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Protein tyrosine phosphatase inhibition down-regulates ligand-induced ABCA1 expression

Winnie Luu, Laura J. Sharpe, Andrew J. Brown*

School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Sydney, NSW 2052, Australia

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ABSTRACT

Objective: ATP-binding cassette transporter (ABC)-A1 is an important protein of cholesterol homoeostasis and atherosclerosis as it is the major lipid transporter responsible for the export of cholesterol from cells. Many studies have examined kinase regulation of ABCA1 expression. In contrast, very little is known about whether dephosphorylation events play a role in ABCA1 expression. In this study, we explored the involvement of phosphatases in the regulation of ABCA1 expression.
Methods and results: We observed that general protein tyrosine phosphatase inhibitors ablated ABCA1 protein and mRNA when stimulated with synthetic ligands. This effect is transcriptional, and appears to involve the nuclear receptor, retinoid X receptor (RXR).
Conclusion: Our data demonstrate that inhibition of protein tyrosine phosphatases down-regulates ABCA1 expression, indicating a new level of regulation of a key protein in cholesterol export.

1. Introduction

High-density lipoprotein (HDL) is a well-established protective factor for heart disease, and is of intense interest as a therapeutic target. Its protective qualities have been attributed to a number of properties, including anti-inflammatory and anti-oxidative effects [1]. A key attribute is HDL's important role in reverse cholesterol transport, the removal of cholesterol from the periphery back to the liver where it can be eliminated from the body as biliary cholesterol and bile acids. ATP-binding cassette transporter (ABC)-A1 mediates the first step of reverse cholesterol transport, exporting cholesterol from macrophage foam cells to lipid-poor apolipoprotein acceptors such as ApoAI. The essentiality of ABCA1 for the formation of HDL is clearly demonstrated in the rare autosomal recessive Tangier disease, where homozygotes with mutations in ABCA1 have extremely low HDL levels and suffer premature atherosclerosis [2]. In addition, genetic variation in ABCA1 predicts ischaemic heart disease in the general population [3]. Studies in mice have confirmed that deletion of ABCA1 is proatherogenic [4], whereas ABCA1 overexpression is atheroprotective [5,6].

ABCA1 is highly regulated at both the transcriptional and post-transcriptional levels. Like several other proteins important in cholesterol efflux, ABCA1 is regulated transcriptionally by the liver X receptor (LXR), a nuclear receptor which forms an obligate heterodimer with retinoid X receptor (RXR). Post-transcriptional processes are also very important in regulating ABCA1 protein levels and activity. In particular, diverse intracellular signalling pathways regulate ABCA1, mostly at the post-translational level, with several kinases implicated, including protein kinase A, protein kinase C, Janus kinase 2, and casein kinase 2 [7,8]. The emphasis thus far has been on protein phosphorylation by kinases, with little attention paid to the reverse process: dephosphorylation by phosphatases.

Since phosphorylation is one of the major and fundamental forms of regulating cellular processes, including the modulation of protein activity, localisation, and stability [9], kinases have been considered the important regulators in signal transduction. However, far from their original reputation as simple housekeeping 'kinase counteracters', phosphatases are now becoming recognised as specific and critical enzymes needed to set the appropriate level of phosphorylation in cells, thereby maintaining homoeostasis [10]. Aberrant phosphatase activity has been implicated in many human diseases, such as cancer and diabetes [11,12].

Phosphatases are classified into two broad families depending on their substrate specificity; protein serine/threonine phosphatases that dephosphorylate phosphoserine and phosphothreonine residues, and the protein tyrosine phosphatases (PTPs) that

Abbreviations: ABC, ATP-binding cassette; CHO-7, Chinese hamster ovary-7; HDL, high-density lipoprotein; LXR, liver X receptor; Na3VO4, sodium orthovanadate; PBGD, porphobilinogen deaminase; PTP, protein tyrosine phosphatase; RXR, retinoid X receptor.

* Corresponding author. Tel.: +61 2 9385 2005; fax: +61 2 9385 1483.
E-mail address: aj.brown@unsw.edu.au (A.J. Brown).

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dephosphorylate phosphotyrosine residues. Dual-specificity phosphatases can dephosphorylate all three phospho-residues [13]. In the human genome, there are approximately 107 PTPs and 40 serine/threonine phosphatases [14]. Although tyrosine phosphorylation accounts for less than 2% of the phosphoproteome [15], PTPs play a disproportionately significant role in human health and disease, with several proposed as attractive therapeutic targets, including PTP1B for obesity and type II diabetes; SHP2 for cancer; and Lyp for rheumatoid arthritis [11,12].

Considering the importance of phosphatases in human health and disease, and that very little is known about them in the context of ABCA1 regulation, we investigated their role in ABCA1 expression. We found that inhibition of an unknown PTP down-regulated LXR/RXR-mediated transcription of *ABCA1*, introducing a novel mode of ABCA1 regulation.

2. Methods

2.1. Materials

Cell-lines were provided as generous gifts; Chinese hamster ovary-7 (CHO-7) cells were from Drs. Michael S. Brown and Joseph L. Goldstein (UT Southwestern Medical Center, Dallas, TX), J774 cells were from The Centre for Vascular Research (UNSW, NSW, Australia), and HeLaT (highly-transfectable HeLa) cells were from Dr. Noel Whitaker (UNSW, NSW, Australia). Anti-ABCA1 antibody was from Abcam (Cambridge, MA). 24*(S)*,25-epoxycholesterol was from Enzo Life Sciences (Farmingdale, NY). Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DMEM/F12), Roswell Park Memorial Institute 1640 medium (RPMI), foetal calf serum (FCS), newborn calf serum, and Opti-MEM I reduced serum medium were from Invitrogen (Carlsbad, CA). Okadaic acid was from Merck (Darmstadt, Germany). LG268 was from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), 9-*cis*-retinoic acid, anti-α-tubulin antibody, BVT948, bovine serum albumin (BSA), compactin (mevastatin), cyclosporin A, ethyl-3,4-dephostatin, GW3965, mevalonate, phenylarsine oxide, protease inhibitor cocktail, sodium fluoride, sodium orthovanadate (Na_3VO_4), and TO901317 were from Sigma–Aldrich (St. Louis, MO). NSC87877 was from Tocris Biosciences (Minneapolis, MN). Lipoprotein-deficient newborn calf serum (LPDS) and lipoprotein-deficient foetal calf serum (FCLPDS) were prepared from newborn calf serum [16] and FCS [17] respectively.

2.2. Cell culture

For experiments, cells were seeded, transfected where required, statin-pretreated (medium contains 5 μM compactin and 50 μM mevalonate) overnight, and treated in fresh media containing 5 μM compactin/50 μM mevalonate in the presence of various compounds. CHO-7 cells were maintained, statin-pretreated, and treated in 5% (v/v) LPDS/DMEM/F12; HeLaT cells were maintained, statin-pretreated, and treated in 10% (v/v) FCS/RPMI; J774 cells were maintained in 10% (v/v) FCS/RPMI, and were statin-pretreated and treated in 10% (v/v) FCLPDS/RPMI.

2.3. Plasmid constructs

Firefly luciferase plasmids consisting of the indicated promoter elements driving the luciferase reporter gene were as follows: hABCA1-luc, mut-hABCA1-luc [18], 3×hLXRE-luc (LXRE-luc) [18], pGL3-TK-luc [18], PPRE3-TK-luc (RXRE-luc; a DR-1 response element common to both the RXR-response element and PPAR-response element), and pGL3-TK2-luc (generated by deletion of PPRE3 from PPRE3-TK-luc). To control for transfection efficiency, we co-transfected with a *Renilla* luciferase plasmid, phRL-PBGD (PBGD-Renilla [19]). A FLAG epitope tag was inserted into the pCMX-hRXR plasmid [20] by polymerase incomplete primer extension [21]. Mutated RXR constructs were created by mega-primed mutagenesis [22].

2.4. Western blot analysis

After treatment, cells were harvested for ABCA1 as described [23], with minor modifications. Briefly, cells were harvested in Triton X buffer [20 mM HEPES (pH 7.4), 5 mM KCl, 5 mM $MgCl_2$, 0.5% Triton X-100] supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate and 10 mM β-glycerophosphate). The cell lysate was incubated on ice for 10 min, followed by a 10 min centrifugation at 10,000 × *g*. Protein concentrations of the Triton-soluble fraction were determined using the bicinchoninic acid assay kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. For experiments where LXR and RXR protein were examined, cells were harvested using SDS lysis buffer as described [24]. Equal amounts of protein were mixed with loading buffer [final conc.: 50 mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v) glycerol, 0.04% (w/v) bromophenol blue, and 1% (v/v) β-mercaptoethanol], and subjected to SDS-PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane, blocked for 1 h, incubated with primary anti-ABCA1 antibody (1:2500) or anti-α-tubulin (1:200,000), and then further incubated with secondary antibody (1:20,000). The antibodies were visualised by the enhanced chemiluminescent detection system, and membranes were exposed to Hyperfilm. Proteins were identified by their predicted molecular weight (ABCA1, 254 kDa; α-tubulin, 50 kDa; LXR: 51 kDa; RXR, 51 kDa). Before reprobing, antibodies were removed with stripping buffer [25 mM glycine, 1.5% (w/v) SDS, pH 2].

Protein band intensities from Western blots were quantified by densitometry using ImageJ (Version 1.45s) [25]. The bands corresponding to ABCA1 were quantified and normalised to the bands corresponding to α-tubulin to yield relative intensities, with the +GW3965 condition set to 1.

2.5. Quantitative reverse-transcriptase PCR (qRT-PCR)

CHO-7, HeLaT, and J774 cells were seeded in triplicate wells per condition in 12-well plates and were statin-pretreated overnight. Cells were refreshed with media containing compactin, mevalonate, and treatments as indicated in the figure legends. Cells were harvested for total RNA using TRI Reagent, according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed to cDNA with Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). qRT-PCR was performed using a Corbett Rotorgene 3000 and analysed using Rotor-Gene Version 6.0 (Build 27) (Qiagen, Doncaster, VIC, Australia). Primers were used to amplify the cDNA of hamster, mouse, or human *ABCA1* [26,27], *ABCG1* [28], *ABCG2* [29], *SREBP-1c* (human: [20], mouse: designed for this study), and the housekeeping control *porphobilinogen deaminase* (*PBGD*) [27,30]. Changes in gene expression levels of *ABCA1*, *ABCG1*, and *ABCG2* were normalised to *PBGD* for each sample by the ΔΔCt method, and made relative to the +GW3965 or +9-*cis*-retinoic acid condition, which was set to 1.

2.6. Luciferase assay

HeLaT cells were seeded in triplicate wells per condition in 24-well plates. Cells were refed with fresh media prior to transfection