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Human ATP-binding cassette transporter-2 (ABCA2) positively regulates low-density lipoprotein receptor expression and negatively regulates cholesterol esterification in Chinese hamster ovary cells

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Abstract

We present evidence that the ATP binding-cassette transporter-2 (ABCA2) is a sterol-responsive gene that has a role in the trafficking of low-density lipoprotein-derived free cholesterol (LDL-FC). In HepG2 cells ABCA2 was coordinately expressed with other sterol-responsive genes. Stable constitutive expression of ABCA2 in Chinese hamster ovary cells (CHOA2) was accompanied by an increase the expression of the low-density lipoprotein receptor (LDLR) and other genes involved in the regulation of cholesterol homeostasis. LDLR mRNA was elevated greater than ninefold and 3-hydroxy-3-methylglutaryl CoA synthase (HMGCoA S) expression was elevated sevenfold in CHOA2 cells. The increase in LDLR expression was regulated at the level of transcription; however, culture of CHO and CHOA2 cells in medium containing lipoprotein-deficient serum (LPDS) results in similar levels of LDLR promoter expression. No differences were measured in the dose-dependent uptake of fluorescently labeled 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchorate-LDL (DiI-LDL) between CHO and CHOA2 cells cultured in medium containing LPDS. Ultraviolet microscopy revealed a similar distribution of the DiI-LDL label in cytoplasmic vesicles. We measured an LDL dose-dependent reduction in esterification of LDL-FC in intact CHOA2 cells cultured in medium containing LPDS, however, no significant difference was measured in acylcoenzyme A:cholesterol acyltransferase (ACAT) activity in cell-free extracts of CHO and CHOA2 cells. CHO cells or CHOA2 cells treated with the hydrophobic amine, U18666A, showed similar filipin staining of unesterified cholesterol in cytoplasmic vesicles. Addition of progesterone or U18666A to CHO cells elevated ABCA2 expression. Finally, we found that ABCA2 expression was elevated in Niemann–Pick type C1 (NPC1) fibroblasts and in Familial Hypercholesterolemia (FHC) fibroblasts.

Keywords: ABC transporter; Low-density lipoprotein receptor; Chinese hamster ovary; U18666A; Progesterone; SREBP2; ACAT; SCAP

1. Introduction

ATP-binding-cassette (ABC) transporters utilize the energy of ATP hydrolysis to pump substrates across cell membranes [1]. ABC transporters have been identified that are specific for a variety of substrates including amino acids, sugars, inorganic ions, polysaccharides, lipids, peptides and proteins [2]. ABC transporters have been implicated in human diseases as well as contributing to the multi-drug resistance phenotype in response to cancer chemotherapy. Diseases with a demonstrated etiology to defects in ABC transporter function include: cystic fibrosis and the cystic fibrosis transmembrane receptor protein (CTFR or ABCC7) [3], X-linked sideroblastic anemia and ataxia and the mitochondrial half-transporter (ABCB7) [4], Stargardt's disease and other retinal disorders and the rod photoreceptor ABC transporter (ABCA4) [5], Tangier disease, affecting reverse cholesterol transport and susceptibility to atherosclerosis and the ABCA1 transporter [6].

Abbreviations: ABC, ATP-binding cassette; LDLR, low-density lipoprotein receptor; CHO, Chinese hamster ovary; U18666A, 3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one; DiI-LDL, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchorate-LDL; NPC1, Niemann–Pick type C1; FBS, fetal bovine serum; LPDS, lipoprotein-deficient serum; SREBP2, sterol response element binding protein-2; SCAP, sterol cleavage activating protein; ACAT, acyl-coenzyme A:cholesterol acyltransferase; PUFA, polyunsaturated fatty acid; AA, arachidonic acid

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A central role for ABCA1 in cholesterol homeostasis has been demonstrated by its facilitation of the removal of excess cholesterol from macrophages. This removal is critical for the formation of high-density lipoprotein (HDL) [7]. The cholesterol content of most cellular membranes is regulated by a feedback system consisting of de novo synthesis from acetyl CoA (synthetic pathway) and through uptake of cholesterol-containing low-density lipoprotein (LDL) by receptor-mediated endocytosis (salvage pathway) [8].

The ABCA2 transporter is a member of the subfamily A of ABC transporters, including ABCA1 described above, with which it shares the highest structural homology [9]. The human gene was identified by this laboratory in investigating mechanisms of acquired resistance of ovarian carcinoma cells to the anticancer drug estramustine [10]. Presumably, the overexpression of the transporter by gene amplification in drug-resistant cells inhibits the accumulation of the drug with a consequent reduction of toxicity and cell death. The human ABCA2 gene comprises 48 exons, which are localized within a genomic region of 21 kb [11]. Studies examining the expression of the gene have detected the greatest abundance in the brain [12–14], and greatest immunoreactivity is detected in oligodendrocytes [15]. Subcellular localization experiments have detected ABCA2 within late-endosomal/lysosomal and trans-Golgi network organelles [14,15]. A recent study has demonstrated that ABCA2 is expressed in peripheral nerves and in spinal cord oligodendrocyte progenitors of the rat and continues to be expressed into adulthood in mature oligodendrocytes during postnatal development [16]. In spite of the original identification of the ABCA2 gene almost a decade ago [12], a clear demonstration of its functional role in cells has not been established. Its structural similarity to family member ABCA1 is suggestive of a role in cholesterol homeostasis.

We undertook experiments to evaluate the function of ABCA2 in cholesterol homeostasis and report that ABCA2 may have an important role in the delivery of low-density lipoprotein-derived free cholesterol (LDL-FC) to the endoplasmic reticulum for esterification. Chinese hamster ovary cells engineered to constitutively express the ABCA2 protein display increased expression of the LDL receptor and 3-hydroxy-3-methylglutaryl CoA synthase (HMGCoA S) genes and reduced esterification of LDL-derived free cholesterol. We found that treatment of CHO cells with the steroid hormone, progesterone [17], or the hydrophobic amine, U18666A [18], which cause accumulation of LDL-derived cholesterol in lysosomes, and also inhibit cholesterol synthesis [19,20], both elevate ABCA2 expression. We also measured an increase in ABCA2 expression in Niemann–Pick and Familial Hypercholesterolemia (FHC) fibroblasts. This report represents the first characterization of a functional role for the expression of the ABCA2 transporter and the regulation of trafficking of LDL-derived free cholesterol in the maintenance of cellular cholesterol homeostasis.

2. Materials and methods

2.1. Radioisotopes

[α-^{32}P] dATP (specific activity 3000 Ci/mmol) and [1-^{14}C] coenzyme A (specific activity 56 mCi/mmol) were purchased from Amersham Biosciences, Piscataway, NJ. [Cholesteryl 1,2,6,7 ^{3}H(N)] oleate (specific activity 60 Ci/mmol) and [1-^{14}C] oleic acid (specific activity 55 Ci/mmol) were purchased from Perkin-Elmer Life Sci., Boston, MA).

2.2. Cell culture

HepG2 cells were obtained from the American Type Tissue Collection (ATCC) and were cultured in DMEM, 2 mM glutamine 10% fetal bovine serum (FBS) and 1% Penstrep at 37 °C and 5% CO_2 (medium A). Medium B consists of medium A, in which the 10% FBS was replaced with 5% lipoprotein-deficient serum (LPDS, Biomedical Technologies Inc., Stoughton, MA). The CHO FlpIn cell line, derived from CHO-K1, was obtained from Invitrogen (Carlsbad, CA) and cultured in DMEM/Ham's F12 (1:1), 2 mM glutamine, 10% FBS, 1% Penstrep (medium A). Medium B consists of medium A, in which the 10% FBS was replaced with 5% LPDS. The normal human (GM05659D), Familial Hypercholesterolemia (FHC, GM02000G) and Niemann–Pick type C1 (NPC1, GM03123A) fibroblast cell lines (passage 15 or less) were obtained from the Coriell Institute for Medical Research (Camden, NJ) and were cultured in MEM Eagle-Earle BSS, 2 × concentration of essential and nonessential amino acids and vitamins, 2 mM L-glutamine, 15% FBS, 1% Penstrep) at 37 °C and 5% CO_2. For studies examining effects of progesterone or 3-β-[2-(diethylamino) ethoxy]androst-5-en-17-one (U18666A), on day 1, CHO or HepG2 cells were seeded at 1×10^6 cells in 100-mm plates in medium A and on day 2 medium A was supplemented with progesterone (10 μg/ml, Calbiochem, San Diego, CA) or U18666A (5 μM, Biomol, Plymouth Meeting, PA) and cells were cultured as described above. On day 5, total RNA was isolated (Nucleospin RNA II, BD Bioscience, Palo Alto, CA) and ABCA2 expression was by real-time PCR (CHO) or Northern blot (HepG2).

2.3. Construction of CHOA2 stable transfectant cell line

A 7434-base-pair (bp) *Hind*III/*Eco*R1 ABCA2 cDNA fragment [14] was subcloned into pcDNA5/FRT vector (Invitrogen), which permits constitutive expression from the cytomegalovirus (CMV) promoter. The CHOA2 cell line was produced using the FlpIn System (Invitrogen) according to manufacturer's instructions. The CHO FlpIn was transfected with the ABCA2 pcDNA5/FRT expression vector and pOG44 vector using Lipofectamine 2000 (Invitrogen) and hygromycin-resistant colonies were selected, expanded and analyzed for ABCA2 expression.

2.4. Northern blot analysis

Total RNA was isolated from HepG2, CHO or fibroblast cells and 5 μg was fractionated in glyoxal loading buffer on agarose gels using the NorthernMax system (Ambion, Austin, TX) and transferred to nylon membranes. All Northern blot hybridization probes were generated using a gene-specific antisense primer, [α-^{32}P] dATP (Amersham) and the StripEZ kit (Ambion) on a gene-specific template generated by RT-PCR. The ABCA2-specific probe was generated from a (727 bp) cDNA template using primer 5′ GGGGACTTCATCTTGTCAAACTGG 3′. The LDLR-specific probe was generated from a (575 bp) cDNA template using primer 5′ CGGTTGGCACTGAAAATGGCTTC 3′. The SREBP2-specific probe was generated from a (450 bp) cDNA template using primer, 5′ AGCGACAGTAGCAGGTCACAGGTG 3′. The GAPDH-specific probe was generated from a (526 bp) cDNA template using primer 5′ CCACCACCCTGTTGCTGTAGC 3′. Blots were hybridized with 1×10^6 cpm/ml of probe in ExpressHyb (Ambion) hybridization buffer. Blots were washed in 2× SSC, 0.1% SDS and 0.1% SSC, 0.1% SDS. Autoradiographs were imaged using a BAS 2000 phosphorimager (Fuji Film, Japan). The experiments were performed several times with similar results. A representative gel is displayed in this report.

2.5. Real-time PCR

Real time PCR was performed on reverse transcribed RNA templates as described in figure legends using a Cephid Smart Cycler. The Sybr Green PCR Mix (Qiagen, Valencia, CA) was used for PCR reactions with the following programs 95 °C for 15 min and 50 cycles of 94 °C for 30 s, 60 °C (LDLR, HMGCoA S, ABCA2, 18S rRNA) for 30 s, 72 °C for 60 s. The primers for amplification of LDLR, sense: 5′ TACTGGTCTGACCTGTCCCAGAGA 3′ antisense: 5′ CGGTTGGCACTGAAAATGGCTTC 3′; HMGCoA S, sense: 5′ GATGGTGTAGATGCTGGAAAGTATA 3′, antisense: 5′ CTGAGGTAGCACTGTATGGAGAG 3′; ABCA2, sense: 5′ AACTACGTGTGGGACATGCTCAA 3′, antisense: 5′ GGGGACTTCATCTTGTCAAACTGG 3′. Following the PCR cycles, melt peaks were determined to be same among all the samples, confirming that a gene-specific product was measured. The quantification of expression for each sample was calculated as described [21] from the threshold cycle value (C_T), which is the cycle where an increase in PCR product is first detected at a statistically significant level. The relative expression value of each gene is obtained by evaluating the C_T values for the unknown reaction using the equation $2^{-\Delta\Delta CT}$. The ΔC_T for the calibrator and samples in each gene expression assay were obtained by subtracting the C_T value of the 18S rRNA from the C_T value for the gene. $\Delta\Delta C_T$ was calculated by subtracting the average ΔC_T (calibrator) values from the ΔC_T (sample). The relative quantification was calculated by the equation: $2^{-\text{Average}\Delta\Delta CT}$.

2.6. Western blot analysis

Protein was isolated from CHO cells by Dounce homogenization (30 strokes, tight pestle) in 250 mM sucrose, 20 mM HEPES-KOH, pH 7.2, 0.5 mM EGTA, pH 7.45 and centrifugation of the cell lysate at $3000 \times g$ for 10 min to pellet nuclei. Protein concentration was determined on the supernatant using the DC protein assay kit (Bio-Rad) and 30 μg was separated by electrophoresis on SDS-6% PAGE and transferred to nitrocellulose. Filters were probed with a rabbit polyclonal antibody raised to the C-terminal 20 amino acids of human ABCA2 at a dilution of 1:500 and donkey anti-rabbit peroxidase-conjugated secondary antibody at a dilution of 1:1000 (Amersham) and developed using the Enhanced Chemiluminescence kit (ECL, Amersham).

2.7. Reporter gene construction (LDLRp and mLDLRp)

The human LDLR promoter construct was constructed by PCR amplification from a human genomic DNA template (Promega, Madison, WI) using sense primer: 5′ TCGGTACCAATTCTGAAACTGAGTCTTAACTG 3′ and antisense primer: 5′ ATCTCGAGGACCTGCTGTGTCCTAGCTGGAAAC 3′. The reaction product, extending from −1563 to −41 relative to the translation start site, was purified and digested with *Kpn*I and *Xho*I and cloned into the pGL3 Basic luciferase reporter vector (Promega) and DNA sequenced to ensure fidelity. The SRE element in the LDLR promoter (mLDLRp) was mutated to generate a transversion from cytosine to adenine at position 10 (A^{10}), which abrogates SREBP factor binding, by using the Quickchange II XL Site-Directed Mutagenesis Kit (Stratagene), according to manufacturers' instructions. Briefly, two complementary oligonucleotides containing the desired mutation are synthesized, flanked by unmodified sequence; sense primer: 5′ TGAAAATCACCCCAATGCAAACTCCTCCCCCTGCTAGA 3′ and antisense primer: 5′ TCTAGCAGGGGGAGGAGTTTGCATTGGGGTGATTTTCA 3′. PCR was performed on 10 ng of the cloned LDLR promoter construct described above using *Pfu* Ultra HF DNA polymerase for 1 cycle at 95 °C and 18 cycles of 95 °C for 50 s, 60 °C for 50 s, and 68 °C for 1 min per kilobase of plasmid length. After PCR, the parental nonmutated supercoiled DNA is digested by addition of 10 units of *Dpn*I restriction enzyme to the amplification reaction and incubation at 37 °C for 1 h. XL-10 Gold Ultracompetent cells (Stratagene) are transformed with 2 μl of the *Dpn*I-treated DNA. Following transformation, plasmid is isolated from several colonies and the DNA is sequenced to ensure fidelity of the mutagenesis procedure.

2.8. Luciferase reporter gene assays

The day before transfection, CHO and CHOA2 cells were seeded at 1.0×10^5 cells in 1 ml of medium, DMEM/F12 (1:1), 2 mM glutamine, 10% FBS, 1% Penstrep. The day of transfection the medium was replaced with 1 ml of

OptiMem I. The cells were transfected with 0.5 μg of LDLRp or mLDLRp reporter vector and 100 ng of pRLCMV Renilla luciferase expression vector (Promega) to control for transfection efficiency. The cells were transfected with polyethylenimine (PEI) transfection reagent (Polysciences, Warrington, PA) at an N/P ratio of 9 and cultured for 4 h at 37 °C 5% and CO_2. The medium was replaced with normal growth media after 4 h. Culture proceeded for 48 h before reporter gene analysis on cell lysates using the Dual Luciferase Assay System (Promega). The values are expressed as the average fold change in expression compared to the CHO parental cell line ($\pm$ S.D.). The results presented are from three independent transfection experiments.

2.9. DiI-LDL uptake in CHO and CHOA2 cells

On day 0, CHO and CHOA2 cells were seeded at 5×10^4 cells on coverslips containing 1 ml of DMEM/Ham's F12 and 10% FBS (medium A). On day 1, the cells were washed with PBS and the media was replaced with DMEM/Ham's F12 supplemented with 5% LPDS (medium B) and cultured for an additional 48 h. On day 3, the cells were incubated with 10 μg/ml 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchorate (DiI-LDL; Biomedical Technologies) in medium B for 5 h at 37 °C. The cells were washed twice in PBS/1.5 mg/ml BSA and once in 2 ml of PBS. Cells were fixed in 4 ml of 3% paraformaldehyde in PBS for 30 min at room temperature and rinsed briefly with distilled water. Coverslips were mounted in 90% glycerol/10% PBS onto a microscope slide and sealed with wax. Fluorescence microscopy was visualized on a Nikon E800 fluorescence microscope using a × 60 Plan apo oil-immersion objective at a 1.4 numerical aperture and 510–560:590 excitation and emission filters. Images were captured using a Quantix (Roper) cooled-color digital camera (CCD). Fluorescence measurements of DiI-LDL uptake were performed as described [22]. Briefly, cells were seeded at 1.5×10^5 cells and cultured as described above. Cells were washed twice in PBS containing 0.4% BSA and three times with PBS. Cells were incubated with 0.5 ml of lysis reagent (0.1% SDS/0.1 N NaOH) and the relative uptake of DiI-LDL into cells was measured on a fraction of the lysate in a Perkin-Elmer LS 50 fluorometer (Shelton, CT) with excitation and emission wavelengths set at 520 and 580 nm, respectively. Emission data were normalized to the protein content (DC protein assay kit).

2.10. Filipin staining of CHO cells

Filipin staining of CHO and CHOA2 cells was performed essentially as described Briefly, on day 0, cells were seeded at 5×10^4 cells on coverslips containing 1 ml of DMEM/Ham's F12 and 10% FBS (medium A). On day 1, the cells were washed with PBS and the media was replaced with DMEM/Ham's F12 supplemented with 5% LPDS (medium B). On day 2, the media is replaced with medium B supplemented with 50 μg/ml of LDL (Biomedical Technologies). On day 3, CHO cells were treated with U18666A (5 μM) for 24 h. On day 4, cells were washed four times in PBS and fixed with 3% paraformaldehyde for 30 min. Cells were washed in PBS and quenched with 1.5 mg/ml glycine in PBS for 10 min and stained with 0.05 mg/ml filipin (Sigma) in PBS for 1 h. After washing in PBS, slides were mounted in Slow-Fade (Molecular Probes). UV fluorescence microscopy was visualized on a Nikon E800 fluorescence microscope using a 60× Plan apo oil-immersion objective at a 1.4 numerical aperture and 330–380: emission filters. Images were captured using a Quantix (Roper) cooled-color digital camera (CCD).

2.11. Incorporation of [^{14}C] oleate into cholesteryl [^{14}C] oleate in intact cells

On day 1, CHO and CHOA2 cells (2.5×10^4 cells/well) were seeded in triplicate in six-well plates and grown in 1 ml of DMEM/Ham's F12, 2 mM glutamine, 1% Penstrep and 10% FBS. On day 2, cells were washed twice with 0.5 ml of PBS refed with 1 ml of medium B (medium A in which 10% FBS (v/v) was replaced by 5% lipoprotein deficient serum (LPDS). On day 4, the cells were washed with PBS and the media was replaced with DMEM/Ham's F12 supplemented with 5% LPDS supplemented with 10, 20, or 30 μg/ml of human LDL (Biomedical Technologies) and incubated for 5 h at 37 °C. Monolayers were pulsed for 2 h with 20 μl of 10 mM [^{14}C] oleate (10,000 dpm/nmol, NEN Life Science, Boston MA) bound to albumin [23]. Each monolayer was washed twice with 2 ml of 150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 2 mg/ml BSA followed by one wash in which BSA was omitted. Lipids were extracted with hexane/isopropanol (3:2) and an internal standard added as a correction for procedural losses containing [^{3}H] cholesteryl oleate, (~ 35,000 cpm), unlabeled triolein (50 μg), and unlabeled oleic acid (50 μg) in chloroform–methanol (2:1). Cholesteryl [^{14}C] oleate was isolated by thin layer chromatography (TLC) in heptane–ethyl ether–acetic acid (90:30:1). After lipid extraction, monolayers were incubated in Passive Lysis Buffer (Promega) and protein content of cell lysates was measured using DC protein assay kit.

2.12. Assay of acyl-CoA:cholesterol acyltransferase activity of cell-free extracts

The transfer of [^{14}C] oleate from [^{14}C] oleoyl-CoA to cholesterol was assayed as described [24]. CHO and CHOA2 cells were cultured as described above for 48 h in medium containing 5% LPDS serum and then supplemented with $\pm$ 50 μg/ml LDL and cultured an additional 24 h. Cells lysates were prepared using a Dounce homogenizer, and aliquots of whole cell extracts (100 μg of protein) were incubated for 60 min in 0.2 ml of solution containing 50

mM potassium phosphate pH 7.4, 2 mM dithiothreitol, 6 mg/ml bovine serum albumin and 0.1 mM [oleoyl-1-^{14}C] coenzyme A (Dupont/NEN, 56 Ci/mmol). The reaction was stopped by addition of 4 ml of chloroform–methanol (2:1) and the [^{14}C] cholesteryl oleate was isolated by TLC and quantified as described above. In control experiments in the absence of protein extract, no [^{14}C] radioactivity was found in the cholesterol ester fraction.

3. Results

3.1. ABCA2 is coordinately expressed with other sterol-responsive genes

To investigate whether ABCA2 has role in cholesterol metabolism, we tested the hypothesis that it is coordinately regulated with other sterol-responsive genes. The expressions of both the LDLR and HMGCoA synthase genes are sensitive to changes in the level of cellular cholesterol. Up-regulation of the LDLR facilitates the uptake and release of free cholesterol from extracellular LDL (salvage pathway). HMGCoA synthase is one of a battery of enzymes whose expression is up-regulated under sterol-depleted conditions to synthesize cholesterol from acetyl CoA and restore cholesterol homeostasis (synthetic pathway). Expression of mRNA for these genes increases when cells are cultured in lipoprotein-deficient medium and is repressed when exogenous sterols (e.g. 25-hydroxycholesterol) are added to the culture medium. HepG2 cells were cultured for 48 h in medium supplemented with 5% LPDS (sterol-deprived) ± 1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol. Total RNA was isolated and Northern blot was performed with specific probes to the LDLR, HMGCoA synthase and ABCA2 genes. The expression of ABCA2 increased under sterol-deprived conditions and was repressed by the addition of exogenous sterols (Fig. 1A). As expected, LDLR and HMGCoA synthase mRNA expression was similarly affected by changes in availability of sterols from the culture medium (Fig. 1B and C). These results suggest that the expression of ABCA2 is coordinately regulated with genes whose expression responds to changes in the level of available cellular cholesterol.

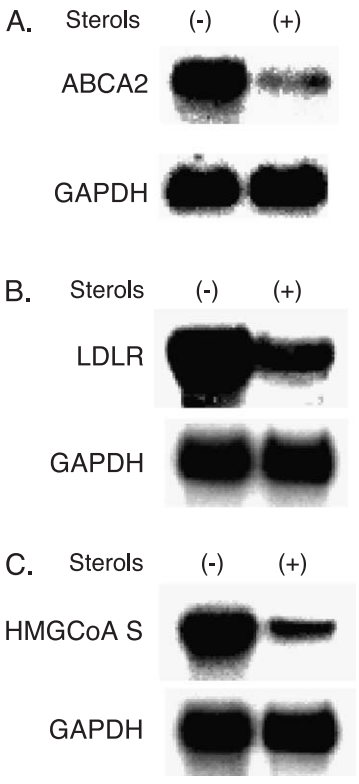

Fig. 1. ABCA2 is coordinately expressed with other sterol-responsive genes. HepG2 cells were cultured for 48 h in complete medium supplemented with 5% LPDS (sterol-deprived) ± 1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol. Total RNA was isolated and Northern blot performed with specific probes to the ABCA2 (A), LDLR (B), HMGCoA synthase (C) transcripts.

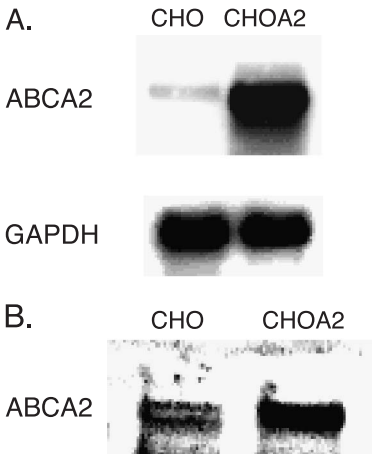

Fig. 2. Stable constitutive expression of ABCA2 in CHO cells. CHO cells were transfected with an ABCA2 expression construct using the FlpIn System and selected in hygromycin. Isogenic clonal cell lines were expanded and analyzed for ABCA2 mRNA expression by Northern blot (A) and for protein expression by Western blot (B).

3.2. Stable constitutive expression of ABCA2 in CHO cells

The Chinese Hamster Ovary (CHO) cell line is an established model system to study cholesterol [18,19,25]. An ABCA2 expression construct was introduced into CHO cells using the FlpIn System to generate stable isogenic cell lines (CHOA2). ABCA2 transcription was driven by the cytomegalovirus promoter to permit constitutive expression independent of changes in cellular cholesterol levels. Total RNA or protein was isolated from cell lysates and ABCA2 mRNA expression was measured by Northern blot or ABCA2 protein expression by Western blot. ABCA2 expression was elevated at both the mRNA (Fig. 2A) and protein level (Fig. 2B) CHOA2 cells. Two independently derived CHOA2 clonal cell lines were analyzed with similar results.

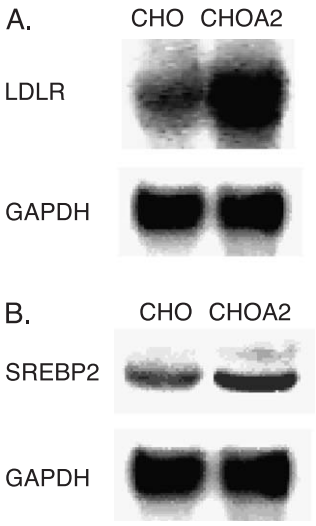

Fig. 3. Sterol-responsive gene expression in CHO and CHOA2 cells. CHO and CHOA2 cells were cultured in medium containing fetal bovine serum (FBS), total RNA was isolated and LDLR (A) and SREBP2 (B) expression was measured by Northern blot. The experiment was repeated and a representative blot is shown.

3.3. Sterol-responsive gene expression in CHO and CHOA2 cells

We tested the hypothesis that stable constitutive expression of ABCA2 in CHOA2 cells would coordinately increase the expression of genes involved in the regulation of cholesterol homeostasis. In response to culture under sterol-depleted conditions in medium containing LPDS, the LDLR expression is elevated through activation of transcription mediated by the sterol cleavage activating protein (SCAP) and the transcription factor, sterol response element binding protein-2 (SREBP2) [26]. CHO and CHOA2 cells were cultured in medium containing fetal bovine serum, a source of LDL lipoproteins. Total RNA was isolated and LDLR and SREBP2 expression was measured by Northern blot. LDLR (Fig. 3A) and SREBP2 (Fig. 3B) mRNA expression was elevated in CHOA2 cells. The experiment was repeated and a representative blot is shown.

If constitutive expression of ABCA2 in CHOA2 cells cultured in medium containing serum as a source of LDL-FC mimics sterol-deprivation and activation of the cholesterol salvage pathway and LDLR transcription, then we would predict that the cholesterol synthetic pathway would also be activated and another sterol-responsive gene should be similarly up-regulated. To validate this hypothesis, we measured expression of the HMGCoA S in CHO and CHOA2 cells. The HMGCoA S gene is activated under sterol-depleted conditions to synthesize cholesterol de novo [27]. Cells were cultured in medium containing serum and

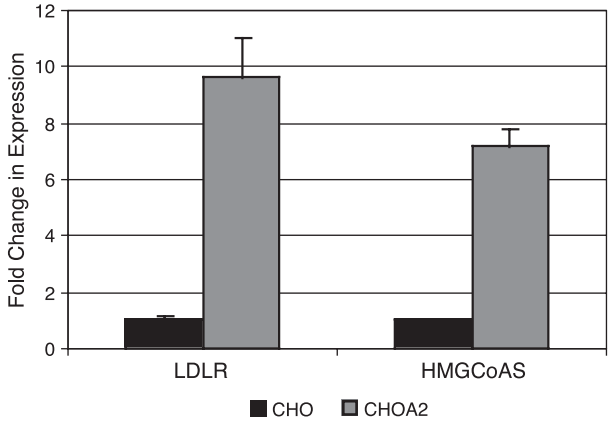

Fig. 4. Real time PCR measurement of LDLR and HMGCoA mRNA levels in CHOA2 cells. Cells were cultured in medium containing FBS for 48 h. Total RNA was isolated and reverse transcribed. Real time PCR was performed using LDLR- and HMGCoA S-specific primers. Quantitation of transcript levels was calculated by the comparative C_T method. The data are expressed as the average fold increase in expression of duplicate determinations relative to the level in CHO cells (± S.D.).

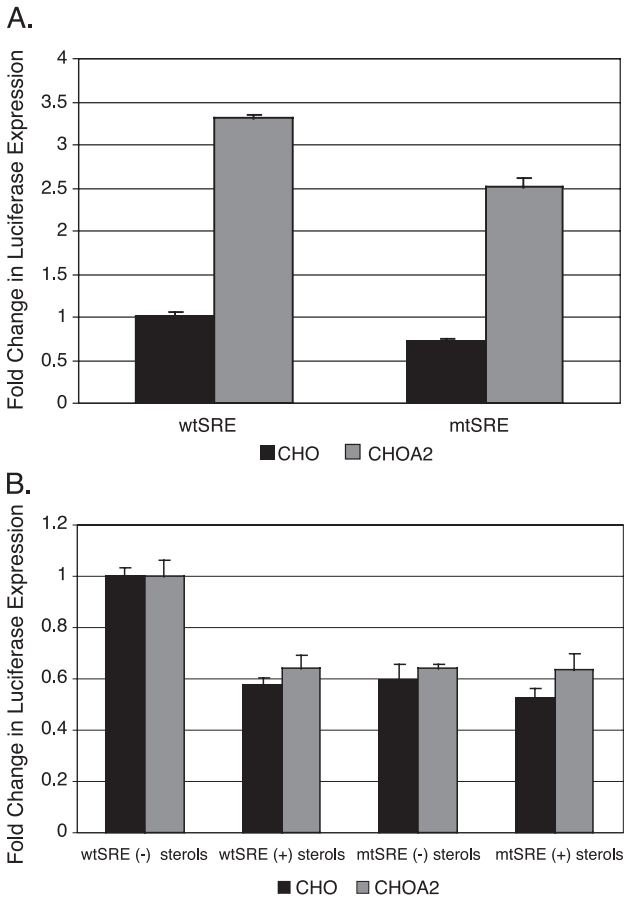

Fig. 5. Analysis of LDLR promoter expression in CHO and CHOA2 cells. (A) CHO and CHOA2 cells are transfected with a luciferase reporter gene construct containing the LDLR promoter extending from −1541 to −41 bp relative to the translation start, or a construct that contains a mutation in the SRE element (position 24–120 bp) that abrogates binding and activation by SREBP2. Cells are cultured in serum containing medium for 48 h and luciferase activity measured in cell lysates. (B) CHO and CHOA2 cells are transfected as above and cultured in complete medium with 5% LPDS for 48 h ± 1 μg/ml 25 hydroxycholestrol and 10 μg/ml cholesterol. Data are expressed as the average fold change in expression in CHO cells (A) or untreated cells (B) of three independent experiments (± S.D.).

LDLR and HMGCoA S mRNA expression was measured by real time PCR. As predicted, the LDLR mRNA was elevated greater than ninefold in CHOA2 cells and HMGCoA S expression was also elevated ~7-fold in CHOA2 cells (Fig. 4).

3.4. LDL-receptor promoter expression is elevated in CHOA2 cells

To determine whether the increase in LDLR expression in CHOA2 cells cultured in medium containing serum is regulated at the level of transcription, we performed reporter gene assays on the LDLR promoter. We measured the expression of an LDLR promoter luciferase reporter construct extending from −1541 to −41 bp relative to the translation start site and observed an increase in transcription of ~3.5-fold in CHOA2 cells compared to the parental cell line (Fig. 5A). When we mutated the serum response element (SRE) located at position −120 bp relative to the translation start site to abrogate SREBP binding and activation, we measured an ~25% decrease in transcription of the LDLR promoter in CHOA2 cells. These results suggest that constitutive expression of ABCA2 induces an increase in LDLR expression at the level of transcription and that the increase, in part, requires an intact SRE element that can bind the sterol-response element binding factors.

If the LDL lipoprotein component present in serum is responsible for the elevation in the transcription of the LDLR gene in CHOA2 cells, then culture of CHO and CHOA2 cells in LPDS should result in similar levels of LDLR transcription in these cells. Cells were cultured for 48 h in lipoprotein-deficient medium following transfection of the reporter gene constructs into CHO and CHOA2 cells. As predicted, we measured similar levels of LDLR promoter expression under these conditions (Fig. 5B). The expression in CHO and CHOA2 cells was reduced about 40% in both CHO and CHOA2 cells by addition of 25-hydroxycholesterol to the culture medium. Addition of 25-hydroxycholesterol inhibits the activity of the sterol cleavage activator protein (SCAP) located in the ER, which mediates the cleavage and activation of SREBP2 [26]. Mutation of the SRE element in the LDLR promoter also results in a similar reduction in transcription. These findings suggest that when cells are sterol-deprived by culture in lipoprotein-deficient media, LDLR expression is subject to similar controls in CHO and CHOA2 cells.

3.5. CHO and CHOA2 cells have similar uptake of fluorescent DiI-LDL

To address whether CHO and CHOA2 cells, cultured under sterol-depleted conditions in medium containing LPDS, express similar levels of functional LDL receptors,

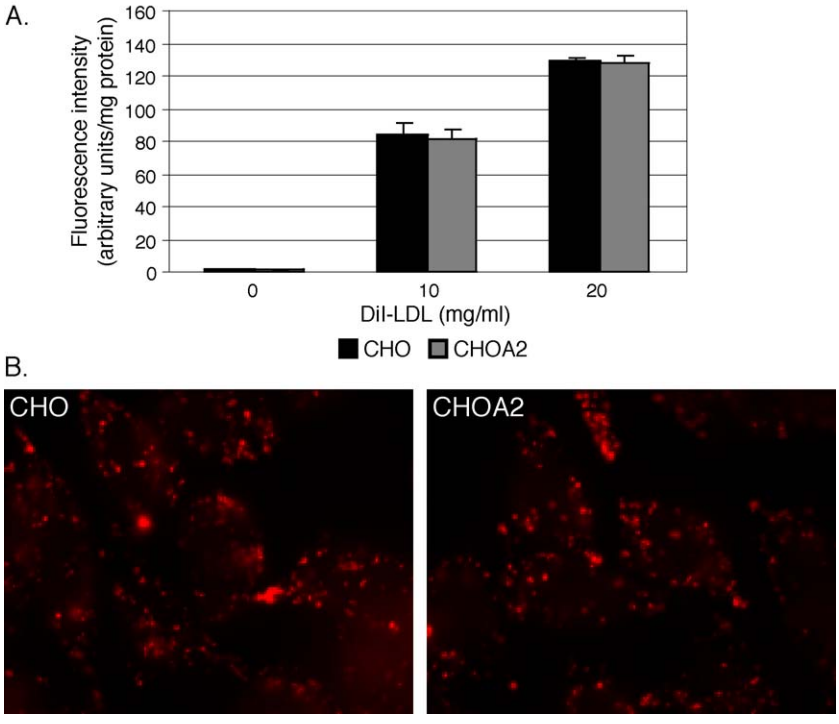

Fig. 6. CHO and CHOA2 cells exhibit similar uptake and distribution of fluorescent DiI-LDL. (A) CHO and CHOA2 cells were cultured in medium supplemented with 5% LPDS for 48 h and subsequently for 5 h in the same media containing 0, 10 or 20 μg/ml DiI-LDL. Cell lysates were prepared and fluorescence measured as described in Section 2. Values represent the average fluorescence of triplicate measurements (± S.D.). (B) Cells were cultured as described above and DiI-LDL fluorescence visualized by UV microscopy as described in Section 2. Shown is a representative field from repeated experiments.

we evaluated the effects of constitutive expression of ABCA2 on uptake and distribution of LDL labeled with the fluorochrome, DiI-LDL. No differences were measured in the dose-dependent uptake of DiI-LDL between CHO and CHOA2 cells as measured by spectrophotometric analysis of cell lysates (Fig. 6A). Ultraviolet fluorescence microscopy revealed a similar distribution of the DiI-LDL label in cytoplasmic vesicles in CHO and CHOA2 cells (Fig 6B). These findings suggest that the uptake and trafficking of LDL to lysosomes is normal in cells constitutively expressing ABCA2.

3.6. CHOA2 cells exhibit reduced esterification of LDL-FC

If constitutive ABCA2 expression in CHOA2 cells leads to an increase in expression of genes activated upon sterol-deprivation, then it may affect the delivery of LDL-FC to the endoplasmic reticulum. We investigated whether constitutive expression of ABCA2 would result in a decline in esterification of [^{14}C] oleate into cholesteryl [^{14}C] oleate with increasing concentrations of LDL in intact cells. CHO and CHOA2 cells were cultured in LPDS medium for 48 h to deplete sterols and then supplemented with increasing amounts of LDL. Following addition of [^{14}C] oleate, the esterification of LDL-FC was measured following lipid extraction and separation by TLC. We measured an almost 60% decline in cholesterol esterification in CHOA2 cells at all concentrations tested compared to the parental cell line (Fig. 7A).

The decrease in LDL-FC esterification measured in CHOA2 cells may have been due to a direct inhibitory effect of ABCA2 expression on the activity of the acyl-coenzyme A:cholesterol acyltransferase (ACAT) enzyme. If constitutive ABCA2 expression in CHOA2 cells does not directly affect ACAT activity, then an assay of ACAT activity in cell-free extracts of CHO and CHOA2 cells should give similar levels of cholesterol ester formation. CHO and CHOA2 cells were cultured in LPDS medium to deplete sterols and then supplemented with 50 μg/ml LDL. Following addition of [^{14}C] oleoyl CoA, lipids were extracted and separated by TLC. No significant difference was measured in the transfer of [^{14}C] oleate from [^{14}C] oleoyl CoA to cholesterol ACAT activity in cell-free extracts of CHO and CHOA2 cells (Fig. 7B) and we infer that constitutive expression of ABCA2 does not affect ACAT activity.

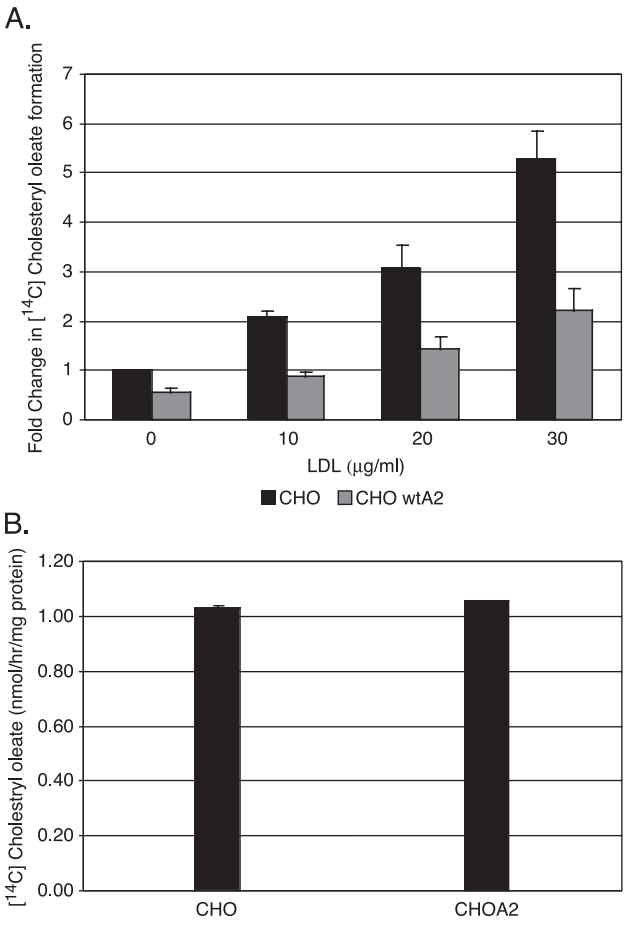

Fig. 7. CHOA2 cells exhibit reduced esterification of LDL-derived cholesterol. (A) CHO and CHOA2 cells were grown as described in Section 2. The growth medium was replaced with DMEM/Ham's F12 supplemented with 5% LPDS and 10, 20, or 30 μg/ml of human LDL and incubated for 5 h at 37O C. Monolayers were pulsed for 2 h with 20 μl of 10 mM [^{14}C] oleate. Lipids were extracted and cholesteryl [^{14}C] oleate formation resolved by TLC. Values represent the average fold change in cholesteryl [^{14}C] oleate formation of triplicate determinations (nmol/h/mg protein ± S.E.) compared to CHO cells incubated in the absence of LDL addition. (B) CHO and CHOA2 cells were grown as described above and ACAT activity determined from 100 μg of cell lysate and 100 μM [1-^{14}C] oleoyl CoA after lipid extraction and TLC. The data are expressed as the average [^{14}C] cholesteryl oleate formation (nmol/h/mg protein ± S.D.) of two independent determinations.

3.7. Filipin staining of unesterified cholesterol in CHOA2 cells and CHO cells cultured with U18666A

The hydrophobic amine, 3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one (U18666A) impairs the intracellular transport of LDL-FC, resulting in free cholesterol sequestration in lysosomes [18]. U18666A can also inhibit cholesterol synthesis [20]. If ABCA2 inhibits the movement of LDL-FC from late-endosomes and lysosomes, then constitutive expression of ABCA2 in CHOA2 cells should exhibit similar filipin fluorescent staining patterns of unesterified free cholesterol as in CHO cells treated with U18666A. CHO cells were cultured in medium containing serum as a source of lipoproteins, treated with U18666A and stained with filipin. As expected, the U18666A-treated cells showed intense staining of perinuclear vesicles and reduced plasma membrane staining as detected by UV microscopy (Fig. 8, panels A and B). CHOA2 cells stained with filipin exhibited a similar pattern of staining, suggesting sequestration of free cholesterol within cytoplasmic vesicles (Fig. 8, panels C and D).

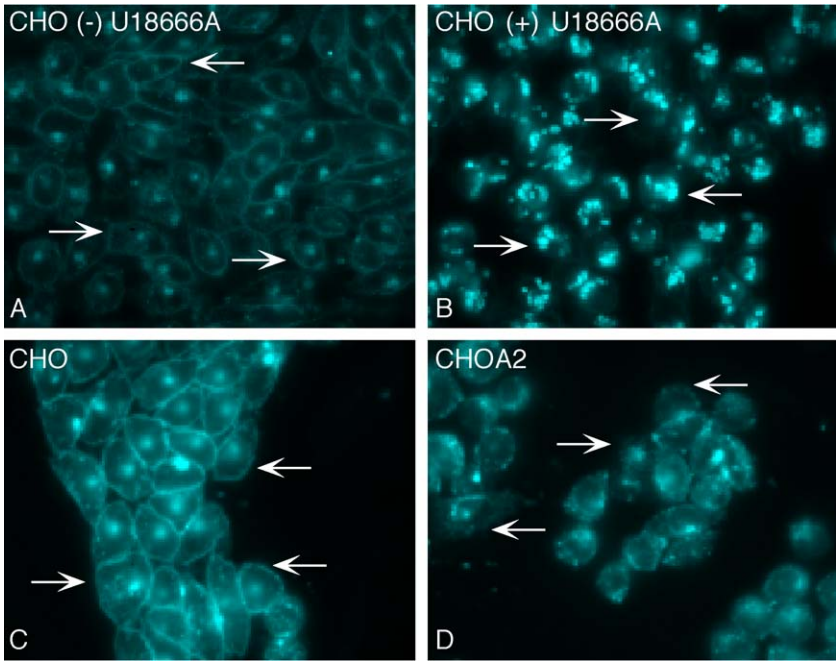

Fig. 8. Filipin staining of unesterified cholesterol in CHOA2 cells and CHO cells cultured with U18666A. (A and B) CHO cells were cultured in medium containing FBS for 24 h ± 5 μM U18666A. (C and D) CHO and CHOA2 cells were cultured in medium containing FBS for 48 h. Cells were stained with filipin and visualized by UV microscopy. The arrows depict the intensity of filipin staining of plasma membrane free cholesterol. Shown are representative fields from repeated experiments.

3.8. Progesterone and U18666A increase expression of ABCA2

The steroid hormone progesterone is reported to block LDL-derived cholesterol translocation from lysosomes and cholesterol esterification and as well as inhibit de novo cholesterol synthesis [17,19]. We hypothesized that the increased lysosomal accumulation of LDL-FC and decreased cholesterol synthesis mediated by progesterone could mimic culture of cells under sterol-deprived conditions. A similar effect would be expected for culture of cells in medium containing U18666A. We measured an elevation in ABCA2 expression (over twofold and 3.5-fold, respectively, by real-time PCR (Fig. 9) in CHO cells cultured with progesterone (10 μg/ml) or U18666A (5 μM). Similar results were observed in HepG2 cells (data not shown).

3.9. ABCA2 expression is elevated in NPC1 and FHC fibroblasts

NPC1 fibroblasts have a defect in the delivery of LDL-FC from a late-endosomal/lysosomal compartment to the endoplasmic reticulum, leading to cholesterol accumulation within these vesicles [28]. The expression of the LDLR and the endogenous cholesterol synthetic genes are elevated in these fibroblasts. In FHC, fibroblasts from patients lack functional LDL receptors [29]. This effect mimics culture of cells in sterol-deficient media with a consequent induction of cholesterol synthetic genes. We hypothesized that decreased delivery of LDL-FC to the endoplasmic reticulum in Niemann–Pick fibroblasts or decreased uptake of LDL in FHC fibroblasts would mimic sterol-deprivation and result in activation of ABCA2 expression. Normal fibroblasts and fibroblasts from FHC patients were cultured in complete medium and following RNA isolation, ABCA2 expression was measured by Northern blot. ABCA2 expression was elevated in FHC fibroblasts compared to normal fibroblasts.

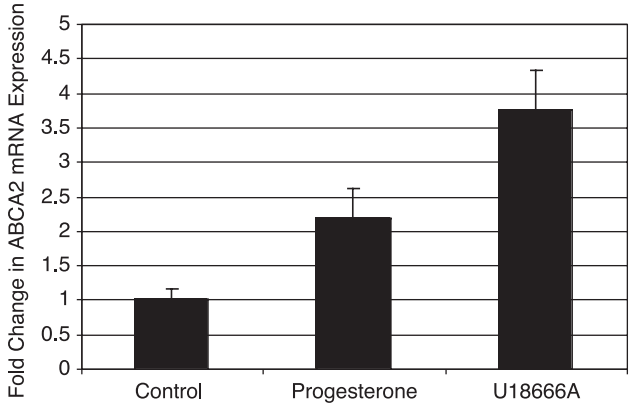

Fig. 9. Progesterone and U18666A increase expression of ABCA2. CHO cells were cultured in complete medium containing FBS and progesterone (10 μg/ml) or U18666A (5 μM) as described in Section 2. Total RNA was isolated and reverse transcribed. Real time PCR was performed ABCA2-specific primers. Quantitation of transcript levels was calculated by the comparative C_T method. The data are expressed as the average fold increase in expression of duplicate determinations relative to the level in untreated CHO cells (± S.D.).

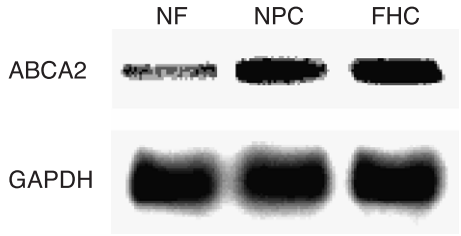

Fig. 10. ABCA2 expression is elevated in NPC1 and FHC fibroblasts. Normal fibroblasts (NF), Niemann–Pick type C1 fibroblasts (NPC) and Familial Hypercholesterolemia (FHC) fibroblasts were cultured as described in Section 2. Total RNA was isolated and ABCA2 mRNA levels were measured by Northern blot. The experiment was repeated and a representative blot is shown.

The experiment was repeated and a representative blot is shown (Fig. 10).

4. Discussion

We hypothesized that due to the high degree of similarity between the ABCA2 and ABCA1 transporters, they may serve similar functions in regulating cholesterol homeostasis. ABCA1 localizes to the plasma membrane and is functional in the efflux of excess cholesterol from the cells, principally macrophages and enterocytes, into high-density lipoprotein particles [7]. The subcellular localization of the ABCA2 transporter in lysosomes suggested a possible role for ABCA2 in regulating the movement or efflux of LDL-derived free cholesterol from the lysosome to the plasma membrane and endoplasmic reticulum. Both immunofluorescent and electron microscopic analyses have detected high levels of ABCA2 expression in late-endosomes/lysosomes [14,15].

In murine macrophages, sustained cholesterol influx mediated by modified LDL (eLDL) induces an up-regulation of ABCA2 mRNA [11]. In addition to up-regulation of ABCA2, expressions of ABCG1 [30], ABCA3 [31] and ABCA7 [32] are also elevated by treatment with eLDL. These authors suggest that ABCA2 may be a component of lipid export machinery that is activated upon excessive cholesterol influx into macrophages. This conclusion is consistent with our findings that ABCA2 expression may be activated to prevent excess free cholesterol flux from the lysosome to other cellular compartments following sustained cholesterol influx from LDL.

In this report we have provided several lines of evidence that support an argument for the coordinated expression of ABCA2 in regulation of low-density lipoprotein-derived free cholesterol metabolism. We have determined that the expression of ABCA2 is coordinately increased along with the expression other sterol-responsive genes, LDLR, HMGCoA S and SREBP2. These sterol-responsive genes are elevated under sterol-deprived conditions and repressed upon oxysterol addition and they constitute components of the cholesterol salvage and cholesterol synthetic pathways (Fig. 11). We employed a constitutively ABCA2 expressing CHO cell line model system to study its effects on LDL-FC metabolism. Real time PCR measurement of LDLR and HMGCoA synthase expression revealed that both salvage and synthetic cholesterol pathways were induced by constitutive ABCA2 expression. This finding suggested that constitutive expression of ABCA2 may mimic sterol-deprivation of cells in culture, leading to activation of genes that restore cholesterol levels. Evaluation of LDLR promoter activity in cells cultured in complete medium containing FBS (a source of LDL-FC) confirmed that LDLR expression was elevated at the level of transcription. Mutation of the SRE element in the LDLR promoter suggested that transcription was partially SRE-dependent but an SRE-independent mechanism was also suggested by the data. It is possible that esterified polyunsaturated fatty acids (e.g. arachidonic acid), a component of LDL [33,34], may contribute to the activation of the LDLR promoter. Dysregulation of LDLR expression has been observed in prostate cancer cell lines and is believed to be due to the delivery of arachidonic acid into cells that promotes persistent elevation of LDLR expression [35,36]. That free cholesterol derived from LDL is required for the differences in LDLR transcription measured in CHO and CHOA2 cells is supported by evidence demonstrating no difference in transcription when these cells are cultured in LPDS. The addition of 25-hydroxycholesterol, which suppresses SREBP2-dependent transcription by inhibition of the SCAP, also reduced LDLR transcription in CHO and CHOA2 cells. In addition, LDLR transcription was SRE-dependent as mutation of the SRE element reduced expression in CHO and CHOA2 cells to similar levels.

We determined that constitutive expression of ABCA2, in cells cultured in medium containing LPDS to deplete sterols, does not affect the level of the LDLR or the uptake and distribution of LDL in CHO and CHOA2 cells. We investigated uptake and trafficking by following the fate of endocytosed fluroescent DiI-LDL. This reagent has been used in many studies to measure uptake and trafficking of LDL in various model systems [23,37–39]. Our measurements of similar LDLR level, DiI-LDL uptake and distribution are consistent with the similar levels of LDLR reporter gene transcription determined under the same sterol-deprived culture conditions.

Our hypothesis that constitutive expression of ABCA2 inhibits delivery of LDL-FC to the endoplasmic reticulum, which mimics culture under sterol-deprived conditions, was tested by measurement of LDL-dependent esterification of [^{14}C] oleate to [^{14}C] cholesteryl oleate. The decline in cholesterol esterification measured in intact CHOA2 cells was in contrast to measurement of ACAT activity in cell-free extracts. Similar rates of transfer of [^{14}C] oleate from [^{14}C] oleoyl CoA to cholesterol in cell-free extracts from CHO and CHOA2 cells indicates that constitutive expression of ABCA2 does not directly inhibit the ACAT enzyme. Fur-

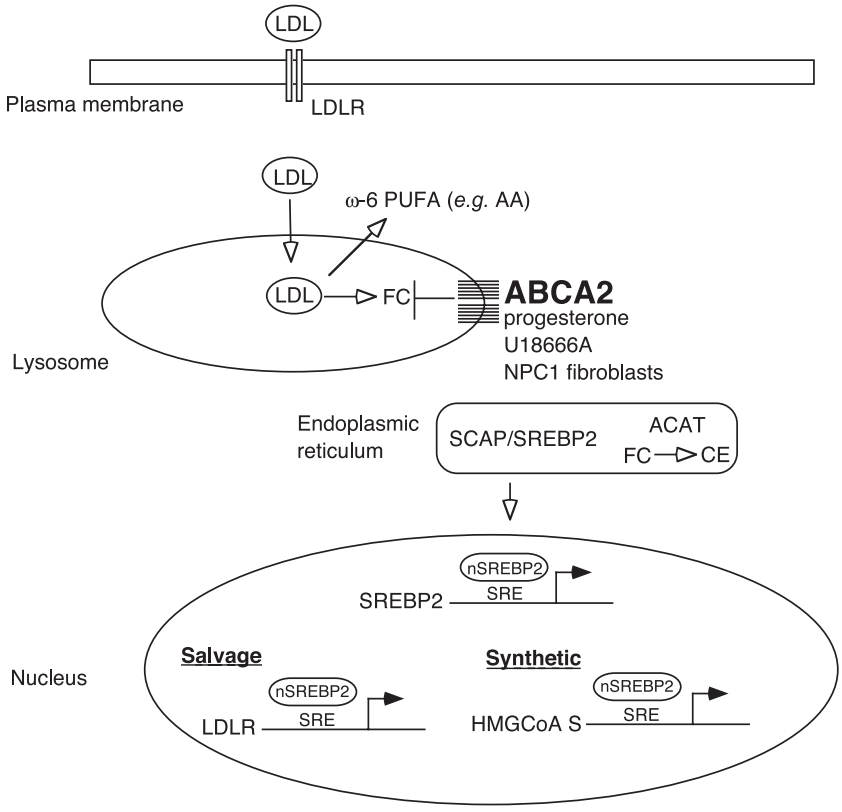

Fig. 11. Model for ABCA2 regulation of LDL-FC trafficking and metabolism. The fate of LDL-FC is depicted following receptor-mediated endocytosis of the bound LDLR. The role of ABCA2 in inhibition of the delivery of FC from the lysosome and eventual trafficking to the endoplasmic reticulum for esterification by ACAT is depicted. The inhibition of LDL-FC movement is similar to the effect observed for progesterone, U18666A and in Niemann–Pick fibroblasts. The reduced LDL-FC delivery to the lysosome leads to the SCAP-mediated activation of the SREBP2 transcription factor, which induces transcription of the LDLR (salvage pathway) and HMGCoA S (synthetic pathway) to restore cholesterol homeostasis. LDL (low-density lipoprotein), LDLR (low-density lipoprotein receptor), PUFA (polyunsaturated fatty acid), AA (arachidonic acid), SCAP (sterol cleavage activator protein), SREBP2 (sterol response element binding protein-2.

ther, these results suggest that the reduction in LDL-dependent cholesterol esterification measured in intact CHOA2 cells is a consequence of a decline in the delivery of free cholesterol to the endoplasmic reticulum.

Since the hydrophobic amine U18666A causes accumulation of LDL-FC in lysosomes, we compared the filipin staining pattern of free cholesterol in U18666A-treated CHO cells to that of CHOA2 cells. A similar filipin staining pattern with intense vesicular staining and less intense plasma membrane staining suggests that constitutive expression of ABCA2 and U18666A treatment exert similar effects on cholesterol sequestration within cytoplasmic vesicles. The subcellular localization of ABCA2 in late-endosomes/lysosomes [14,15] suggests that LDL-FC trafficking from this compartment may be retarded by constitutive expression of ABCA2.

As discussed, the hormone progesterone, in addition to U18666A, acts to sequester LDLFC into late-endosomes/lysosomes. Culture of cells in the presence of these agents also results in a decline in LDL-FC esterification, similar to the effect observed for constitutive ABCA2 expression. Both of these agents are also reported to block de novo cholesterol synthesis. The net effect results in a condition of sterol depletion and activation of the cholesterol salvage and synthetic pathways (Fig. 11). We anticipated that when CHO cells were treated with progesterone or U18666A, ABCA2 expression would increase as a result of sterol deprivation and we in fact measured these effects. We may speculate that up-regulation of ABCA2 expression and its transporter activity may be a component of the mechanism for how LDL-FC is sequestered in late-endosomes/lysosomes.

In Niemann–Pick disease, a mutant NPC1 protein inhibits cholesterol trafficking out of the lysosome and results in lysosomal and Golgi accumulation of cholesterol [28]. Both the NPC1 protein and ABCA2 co-localize to LAMP1 and LAMP2 containing lysosomal compartments [40] and we suggest that these proteins may be functionally antagonistic in regulating the movement of LDL-FC from the late-endosomes/lysosomes. In FHC, defective LDLR function deprives cells of cholesterol from the LDL salvage pathway. In both diseases, cells are compromised in their ability to utilize LDL-FC and compensatory mechanisms (i.e., salvage pathway) are activated to provide cholesterol. We anticipated that under these sterol-deprived conditions, ABCA2 expression would be elevated, and we in fact

measured an increase in fibroblasts from NPC1 and FHC patients.

In summary, we have established that ABCA2 is a sterol-responsive gene whose expression is correlated with other genes that regulate cholesterol homeostasis. Constitutive expression of the ABCA2 transporter in CHO cells is correlated with elevated expression of the LDL receptor and other genes that increase cellular cholesterol levels. Evidence suggests that constitutive expression of ABCA2 may inhibit the delivery of LDL-FC to the endoplasmic reticulum, which leads to increased synthesis and activation of transcription factors that positively regulate the expression of sterol-responsive genes. Future studies will examine the mechanisms responsible for the activation of the LDL receptor expression in CHOA2 cells and investigate the role of ABCA2 in diseases that manifest a pathological dysregulation of LDL receptor metabolism.

Acknowledgements

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