Short Communication

The impact of severe LDL receptor mutations on SREBP-pathway regulation in homozygous familial hypercholesterolemia (FH)

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ABSTRACT

Familial hypercholesterolemia (FH), Niemann–Pick disease type C (NPC) and Tangier disease (TD) are genetic inherited disorders with impaired processing of cholesterol, caused by mutations in genes that regulate cellular uptake, intracellular movement and transport of cholesterol. Various studies have shown a crucial regulatory role of the SREBP-pathway for cellular cholesterol homeostasis in these diseases. Since cholesterol is an essential structural component of cells, we assessed the impact of a severe FH causing LDLR mutation (FH p.W556R) on the SREBP pathway in primary FH fibroblasts. Primary FH fibroblasts derived from patients with the LDL receptor mutation p.W556R were used for gene expression experiments. Gene expression studies revealed increased expressions of SREBP regulated genes HMGCR, LDLR, SREBP-2, SREBP-1, SR-BI, INSIG-1, but interestingly not SCAP. In contrast expression of ABCA1, was strongly decreased in homozygous, but not in heterozygous p.W556R fibroblasts. Gene expression experiments with LDL receptor lacking primary FH fibroblasts, revealed that SR-BI and ABCA1 are important regulators for cholesterol acquisition in FH cells, consistent with findings in cells from NPC and TD patients.

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1. Introduction

The LDL receptor (LDLR) (Brown and Goldstein, 1986) is an essential receptor for the uptake of LDL into cells by receptor mediated endocytosis, mainly in hepatocytes, and accounts for the clearance of 70% of all plasma circulating LDL. Homozygous familial hypercholesterolemia (FH) is a rare autosomal dominant disorder with a frequency of one per one million in the general population (Austin et al., 2004; Hobbs et al., 1992; Soutar, 2010) that is characterized by extremely elevated serum LDL cholesterol levels (> 600 mg/dl). Clinically homozygous FH patients have xanthomas, thickened Achilles tendons, carotis stenosis and aortic valve stenosis that develop in the first decade of life, leading to premature death from stroke or myocardial infarction in early childhood without early intervention (Rader et al., 2003). The molecular basis of FH has been elucidated by the Nobel prize awards Brown and Goldstein who showed that FH is caused by mutations within the LDL receptor gene (Goldstein and Brown, 1989; Hobbs et al., 1992). Despite FH several other rare inborn errors of cholesterol metabolism such as Niemann–Pick disease type C (NPC) and Tangier disease (TD) have been identified. Niemann–Pick disease type C (NPC) is caused by mutations in the late endosomal membrane protein NPC1 and accompanied with impaired cholesterol esterification in the ER. As a consequence accumulation of unesterified cholesterol in late endocytic organelles and defective suppression of cholesterol synthesis and LDLR activity occurs (Liscum and Klaneck, 1998; Rosenbaum and Maxfield, 2011). In Tangier disease mutations of the plasma membrane protein ABCA1 (ATP binding cassette transporter A1) result in decreased SR-BI mediated efflux of cellular cholesterol to lipid–poor apoA-I, thus leading to HDL deficiency and cholesterol accumulation in tissues (Fitzgerald et al., 2010). As cholesterol is a crucial component of cells, the amount of intracellular cholesterol biosynthesis and LDL receptor mediated uptake of lipoprotein derived cholesterol is tightly controlled by a feedback regulatory system. This regulation is modulated by membrane-bound transcription factors termed sterol regulatory element-binding proteins (SREBPs), that senses the amount of cholesterol in cell membranes and modulates transcription of genes encoding enzymes and proteins of cholesterol metabolism (Brown and Goldstein, 1999; Horton et al., 2002; Sato, 2010). Upon cellular cholesterol deprivation, the membrane SREBP proteins associate with the escort protein SREBP cleavage-activating protein (SCAP) that transports SREBPs to the Golgi, where sequential proteolytic cleavage liberates the mature NH2-terminal transcription-activation domain of the SREBP proteins that translocate to the nucleus, where they bind to sterol-regulatory elements (SREs) in promoters of...
various lipid regulatory genes and activate transcription, which results in increased LDL receptor-mediated uptake of LDL derived plasma cholesterol and intracellular cholesterol biosynthesis (Bengochea-Alonso and Ericsson, 2007). This shows the significant role of the SREBP/SCAP pathway for the regulation of cholesterol metabolism under normal steady state conditions in FH fibroblasts. As there is functional cooperativity and crosstalk between ABCA1, NPC1, SR-BI and the LDL receptor pathway (Boadu et al., 2008; Schmitz and Grandl, 2009), we examined the regulatory consequences of complete cellular LDL deficiency and its impact on SREBP-pathway regulation in primary FH fibroblasts.

2. Materials and methods

2.1. Cell culture of human fibroblasts

Fibroblast cultures were established from a normocholesterolemic healthy subject and 2 male homozygous FH monozygotic twins with the p.W556R LDLR mutation (Soufi et al., 2009) and their parents. Informed consent was given by the patients for genetic screening of their disorder and all procedures were in accordance with the Helsinki Declaration of 1975, as revised in 1996 and the study was approved by the local ethics committee at the Philipps-University, Marburg. Skin biopsies were obtained from the proximal part of the volar side of the forearm. Cells were grown in monolayer in 75 cm² stock flasks containing 15 ml DMEM (Invitrogen, Carlsbad) supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 5% CO2. The cells were harvested when they had almost reached confluency. For all experiments cells between passages 3 and 10 were used.

2.2. Cellular fractionation for Western blotting

For the detection of scavenger receptor class B type 1 (SR-BI) and Na+/K⁺-ATPase in western blotting experiments, membrane protein extracts from human fibroblasts cultured in DMEM 10% FCS, were prepared using the Qproteome Cell Compartment Kit (QIAGEN, Hilden, Germany) as described by the manufacturer’s instructions. Protein concentrations were determined using the CB-X™ protein assay kit (G Biosciences, St. Louis, MO, USA) as described by the manufacturer. Equal amounts of protein samples were separated in 8% SDS-PAGE and transferred to nitrocellulose membranes using the BIO-RAD™ electroblotting transfer system. The membranes were blocked with Tris-buffered saline containing 5% (w/v) skimmed milk and 0.05% (v/v) Tween 20, and either incubated with rabbit anti-human Na⁺/K⁺-ATPase polyclonal antibody or goat anti-human SR-BI polyclonal antibody (Santa Cruz, Heidelberg, Germany) then treated with anti-rabbit/anti-goat IgG-horseradish peroxidase secondary antibody. Proteins bound to the antibodies were then visualized with super enhanced chemiluminescence kit (SECL, Amersham, Arlington Heights, IL, USA).

2.3. Gene expression analysis in FH-fibroblasts

To measure the expression of genes involved in the regulation and biosynthesis of cholesterol metabolism in real-time rtPCR experiments, total RNA from human fibroblasts cultured in DMEM 10% FCS between passages 3 and 10, was isolated using QIAGEN RNeasy Kit (Qiagen Hilden, Germany) according to the manufacturer’s instructions. For reverse transcription 1 μg of total cellular RNA was used with the SuperScript II first-strand synthesis system (Invitrogen, Carlsbad, CA, USA) in a total reaction volume of 20 μl. After reverse transcription, the first strand cDNA reaction was diluted 1:5 with DEPC-water and 2.5 μl of diluted cDNA were subjected to real time PCR in a 25-μl reaction mixture with the IQ™ SYBR green supermix (Bio-Rad, Munich, Germany) containing 0.6 μM of each gene specific forward and reverse primer. All PCR reactions were carried out in triplicates from 4 independent cellular fractionation preparations using the Bio-Rad iCycler™ system. Cycling conditions were: initial denaturation at 95 °C for 3 min, followed by 40 cycles with 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 45 s. The relative expressions of the genes for LDLR (LDL receptor), SR-BI (scavenger receptor class B type 1), ABCA1 (ATP-binding cassette transporter 1), HMGR (HMG-CoA reductase), SREBP-1 (Sterol regulatory element-binding protein 1), SREBP-2 (Sterol regulatory element-binding protein 2), INSIG-1 (Insulin induced gene 1) SCAP (SREBP cleavage-activating protein) and the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase), were calculated as the ratio between the level of the examined genes and the level of control. Using the Bio-Rad iCycler system software, the threshold (CT) at which the cycle numbers were measured was adjusted to areas of exponential amplification of the traces. The ΔΔ-method of Pfaffl et al. was used to determine comparative expression level by applying the formula 2(-ΔΔCT) = 2(-ΔΔCT) [13]. For statistical evaluation the unpaired Students t test and the Mann-Whitney Rank Sum Test were adopted, p values <0.05 were considered as significant. The sequences of all oligonucleotides used in real time rtPCR experiments are listed in Table 1.

3. Results

3.1. Real time PCR analysis of SREBP regulated genes in FH-fibroblasts

To assess the expression levels of genes that control the regulation, biosynthesis and transport of cholesterol quantitative real time rtPCR experiments were done. For this purpose fibroblasts from wild-type, homozygous and heterozygous p.W556R patients were cultured for 48 in DMEM 10% FCS, under non-sterol depletion conditions. As demonstrated in Fig. 1, the normalized gene expressions of HMGR (HMG-CoA reductase), LDLR (LDL-receptor), SREBP-2 (sterol element binding protein 2), SREBP-1 (sterol element binding protein 1) and INSIG-1 (Insulin induced gene 1), were strongly increased in homozygous and heterozygous FH p.W556R fibroblasts compared to wild-type cells. No different regulation of gene transcription was found for SCAP (SREBP cleavage-activating protein).

3.2. Expression of SR-BI but not ABCA1 is increased in fibroblasts from homozygous p.W556R FH patients

Despite the expected increases of mRNA expressions of SREBP regulated genes that control cellular de novo cholesterol biosynthesis and uptake of LDL derived cholesterol, we also observed a strong increase in SR-BI (scavenger receptor type B1) mRNA and protein levels in fibroblasts from homozygous and heterozygous p.W556R carriers, but not in wild type cells, indicating that SR-BI serves as a bypassing pathway (Fig. 2A), that is used for acquisition of non-lysosomal derived cholesterol in FH-fibroblasts. Although SR-BI transcription and translation was strongly increased in fibroblasts from heterozygous and homozygous p.W556R subjects, one striking difference in homozygous and heterozygous W556R fibroblasts was the level of ABCA1 (ATP-binding cassette transporter 1) gene expression, which was increased in heterozygous and strongly decreased in homozygous p.W556R fibroblasts (Fig. 2B). This might be a direct consequence of the completely different cellular cholesterol contents in both cell types (Figs. 3A and B).

4. Discussion

The rationale for our study was to examine the regulation of cholesterol homeostasis in fibroblast cells from FH patients with the p.W556R LDLR mutation in the context of recent findings from other disorders of cholesterol metabolism such as Niemann–Pick disease type C (NPC) and Tangier disease (TD). It is known that SREBP mediated functional cooperativity and crosstalk between SR-BI,
Table 1

Genes, nucleotide sequences of primers and product sizes of fragments that were examined in real time rt-PCR experiments.

<table>
<thead>
<tr>
<th>Gene/accession no.</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
<th>Fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDLR (NM_001195802)</td>
<td>TGGACTGAACTTGTTGACCTCAAC</td>
<td>GACTCTCTCTGCGCTGCAGCTCCT</td>
<td>280</td>
</tr>
<tr>
<td>SR-BI (NM_001082959)</td>
<td>GGGGCCAATCTTGGCGCAAG</td>
<td>CCGTCAACGTCACTGAGCCTT</td>
<td>330</td>
</tr>
<tr>
<td>ABCA1 (NM_005502)</td>
<td>AACACCGTGTCGGCCTTTTG</td>
<td>AGTICCGTGGCGCCCTACTG</td>
<td>156</td>
</tr>
<tr>
<td>HMGCR (NM_00109827)</td>
<td>ACAAGAATTGAGCTGCTTCGTG C</td>
<td>CTGACAGCACTAGCTGCAAGCTGCA</td>
<td>273</td>
</tr>
<tr>
<td>SREBP-1 (NM_001005291)</td>
<td>TGCCATGGAGCTGAAACCCAG</td>
<td>TCTAGAGAATGGCCTCCCAAGGAT</td>
<td>267</td>
</tr>
<tr>
<td>INSIG-1 (NM_005542)</td>
<td>CAGGCTCTGCTTGTGCTTC</td>
<td>CCATGCTGCTGCTGCTGCT</td>
<td>242</td>
</tr>
<tr>
<td>SCAP (NM_012235)</td>
<td>GGCCGCCAATCTTGGCGCAAG</td>
<td>GCTACTGTCCTGGGCTGTTCTGCT</td>
<td>279</td>
</tr>
<tr>
<td>GAPDH (NM_002046)</td>
<td>TGATGCTCCTGCTAATG</td>
<td>AACACGCCCAGGCTGCTGCCCTC</td>
<td>256</td>
</tr>
</tbody>
</table>

LDLR, LDL receptor; SR-BI, scavenger receptor class B type 1; ABCA1, ATP-binding cassette transporter 1; HMGCR, HMG-CoA reductase; SREBP-1, sterol regulatory element-binding protein 1; SREBP-2, sterol regulatory element-binding protein 1a; INSIG-1, insulin induced gene 1; SCAP, SREBP cleavage-activating protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

ABCA1, NPC1, and the LDL receptor in NPC and TD exists. But few about this is known in FH, therefore we examined the impact of complete LDLR deficiency on SREBP-pathway regulation in FH.

Much insights about the molecular mechanisms and transcriptional control of cellular cholesterol biosynthesis underlying the SREBP-pathway came from in vitro experiments with transfected cells over expressing the SREBP proteins (Brown and Goldstein, 1999; Gong et al., 2006; Horton et al., 2002). As few comparable experimental data concerning the regulatory consequences in LDLR deficiency on SREBP-pathway regulation in FH.

To examine the expression of genes from members of the SREBP-family and its regulated genes of cholesterol metabolism, upon a 48 h incubation in non-sterol depleted media, a strong increase in the expressions of LDLR, SREBP-2, INSIG-1 and HMRG, was observed in homozygous and heterozygous p.W556R fibroblasts. Surprisingly the expression of SCAP was not increased, indicating that SCAP function might be regulated additionally by other mechanisms (e.g. at the post transcriptional/translational level) (Irisawa et al., 2009).

We performed all our experiments in the presence of serum, as this condition best resembles the physiological situation in FH, where lipoprotein derived cholesterol is accessible for cellular uptake. In lipoprotein deficient media genes of cholesterol metabolism are up regulated per se due to cholesterol starvation and this condition completely distorts the real physiological situation present in FH. Under this condition a novel finding of our study was increased gene expression and translation of SR-BI (scavenger receptor type B1) (Acton et al., 1996; Krieger, 1999) in p.W556R fibroblasts, that points out towards an alternative shuttle for acquisition of cholesterol in FH cells. Treguier et al. identified a unique sterol responsive element (SRE) in the SR-BI promoter and showed that SR-BI protein levels were increased in direct proportion to SREBP-2 mediated expression in transfected human kidney 293 cells. In addition they showed that SREBP-2 is a more potent inducer of LDLR and SR-BI gene expression than SREBP-1 (Treguier et al., 2004). This is consistent with our observations, as we detected a 3-fold higher expression of SREBP-2 compared to SREBP-1, indicating that SREBP-2 is the key regulator of SR-BI expression. As the LDLR pathway is completely...
lost in homozygous p.W556R cells, de novo cellular synthesis of cholesterol is the primary way for cholesterol supply. However, an alternative way that bypasses the LDLR pathway is direct uptake of cholesteryl ester from HDL and LDL particles by SR-BI. In this process cholesteryl esters are translocated into cells without internalization, hydrolyzed by a neutral cholesteryl ester esterase and the released unesterified cholesterol is delivered directly into the metabolically active cellular pools. In support with this hypothesis is a study by Xie et al. which revealed that the majority of cholesterol acquisition in NPC1−/− mouse came from de novo synthesis and selective uptake of cholesteryl ester from HDL via SR-BI (Xie et al., 2006; Zannis et al., 2006). This metabolic condition is comparable with homozygous FH where acquisition of LDL derived cholesterol is completely blocked due to the loss of the LDLR pathway. In our gene expression studies we detected increased SR-BI mRNA and protein levels in heterozygous and homozygous FH p.W556R fibroblasts. However, one striking difference was the level of ABCA1 (ATP binding cassette transporter 1) expression, which was strongly downregulated in homozygous, but upregulated in heterozygous p.W556R fibroblasts and thus might be directly linked to different levels of unesterified cholesterol.

Fig. 3. Proposed model system for different SREBP-2 mediated regulation of ABCA1 expression in heterozygous and homozygous FH p.W556R fibroblasts. A. In heterozygous FH p.W556R cells, acquisition of cholesterol is available through SREBP-2 induced upregulation of de novo cellular cholesterol biosynthesis, uptake of LDL derived cholesterol via the LDLR pathway, and acquisition of non-lysosomal cholesterol by SR-BI. The high intracellular levels of cholesterol are sufficient to generate enough LXR ligands for activation of ABCA1 gene transcription. B. In homozygous FH p.W556R cells the SREBP-2 induced de novo cellular cholesterol biosynthesis and SR-BI mediated cholesterol uptake are the primary ways for cholesterol acquisition, due to the complete loss of the LDLR pathway. However the lower levels of intracellular cholesterol are not sufficient to generate enough LXR ligands to activate ABCA1 gene transcription. The model may helps to explain the conflicting results obtained with SREBP-2 mediated regulation of the ABCA1 promoter (Wong et al., 2006; Zeng et al., 2004), and suggests that SREBP-2 mediated repression/activation is dependent on the level and availability of unesterified sterols in the cells, that control SREBP-2 degradation/dissociation or binding to the E-box element on the ABCA1 promoter (figure adapted from Xie et al., 2006).
in the metabolically active pools of the cells. The potential role of SREBP-2 in ABCA1 gene transcription has been examined with conflicting results. Zeng et al. showed that sterol depletion in SREBP-2 transfected vascular endothelial cells increased LDLR and decreased ABCA1 gene transcription via direct binding of SREBP-2 to the E-box element of the ABCA1 promoter (Zeng et al., 2004). In contrast Wong et al. showed that SREBP-2 is required to induce the mevalonate pathway for generating LXR ligands in transfected CHO-cells, and by this acts as a positive indirect regulator of ABCA1 gene expression (Wong et al., 2006). The results from our experiments with non-transfected primary p.W556R FH-fibroblasts support both hypotheses, and show that increased or decreased ABCA1 expression seems to be dependent on the level and availability of unesterified sterols in the cells. This availability is controlled by SREBP-2 as the key regulator, that governs de novo synthesis, LDLR mediated uptake, and acquisition of non-lysosomal cholesterol via SR-BI. In homozygous FH p.W556R cells, sufficient cellular cholesterol is available to generate enough LXR ligands for SREBP-2 mediated ABCA1 upregulation through induction of the mevalonate, LDLR, and SR-BI pathways (Boadu et al., 2008; Schmitz and Langmann, 2005). In contrast LDLR mediated uptake of cholesterol is completely blocked in homozygous p.W556R FH cells, thus de novo synthesis and uptake of cholesterol by SR-BI are the primary ways for cholesterol acquisition. However, under these conditions the low levels of unesterified cholesterol are not sufficient to generate enough LXR ligands for activation of ABCA1 gene transcription (Fig. 3). Our study with the FH p.W556R mutation may also explain why HDL-cholesterol levels remain unchanged or only slightly increase after statin treatment in FH patients. Although statins activate the SREBP-2 pathway, they inhibit the mevalonate pathway and by this subsequent formation of LXR ligands (Du et al., 2006; Wong et al., 2006), which results in decreased ABCA1 expression and lipid efflux to apoA-I (Boadu and Francis, 2006; von Eckardstein et al., 2001). As a result lipid poor apoA-I is rapidly catabolized trough the kidney like in Niemann–Pick disease type C (NPC) and Tangier disease (TD) (Lawn et al., 1999; Schaefer et al., 1992).

Our study has several limitations at present. First, additional studies with primary FH cells in the context of cellular cholesterol status and the exact knowledge about the underlying FH mutations will be required. Secondly, our data have to be extended with western blot data for all assessed SREBP-2 regulated genes to verify all gene expression experiments at the protein levels. And third, functional studies for ABCA1/SR-BI mediated cholesterol efflux have to be done. In summary, we studied the regulation of cholesterol homeostasis by the SREBP-pathway in LDLR deficient primary FH fibroblasts. By this we found for the first time, that SR-BI plays an important role for cholesterol acquisition and ABCA1 regulation in FH like in NPC and TD. Our findings reveal the tight metabolic conjunction of these three genetic inherited cholesterol processing disorders.

Conflict of interest statement

JRS serves as a scientific advisor for MSD and ESSEX pharma. JRS received research grants and/or lecture fees by MSD, ESSEX, Bayer Healthcare, Takeda Pharma, Genzyme, B. Braun Melsungen. None of the other authors declare any potential conflict of interest.

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