They activate nuclear liver X receptors (LXRs) LXRα and LXRβ [18]. LXRα is mainly expressed in the liver, spleen, kidney, adipose tissue, small intestine and macrophages [19], while LXRβ is expressed ubiquitously [20]. LXRs form obligate heterodimers with the retinoid X receptor (RXR). The LXR/RXR heterodimer binds to lipogenic target gene promoters such as 7α-hydroxylase (CYP7A), apoE, cholesterol ester transfer protein (CETP), SREBP1c, ABCA1 and ABCG1, which all contain a direct repeat 4 (DR4) element in their promoter, i.e. two direct hexameric repeats separated by four nucleotides [19].

We demonstrate here that free unsaturated fatty acids (USFAs) strongly suppress the activity of both the ABCG1 and ABCA1 promoters, and elucidate the mechanism by which USFAs suppress transcription of the ABCG1 and ABCA1 genes.

2. Materials and methods

Materials. 22(R)-hydroxycholesterol, 9-cis-retinoic acid, Phorbol 12-myristate 13-acetate (PMA), eicosapentaenoic acid (EPA), linoleic acid (LA), arachidonic acid (AraA), oleic acid (OA), palmitic acid (PA), and stearic acid (SA) were purchased from Sigma (St. Louis, MO). Fatty acid- and endotoxin-free bovine serum albumin (BSA) was purchased from Calbiochem.

Cell culture. Cells from the murine macrophage cell line RAW264 and THP-1 human monocyte (RIKEN, Japan) were cultured in DMEM or RPMI1640 medium containing 10% fetal bovine serum (GIBCO-BRL) 100 units/ml penicillin G and 100 μg/ml streptomycin. The THP-1 cells were treated with PMA for 72 h before experiment for differentiation to macrophage. For all experiments, cells were maintained in serum-free medium which contained 10 g/l bovine serum albumin (BSA) and was supplemented with or without additives (22(R)-hydroxycholesterol, 9-cis-retinoic acid, or fatty acids) at concentrations and time periods described in Section 3 and in the figure legends. A 100 mmol/l stock solution of each fatty acid was prepared by diluting the fatty acid in DMSO. Fatty acid was added from the stock solution bound to 100 times volume of 10% BSA/medium solution and was adjusted to 10 g/l of BSA at final concentration by adding medium.

2.1. Reporter plasmid constructs

2.1.1. Human ABCG1 promoter constructs

Firefly luciferase reporter constructs were generated by amplifying a 1141-bp fragment of the human ABCG1 promoter A (−1104/+37) and a 1330-bp fragment of the human ABCG1 promoter B (−1264/+66) with *Kpn*I and *Xho*I restriction sites out of genomic DNA by PCR amplification with primer hABCG1 promoter A and hABCG1 promoter B, respectively (primer sequences are given in Table 1). PCR products were digested with *Kpn*I and *Xho*I, gel-purified, and ligated to *Kpn*I- and *Xho*I-digested pGL3 Basic vectors (Promega, Madison, WI).

2.1.2. Truncated human ABCG1 promoter constructs

The deleted constructs, −607/+37, −530/+37, −413/+37, −303/+37, −233/+37, −163/+37 and −233/−122 of the human ABCG1 promoter A upstream of exon 1 were generated by PCR with hABCG1 promoter A-607, A-530, A-413, A-303, A-233 or A-163 for the sense primer and hABCG1 promoter A for the antisense primer, and hABCG1 promoter A-233 for the sense primer and hABCG1 promoter A-122 for the antisense primer, respectively (primer sequences are given in Table 1).

2.1.3. Human ABCA1 promoter constructs

Firefly luciferase reporter constructs were generated by amplifying a 980-bp fragment of human ABCA1 promoter as described previously [10].

Table 1
Sequences of PCR primers

Primer name	Primer sequence
hABCG1 promoter A sense	5′-aag ggg tac cat gaa tga aag aag cca gac aca aa-3′
hABCG1 promoter A-607 sense	5′-aag ggg tac ctt gtt acc agg ttt ctg cta agc tc-3′
hABCG1 promoter A-530 sense	5′-aag ggg tac cgc tgc aat aat cat tgg cta gag g-3′
hABCG1 promoter A-413 sense	5′-aag ggg tac ccc gta att gtt ttc aat gtg tgt t-3′
hABCG1 promoter A-303 sense	5′-aag ggg tac ccc ttc tgt gga cag gta cta ggt g-3′
hABCG1 promoter A-233 sense	5′-aag ggg tac cta acc ctg ctc act tcc tgt ttt c-3′
hABCG1 promoter A-163 sense	5′-aag ggg tac ctc atg ctg att tta aaa caa tcc-3′
hABCG1 promoter A antisense	5′-cct atc cgc tcg agc aca aac ata ggt agt cca gct gc-3′
hABCG1 promoter A-122 antisense	5′-cct atc cgc tcg agg tgt aat gct aca ggg agg attg-3′
hABCG1 promoter B sense	5′-aag ggg tac cgg atg act ctt gag aca aca cca tt-3′
hABCG1 promoter B antisense	5′-cct atc cgc tcg agc ggt gcc gac cga gaa agc-3′
hABCG1 DR4mut sense	5′-ggg agg gaa cag aac ccc cct tta gaa acc ctg ctc ac-3′
hABCG1 DR4mut antisense	5′-gtg agc agg gtt tct aaa ggg ggg ttc tgt tcc ctc cc-3′
hABCA1 DR4mut sense	5′-gag agc aca ggc ttt gtg tga tag tac tat ctg cgc tcg g-3′
hABCA1 DR4mut antisense	5′-ccg agc gca gat agt act atc aca caa agc ctg tgc tct c-3′
ABCG1 sense	5′-gga gaa tgc gaa gct gta cc-3′
ABCG1 antisense	5′-gga ggc ggt ttt tac ctc tc-3′
LXRα sense	5′-gat ctg gga tgt cca cga gt-3′
LXRα antisense	5′-ccc ttc tca gtc tgc tcc ac-3′
RXRα sense	5′-tcc ttc tcc cac cgc tcc atc-3′
RXRα antisense	5′-cag ctc cgt ctt gtc cat ctg-3′
GAPDH sense	5′-aac gac ccc ttc att gac-3′
GAPDH antisense	5′-tcc acg aca tac tca gca c-3′

2.1.4. Site-directed mutagenesis

Mutant plasmids were obtained for the human ABCG1 promoter A (1141 bp) and human ABCA1 promoter (980 bp) by using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and each of the following double-stranded oligonucleotides: ABCG1; hABCG1 DR4mut sense, hABCG1 DR4mut antisense, ABCA1; hABCA1 DR4mut sense, hABCA1 DR4mut antisense (the DNA sequences are shown in Table 1). The following point mutations were made at the DR4 site (underlined), ABCG1: TGCCCT TTAG TAACCC → CCCCCT TTAG AAACCC; ABCA1: TGACCG ATAG TAACCT → TGTGTG ATAG TACTAT. The ABCA1 promoter variant containing a mutated DR4 site was obtained as described by Costet et al. [21]. Briefly, 25 ng of the wild-type human ABCG1 promoter A (1141 bp) and ABCA1 promoter (980 bp) plasmids were used in a PCR reaction containing 2.5 μl of *Pfu* polymerase buffer, 1.25 U *Pfu* turbo DNA polymerase, 10 mmol/l dNTPs, 62.5 ng of each oligonucleotide and water to give a reaction volume of 25 μl. The cycle setting for mutagenesis was selected according to the manufacturer's protocol, and the sample was treated with 5 U of *Dpn*I to cleave parental DNA and to improve the efficiency of mutant plasmid screening. The reaction was transferred to XL-1 Blue competent cells, and the transformation mixture was plated on LB ampicillin plates. The identity of all DNA constructs was confirmed by DNA sequencing. All plasmids were purified using Hi-speed Plasmid Midi kit columns (Qiagen).

2.2. Reporter assay

For transfection, 50–80% confluent RAW 264 cells were grown on 24-well plates in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin G and 100 μg/ml streptomycin. Cells were transiently transfected with 1 μg of the firefly luciferase reporter plasmids and co-transfected with 5 ng of phRL-SV40 vector (Promega, Madison, WI) for the control Renilla luciferase by lipofection with 2 μl of LipofectAMINE™ 2000 reagent (Invitrogen, Carlsbad, CA) using 5×10^5 cells per well. After incubation with LipofectAMINE™ 2000 for 6 h, the cells were incubated with 22(R)-hydroxycholesterol, 9-cis-retinoic acid or fatty acids for 6–24 h, as indicated. The luciferase activities were measured using the Dual-Luciferase Assay System™ (Promega). Firefly luciferase activity was divided by Renilla activity to obtain a normalized value: relative luciferase unit (RLU).

2.3. Quantitative real-time PCR assay

2.3.1. RNA isolation and RT-PCR

Total RNA was extracted from cells in 9.6 cm^2 dishes using the RNeasy kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol, and treated with DNase to remove residual contaminating DNA. The RNA was reverse-transcribed and cDNA was amplified by PCR using Superscript II reverse transcriptase and Oligo-dT primer from Invitrogen. The sequences of the primers are listed in Table 1. Mouse ABCG1, LXRα, RXRα and GAPDH gene expression was quantified by real-time PCR using the Applied Biosystems GeneAmp 5700 sequence detection system. Amplification was detected with SYBR Green as a fluorogenic probe specific for double-stranded DNA by using a qPCR-core kit for SYBR-Green (Eurogentec, Herstal, Belgium). Threshold cycle, C_t, which correlates inversely with the target mRNA level, was measured as the cycle number at which the reporter

fluorescent emission increases above a threshold level. Melting curves were recorded and the size and specificity of PCR products were checked on 3.5% agarose gel. Only reactions that produced a single band of the expected size were used for analysis. The mRNA levels for ABCG1, LXRα and RXRα were corrected for GAPDH.

2.4. Detection of ABCG1 by Western blotting

THP-1 cells of two 15 cm dishes were washed in HANKS' balanced salt solution (HBSS) and collected in a 10 mmol/l HEPES with Complete protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). Cell membrane fractions were prepared by three times handling of freeze and thaw. The extracted membrane fraction were lysed in a buffer containing 1% (w/v) Triton-X 100 (Sigma), 10 mmol/l Tris (pH 7.4), 150 mmol/l sodium chloride, 0.5% (w/v) sodium deoxycholic acid, 10 mmol/l PMSF and Complete® cocktail. The lysed cells were denatured by heating at 95 °C for 5 min in buffer containing 100 mmol/l Tris, 8% glycerol and 2% SDS. An equal amount of proteins were electrophoresed in a 7.5% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) microporous membrane (Millipore, MA) by electroblotting. After blocking with 3% skin milk, ABCG1 was probed with a goat anti-human ABC8 antibody (SC-11150) (Santa Cruz Biotechnology, Santa Cruz, CA) and a rabbit anti-mouse ABCG1 antibody (Alpha Diagnostic International, San Antonio, TX). The immunoreaction was visualized after incubation of the PVDF sheets with secondary horseradish peroxidase conjugated anti-rabbit IgG antibodies (Amersham Biosciences, Buckinghamshire, UK) using a ECL Western blotting detection reagent (Amersham).

2.5. Electrophoretic mobility shift assays

Double-stranded oligonucleotide (fragment spanning −264 to −251) containing a LXR/RXR binding site was digested from ABCG1 promoter A construct with *Bst*XI and *Dra*I. The digested 114 bp double-stranded oligonucleotide probe was labeled with biotin using Biotin 3′ End DNA Labeling Kit (Pierce, Rockford, USA). Electrophoretic mobility shift assays (EMSA) were performed by using LightShift® Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's instruction. Labeled double-stranded oligonucleotide probe was incubated with 2.5 μg of nuclear extract from HeLa cells in a buffer containing 100 mM Tris, 500 mM KCl, pH 7.9, 5 mM $MgCl_2$, 10 mM dithiothreitol, 2.5% glycerol, 0.05% Nonidet P-40, and 50 ng/μl of poly(dI-dC) at room temperature for 20 min.

In supershift experiment, nuclear extracts were preincubated with antibodies against LXRα/β (Santa Cruz), on ice for 30 min before addition of probe.

DNA–protein complexes were electrophoresed on a native 5% polyacrylamide gel in 0.25 X tris-boric acid-EDTA (TBE) buffer at 100 V and transferred to a nylon membrane (Amersham Biosciences, Buckinghamshire, UK). The DNA–protein complexes were visualized with chemiluminescent substrate after incubation of the nylon membrane with stabilized streptavidin-horseradish peroxidase conjugate.

3. Results

3.1. Responses of human ABCG1 promoters to agonists for LXR and RXR

ABCG1 is a target gene for LXR [22,23]. The ABCG1 gene has been shown to have two separate promoters which are located upstream of exon 1 and exon 5 and have been designated as promoter A and promoter B, respectively [22]. RAW264 cells were transiently transfected with two kinds of pGL3Basic-wild-type human ABCG1 promoter constructs, and promoter activities were then measured by using a dual-luciferase assay system. Ten μmol/l of 22(R)-hydroxycholesterol and 10 μmol/l of 9-cis-retinoic acid dramatically increased wild-type human ABCG1 promoter A (−1104/+37) activity (Fig. 1A), whereas the LXR/RXR ligands did not affect promoter B (−1264/+66) activity (Fig. 1B). These results indicate that LXR/RXR response element exist only in the promoter upstream of exon 1, but not in that upstream of exon 5. Recently, Kennedy et al. identified three more exons (exons 8–10), and a specific promoter with an LXR responsive element is also found upstream of exon 8 [24]. This promoter may also control transcription from exon 5 on the human ABCG1 gene.

3.2. Effects of various fatty acids on human ABCG1 gene transcription

We next investigated whether fatty acids alter ABCG1 gene transcription. Saturated and unsaturated fatty acids were incubated for 20 h with human ABCG1 promoter-transfected RAW264 macrophages. Mono- and poly-unsaturated fatty acids significantly suppressed human wild-type ABCG1 promoter A activity (eicosapentaenoic acid, −69.6%; linoleic acid, −62.2%; arachidonic acid, −56.7%; oleic acid, −60.0%, respectively), whereas saturated fatty acids did not suppress ABCG1 promoter A activity (Fig. 1C). Furthermore, ABCG1 promoter B activity was not suppressed by unsaturated fatty acids (Fig. 1D). Similar to our previous finding that unsaturated fatty acids downregulate ABCA1 gene transcription [10], unsaturated fatty acids suppressed the human ABCG1 promoter located upstream of exon 1. On the other hand, saturated fatty acids stimulated the transcription activities on both promoter A and promoter B of ABCG1 gene. Stearic acid significantly increased the activities on both promoter A and B, whereas palmitic acid significantly elevated it only in promoter B (Fig. 1C and D).

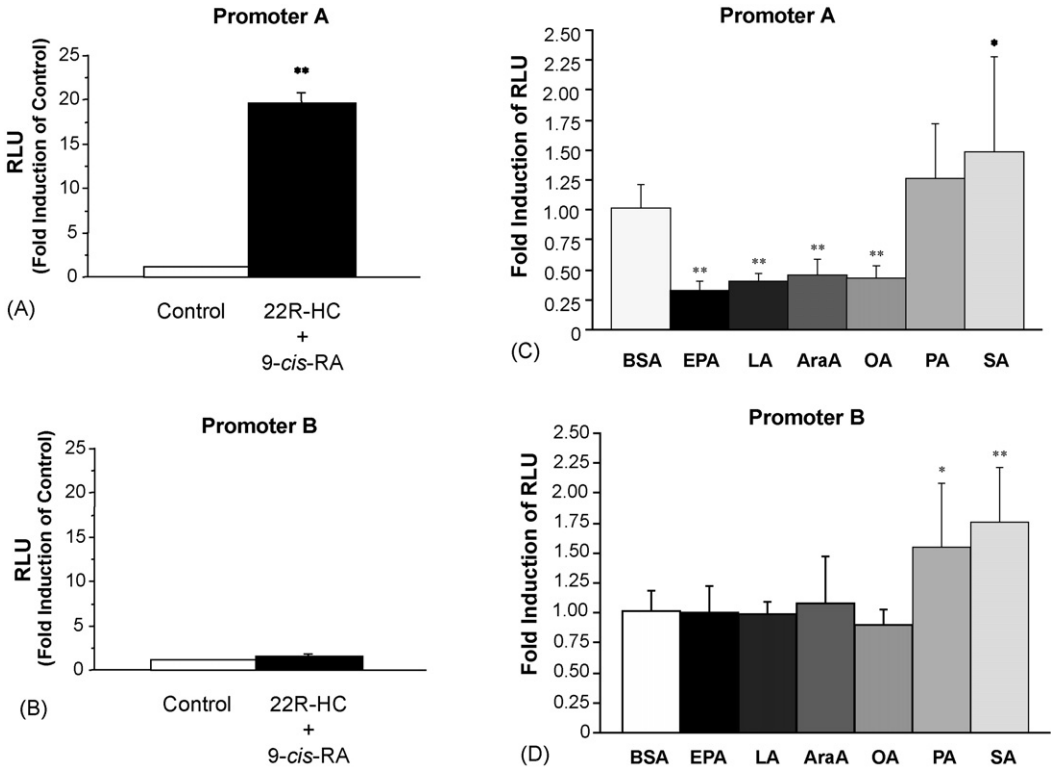

Fig. 1. Effects of 22(R)-hydroxycholesterol, 9-cis-retinoic acid and various fatty acids on the activity of human ABCG1 promoters in RAW264 cells. Wild-type 1141 bp human ABCG1 promoter A upstream of exon 1 (A and C). Wild-type 1330 bp human ABCG1 promoter B upstream of exon 5 (B and D). For experimental details, see Section 2. After transfection, the cells were incubated with 10 μM of 22(R)-hydroxycholesterol and 9-cis-retinoic acid in the presence of 0.5% BSA for 24 h (A and B), and 100 μM of eicosapentaenoic acid (EPA), linoleic acid (LA), arachidonic acid (AraA), oleic acid (OA), palmitic acid (PA) or stearic acid (SA) in the presence of 10 g/l BSA for 20 h (C and D). Control cells were treated with 0.1% DMSO. The luciferase activities were measured using the Dual-Luciferase Assay System™ (Promega). Firefly luciferase activity was divided by Renilla activity to obtain a normalized value as a relative luciferase unit (RLU). Results represent means ± S.D. of at least four independent transfection experiments. $^{**}p < 0.01$, $^{*}p < 0.05$ vs. control; unpaired Student's *t*-test (A and B); one-way ANOVA (C and D).

3.3. mRNA suppressions of ABCG1 by unsaturated fatty acid

Unsaturated fatty acids appeared to suppress ABCG1 gene transcription. To analyse the effects of unsaturated fatty acids, endogenous mRNA levels of ABCG1 were determined in the presence or absence of eicosapentaenoic acid or linoleic acid by real time RT-PCR. Both eicosapentaenoic acid and linoleic acid significantly ($p < 0.01$) suppressed the ABCG1 gene expression (eicosapentaenoic acid, −48.6% and linoleic acid, −49.6%, respectively) (Fig. 2A). Mono-unsaturated fatty acid, oleic acid slightly, but not significantly, decreased the ABCG1 mRNA level.

3.4. Protein suppressions of ABCG1 by unsaturated fatty acid

ABCG1 proteins were analysed by Western blot analysis with two kind of ABCG1 antibodies. Both specific antibodies against ABCG1 were detected ABCG1 proteins in the same molecular weight (Fig. 2B). Mono- and poly-unsaturated fatty acids significantly ($p < 0.01$) suppressed the endogenous ABCG1 protein expressions (eicosapentaenoic acid, −40.5%; linoleic acid, −45.8% and oleic acid, −35.6%, respectively) (Fig. 2B).

3.5. LXR/RXR responsible element on human ABCG1 promoter

As shown in Fig. 1A, the activation of LXR and RXR increased ABCG1 promoter A transcriptional activity. To ascertain the presence and location of a response to LXR/RXR on ABCG1 promoter A, seven different truncated ABCG1 promoters (promoter A) were prepared: six were cut from the 5′ end and one was cut from both 5′ end and 3′ end (Fig. 3). As shown in Fig. 3, while the three shorter promoter constructs (−233/+37, −163/+37 and −233/−122) showed low promoter responses to the LXR and RXR ligands, the response of promoter activities was increased more than 7.7-fold over the control value for the five longer promoter constructs from −1104 to −303 in the presence of LXR and RXR ligands. These results indicate that the LXR/RXR responsible region is located in the human ABCG1 promoter A between −303 and −233.

3.6. Effects of unsaturated fatty acids on several truncated human ABCG1 promoters

To determine the region within the human ABCG1 promoter A that is responsible for unsaturated fatty acid-dependent transcriptional repression, several ABCG1 promoter constructs were prepared and transiently transfected in murine RAW264 macrophage. Two polyunsaturated fatty acids, EPA and LA, were incubated with cells in the presence of 10 g/l of BSA for 20 h after transfection. Interestingly, EPA and LA reduced the transcriptional activities of the five longer promoter constructs, −1104/+37 (EPA, −48.3%; LA, −25.3%), −607/+37 (EPA, −49.9%; LA, −17.3%), −530/+37 (EPA, −77.1%; LA, −54.0%), −413/+37 (EPA, −70.4%; LA, −50.3%) and −303/+37 (EPA, −64.8%; LA, −49.0%). In contrast, EPA and LA did not decrease the activities of the three shorter truncated promoters: −233/+37 (EPA, −5.3%; LA, +33.8%), −163/+37 (EPA, +6.3%; LA, +29.3%) and −233/±122 (EPA, −0.1%; LA, +3.1%), respectively (Fig. 4).

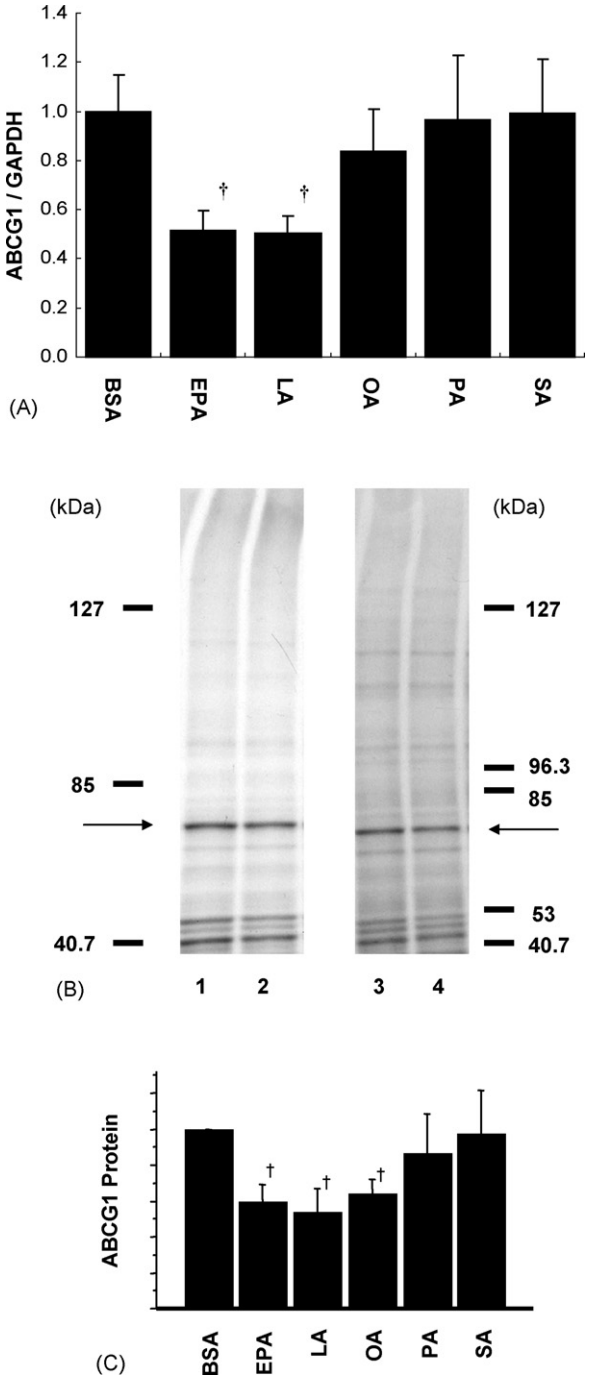

Fig. 2. Effects of saturated and unsaturated fatty acids on the expressions of ABCG1 mRNA and protein. RAW264 and THP-1 cells (A and B) were incubated for 24 h with serum-free medium in the presence of 10 g/l BSA with or without 100 μmol/l of eicosapentaenoic acid (EPA), linoleic acid (LA), oleic acid (OA), palmitic acid (PA) or stearic acid (SA). The mRNA expression of ABCG1 was demonstrated by quantitative real-time PCR. Results represent the means ± S.D. from at least three experiments. $^{\dagger}p < 0.01$ vs. BSA group (one-way ANOVA) (A). ABCG1 protein was analysed by SDS-PAGE and Western blotting of proteins from PMA-treated THP-1 cells. Lanes 1 and 2 represent ABCG1 proteins detected by using the ABCG1 antibody from Santacruz Biotechnology. Lanes 3 and 4 show ABCG1 proteins detected by using the ABCG1 antibody from Alpha Diagnostic International. The PMA-treated cells were incubated in the presence of 10 g/l BSA with or without 100 μmol/l of EPA, LA, OA, PA or SA. The ABCG1 proteins were blotted with the specific antibody of Santacruz Biotechnology. Results represent the means ± S.D. from at least three experiments. $^{\dagger}p < 0.01$ vs. BSA group (one-way ANOVA) (B).

3.7. LXR/RXR responsive element on point-mutated human ABCG1 promoter

As shown in Fig. 3, we investigated the LXR/RXR responsive region located in human ABCG1 promoter A between −303 and −233. To determine more precisely the nucleotide sequence required for binding of LXR/RXR heterodimer, we prepared a point-mutated ABCG1 promoter A construct.

The DR4 element, LXR/RXR binding site is characterized by two direct hexameric repeats as AGGTCA or its similar arrangement separated by four nucleotides, i.e. AGGTCA XXXX AGGTCA. Therefore, the full-length 1141 bp wild-type and mutant promoter constructs (−1104/+37) are described in Fig. 5A. The sequence $^{-243}$ccC CCT TTA Ga^{-233} has three single mutations compared to the wild-type sequence $^{-243}$TGC CCT TTA GT^{-233} to leave the function out completely. 22(R)-hydroxycholesterol and 9-cis-retinoic acid stimulated the luciferase activity of wild-type human ABCG1 promoter A. However, DR4 (an LXR/RXR binding element)-mutated promoter construct did not respond to the LXR and RXR ligands (Fig. 5B).

3.8. Effect of eicosapentaenoic acid on the point-mutated human ABCG1 promoter

As mentioned above, unsaturated fatty acids suppressed ABCG1 promoter activity of wild-type and truncated promoter constructs containing a DR4 element. Moreover, ABCG1 promoter B or deleted promoter A constructs (−233/+37, −163/+37 and −233/−122), which do not have

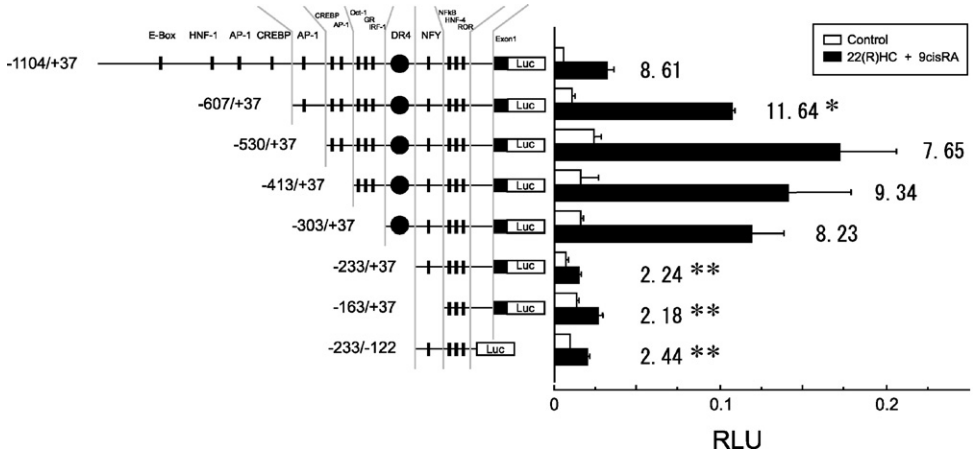

Fig. 3. Effects of LXR and RXR ligands on deletion mutants of human ABCG1 promoters. RAW264 cells were transfected with 5 ng of phRL-SV40 vector and 1 μg of pGL3 basic-ABCG1 promoter (promoter A; upstream of exon 1) vectors containing 1141 bp promoter and truncated 5′-region of the ABCG1 gene. After transfection, the cells were incubated with 5 μM of 22(R)-hydroxycholesterol and 9-cis-retinoic acid in the presence of 0.5% BSA for 20 h. Control cells were treated with 0.1% DMSO. The luciferase activities were measured using the Dual-Luciferase Assay System™ (Promega). Firefly luciferase activity was divided by Renilla activity to obtain a normalized value as a relative luciferase unit (RLU). Results represent means ± S.D. of at least four independent transfection experiments. $^{**}p < 0.01$, $^{*}p < 0.05$ vs. full length promoter construct, −1104/+37 (one-way ANOVA).

the LXR/RXR response element, are not activated by LXR or RXR ligands. These observations suggest that the mechanism of the suppression by unsaturated fatty acids may be related to the stimulation of the promoter by LXR/RXR activators. To identify the domain responsive to the suppressive effects of unsaturated fatty acid, a point-mutated ABCG1 promoter construct was obtained. As expected, eicosapentaenoic acid reduced the luciferase activity of wild-type promoter, but not that of DR4 element-mutated ABCG1 promoter A (Fig. 5C). Unsaturated fatty acids appeared to suppress ABCG1 gene transcription in relation to the specific region for binding to LXR/RXR. Thereafter, the mRNA levels of LXRα and RXRα were determined. Neither unsaturated nor saturated fatty acids affected the gene expression of LXRα and RXRα in murine RAW264 macrophage (data not shown).

3.9. Effect of eicosapentaenoic acid on human ABCA1 promoter with or without mutation of the DR4 site

We previously reported that unsaturated fatty acids inhibit human ABCA1 gene transcription [10], but the exact mechanisms are still unclear. Therefore, to determine more precisely the mechanisms by which unsaturated fatty acids suppress ABCA1 gene transcription, the point-mutated 0.98 kb ABCA1 promoter construct was prepared as described previously. 22(R)-hydroxycholesterol elevated the transcriptional

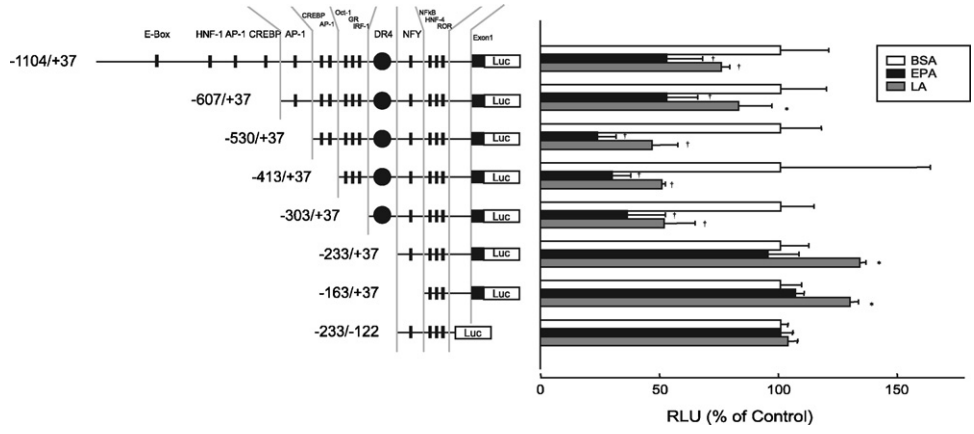

Fig. 4. Effects of unsaturated fatty acids on deletion mutants of human ABCG1 promoters. RAW264 cells were transfected with 5 ng of phRL-SV40 vector and 1 μg of pGL3 basic-ABCG1 promoter (promoter A; upstream of exon 1) vectors containing 1141 bp promoter and truncated 5′-region of the ABCG1 gene. After transfection, the cells were incubated with 100 μmol/l of eicosapentaenoic acid (EPA) and linoleic acid (LA) in the presence of 10 g/l BSA for 20 h. The luciferase activities were measured using the Dual-Luciferase Assay System™ (Promega). Firefly luciferase activity was divided by Renilla activity to obtain a normalized value as a relative luciferase unit (RLU). Results are expressed as the fold induction over the control value for each construct and represent means ± S.D. of at least four independent transfection experiments. $^{\dagger}p < 0.01$, $^{*}p < 0.05$ vs. control (one-way ANOVA).

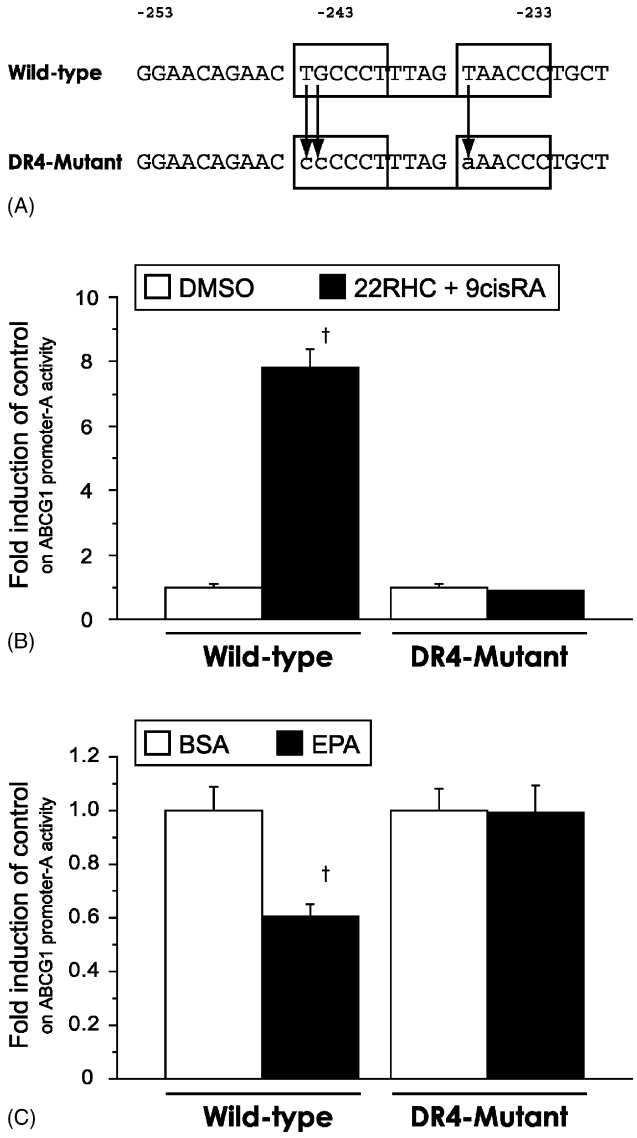

Fig. 5. Mutational analysis of *hABCG1* promoter. RAW264 cells were transfected with wild-type *hABCG1* promoter (from −1104 to +37 bp) or DR4-mutated promoter. The mutations are shown in Fig. 5A and Section 2. After transfection, the cells were incubated with 10 μmol/l of 22(R)-hydroxycholesterol and 9-cis-retinoic acid in the presence of 0.5% BSA for 20 h. Control cells were treated with 0.2% DMSO (B), the cells were incubated with 100 μmol/l of eicosapentaenoic acid (EPA) in the presence of 10 g/l BSA for 20 h. Control cells were treated with 10 g/l BSA and 0.1% DMSO (C). The luciferase activities were measured using the Dual-Luciferase Assay System™ (Promega). Firefly luciferase activity was divided by Renilla activity to obtain a normalized value as a relative luciferase unit (RLU). The RLU are shown in fold change compared with control (DMSO or BSA). The results represent four independent transfection experiments of duplicates. Bars indicate mean ± S.D. $^{\dagger}p < 0.001$ vs. treatment with BSA and DMSO (unpaired Student's *t*-test).

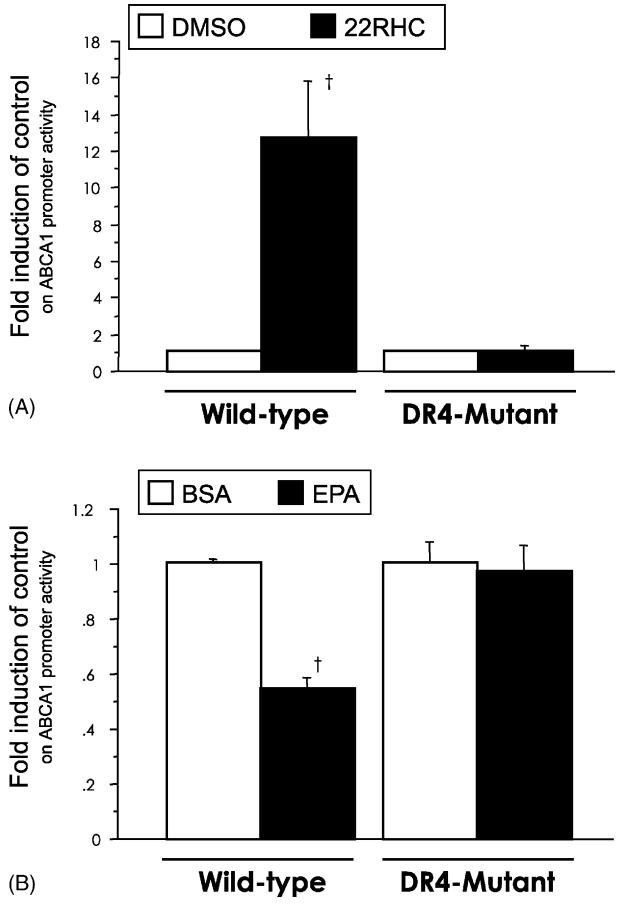

Fig. 6. Mutational analysis of *hABCA1* promoter. RAW264 cells were transfected with wild-type *hABC1* promoter (from −811 to +172 bp) or its mutated version at the DR4 site. The mutations are presented in Section 2. After transfection, the cells were incubated with 10 μmol/l of 22(R)-hydroxycholesterol in the presence of 0.5% BSA for 20 h. Control cells were treated with 0.1% DMSO (B), the cells were incubated with 100 μmol/l of eicosapentaenoic acid (EPA) in the presence of 10 g/l BSA for 20 h. Control cells were treated with 10 g/l BSA and 0.1% DMSO (C). The luciferase activities were measured using the Dual-Luciferase Assay System™ (Promega). Firefly luciferase activity was divided by Renilla activity to obtain a normalized value as a relative luciferase unit (RLU). The RLU are shown in fold change compared with control (DMSO or BSA). The results represent three independent transfection experiments of duplicates. Bars indicate mean ± S.D. $^{\dagger}p < 0.01$ vs. treatment with BSA and DMSO (unpaired Student's *t*-test).

activity of the wild-type human ABCA1 promoter construct but not of a mutant human ABCA1 promoter construct lacking the DR4 element (Fig. 6A). Similar to the results shown for the ABCG1 promoter, eicosapentaenoic acid suppressed the transcriptional activity of the human wild-type ABCA1 promoter construct but not the activity of a variant ABCA1 promoter construct containing a mutated DR4 site (Fig. 6B).

3.10. LXR protein binds to a DR4 element on ABCG1 promoter located upstream of exon 1

To determine whether the DR4 element binds to LXR, oligonucleotide probes containing DR4 site were used to perform electrophoretic mobility shift assay. The DNA–protein complex was detected as the shifted bands in the presence of nuclear extracts from HeLa cells (Fig. 7, lanes 2 and 3). Interestingly, the nuclear extracts incubated with polyclonal

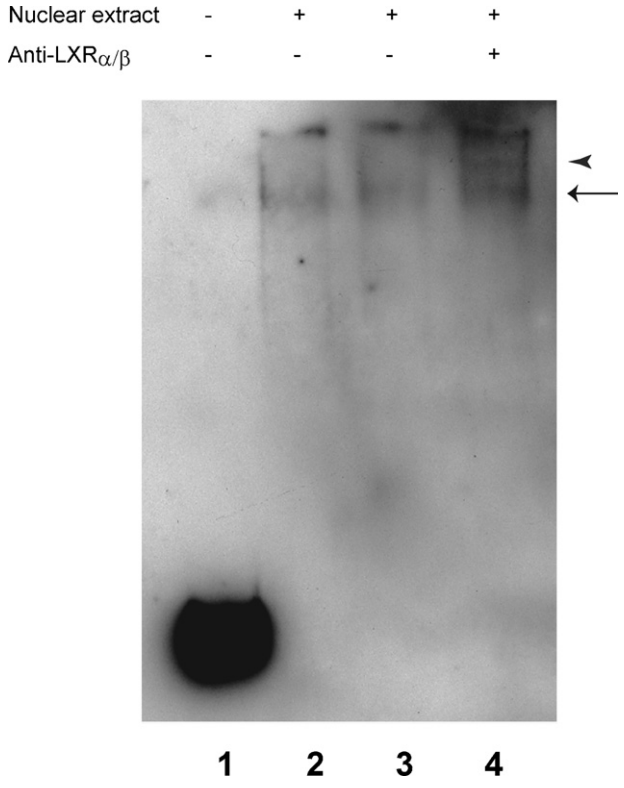

Fig. 7. Electrophoretic mobility shift assay of human ABCG1 promoter fragment. Double-stranded oligonucleotide (fragment spanning −264 to −251) containing a LXR/RXR binding site was digested from ABCG1 promoter A construct. The digested 114 bp double-stranded oligonucleotide probe was labeled with biotin. Labeled double-stranded oligonucleotide probe was incubated with (lanes 2–4) or without (lane 1) HeLa nuclear extract. A sample was pre-incubated with antibodies against LXRα/β before addition of probe (lane 4). DNA–protein complexes were electrophoresed on a native 5% polyacrylamide gel and transferred to a nylon membrane. The DNA–protein complexes were visualized with chemiluminescent substrate after incubation of the nylon membrane with stabilized streptavidin-horseradish peroxidase conjugate. The *arrow* and *arrow head* indicate the band for DNA–protein complex and complex of DNA–protein with LXRα/β antibody, respectively.

LXRα/β antibodies produced supershifted band (Fig. 7, lane 4).

4. Discussion

Patients with diabetes mellitus have a tremendously increased risk of atherosclerotic vascular disease which in part appears to be related to low HDL cholesterol levels. ABC transporters A1 and G1 play an important role in both HDL metabolism and the regulation of cholesterol homeostasis in various cells, notably in macrophages which unlike other cells do not limit cholesterol uptake. It has been well documented that ABCA1 plays a pivotal role in mediating phospholipid and cholesterol efflux to lipid-free apoA-I and, thereby, in the formation of discoidal HDL precursors. Mature HDL particles, which are spherical and transport almost all HDL cholesterol, appear to induce cholesterol efflux via two other ABC transporters, ABCG1 and ABCG4, rather than ABCA1 [17,25]. In a positive feed-forward regulation process, both ABCA1 and ABCG1 gene expression and thereby cholesterol efflux are strongly induced by oxidized cholesterol derivatives, so-called oxysterols. Oxysterols are activators of the nuclear transcription factor LXRα which together with its obligatory heterodimeric partner RXR binds to DR4 elements which are present in both the ABCA1 and the ABCG1 promoters.

We have previously shown that the stimulatory effect of oxysterols on ABCA1 gene transcription is inhibited by free unsaturated fatty acids which interestingly accumulate in patients with diabetes mellitus and insulin resistance. In the present study we extend these observations by three novel findings: first, unsaturated fatty acids suppress the ABCG1 mRNA and protein expressions. Second, the activity of the ABCG1 promoter is also suppressed by unsaturated fatty acids. Third, the suppressive effect of free unsaturated fatty acids depends on the presence of an intact DR4 element and probably due to the binding of the LXRα/RXR heterodimer.

The human ABCG1 gene has at least two promoters, one upstream of exon 1 and another upstream of exon 5 and encodes for several transcripts [22,23]. A previous study demonstrated that the activation of LXRα and RXR increases the expression of ABCG1 transcripts starting from both exon 1 and exon 5 [22]. Interestingly we found that ligands for LXR and RXR dramatically increase activity of ABCG1 promoter A located upstream of exon 1 (Fig. 1A), but do not change the activity of ABCG1 promoter B located within intron 4 (Fig. 1B). These findings suggest that the transcription of exon 5 and subsequent exons may be controlled, at least in part, by the ABCG1 promoter A located upstream of exon 1. To confirm this finding, electrophoretic mobility shift assay was performed. Existence of DNA-binding nuclear proteins were ascertained on the extracted ABCG1 promoter A containing DR4 element. In addition, one of the proteins was identified as LXRs by using the supershifted assay on EMSA experiment. We also show that unsaturated fatty acids do only suppress the activity of ABCG1 promoter A.

In agreement with these findings only the ABCG1 promoter A located upstream of exon 1 contains a DR4 element which is needed for LXRα/RXR binding. In fact, the promoter response to LXR/RXR ligands was not seen in truncated and mutated ABCG1 promoter constructs which lack a functional DR4 site (Figs. 3 and 5B). These mutants were neither responsive to the suppressive effects of free unsaturated fatty acids (Fig. 5C). Likewise the knock-out of the LXR/RXR response element in the ABCA1 promoter eliminated the suppressive effect of USFA on ABCA1 gene transcription (Fig. 6B). Our observation suggests that USFA has a potential for suppressive effect of ABCG1 gene transcription.

Although we show that the suppressive effects of free fatty acids depend on the presence of a LXR/RXR binding site, the mechanism by which free fatty acids suppress the promoter

activities of ABCA1 and ABCG1 is not resolved. Neither in this nor in our previous study, we found any transcriptional effect of unsaturated fatty acids on the expression of LXRα or RXR. By contrast other researchers found either stimulatory effects of USFAs on LXRα but not LXRβ in cultivated hepatoma cells [26] or suppressive effects on LXRα gene expression [27]. Furthermore, fatty acids also play a role in the activation of PPARα and γ [28] which indirectly upregulate ABCA1 and ABCG1 gene expression by elevating LXR gene expression. On the posttranslational level, USFAs were reported to activate RXR [29] to form a heterodimer with LXR. However, USFAs were also found to compete the binding of *oxysterols* to LXRα and thereby to suppress the oxysterol-induced upregulation of SREBP1c [30]. On the other hand, DNA binding transcription factors, LXR and RXR are capable of interacting with coregulators [31,32]. It may be possible that free fatty acids regulate these coregulators of LXR or RXR.

In the present results, saturated fatty acids stimulated the ABCG1 transcription activities. Whereas, the effects of stearic acid did not change on ABCG1 promoter A with DR4 mutation (data not shown). These results suggest that saturated fatty acids stimulate the ABCG1 transcriptions independent of LXR or RXR.

In conclusion, this study shows that ABCG1, like ABCA1, is suppressed by unsaturated fatty acids at the transcriptional level, and describes the structural requirements for this suppressive effect within the human ABCG1 promoter. The suppressive effects of USFAs on promoter activities of both ABCA1 and ABCG1 indicate that elevated levels of unsaturated fatty acids inhibit both apoA-I and HDL-induced cholesterol efflux from macrophages. This impairment of reverse cholesterol transport may contribute to the increased cardiovascular risk of patients with diabetes mellitus.

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