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Regulation of retinoid mediated cholesterol efflux involves liver X receptor activation in mouse macrophages

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ABSTRACT

Removal of cholesterol from macrophage-derived foam cells is a critical step to the prevention of atherosclerotic lesions. We have recently demonstrated the functional importance of retinoids in the regulation of the steroidogenic acute regulatory (StAR) protein that predominantly mediates the intra-mitochondrial transport of cholesterol in target tissues. In the present study, treatment of mouse macrophages with retinoids, particularly all-trans retinoic acid (atRA) and 9-cis RA, resulted in increases in cholesterol efflux to apolipoprotein AI (Apo-A1). Activation of the PKA pathway by a cAMP analog, $(Bu)_2$cAMP, markedly augmented retinoid mediated cholesterol efflux. Macrophages overexpressing hormone-sensitive lipase increased the hydrolysis of cholesteryl esters and concomitantly enhanced the efficacy of retinoic acid receptor and liver X receptor (LXR) ligands on StAR and ATP-binding cassette transporter A1 (ABCA1) protein levels. RAs elevated StAR promoter activity in macrophages, and an increase in StAR levels augmented cholesterol efflux to Apo-A1, suggesting retinoid-mediated efflux of cholesterol involves enhanced oxysterol production. Further studies revealed that retinoids activate the LXR regulated genes, sterol receptor-element binding protein-1c and ABCA1. These findings provide insights into the regulatory events in which retinoid signaling effectively enhances macrophage cholesterol efflux and indicate that retinoid therapy may have important implications in limiting and/or regressing atherosclerotic cardiovascular disease.

1. Introduction

The deposition of excess lipids/cholesterol esters (CEs) in the arterial walls is a hallmark of atherosclerosis, in which macrophages play a pivotal role in forming foam cells. Atherosclerosis is the primary event in cardiovascular diseases and has become the leading cause of morbidity and mortality worldwide [1,2]. Numerous processes, involving dysfunctional macrophage cholesterol homeostasis, contribute to the initiation and progression of atherosclerotic lesions [3,4]. Removal of excess CEs/lipids from macrophage-derived foam cells is crucial in limiting plaque stability and regression of atherosclerosis and associated cardiovascular diseases [2,5–7]. Noteworthy, the StAR protein (also known as StARD1) primarily regulates the delivery of cholesterol from the outer to the inner mitochondrial membrane and, thus, plays an important role in cholesterol coupled events.

Vitamin A (retinol) and its derivatives, especially atRA and 9-cis RA, (collectively referred to as 'retinoids'), play important roles on a spectrum of developmental and physiological processes [8–10]. Retinoids are hormone-like molecules that principally act through two families of ligand-activated nuclear receptors, the retinoic acid receptor (RAR) and retinoid X receptor (RXR), each of which have three major subtypes (α, β and γ), which form either hetero- or homo-dimers [11,12]. Whereas RARs are activated by both atRA and 9-cis RA, RXRs are induced exclusively by 9-cis RA. These receptors are expressed in a wide variety of tissues, suggesting they may be independently regulated, respond to discrete ligands, and perform distinct cellular functions [8,9,11]. We have reported that retinoids, functioning primarily through cAMP/PKA signaling, synergistically

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activate $(Bu)_2$cAMP-induced StAR expression and steroidogenesis [9], a process that may be effective in enhancing cholesterol clearance from macrophage foam cells.

Regulation of cellular cholesterol metabolism and balance involves multiple processes, including *de novo* synthesis of cholesterol, lipoprotein-derived uptake of cholesteryl esters, cholesterol esterification and reverse cholesterol transport [2,13]. Hormone-sensitive lipase (HSL) catalyzes the hydrolysis of CEs in macrophages and steroidogenic tissues and, by doing so, it plays an essential role in regulating intracellular cholesterol homeostasis [5,14,15]. HSL also mediates retinyl ester hydrolysis, and male sterility in HSL knockout mice is associated with perturbed retinoid metabolism [16,17]. Both retinoid and/or cAMP/PKA signaling enhance HSL activity, and HSL mediated up-regulation of StAR expression has been tightly linked with LXR pathways [9,15,18]. LXRs (α and β) bind to oxysterol ligands, form obligate heterodimers with RXRs, and have been shown to regulate the transcription of a number of genes involved in cholesterol utilization, metabolism and balance [13,19]. In this report we demonstrate that retinoid signaling enhances macrophage cholesterol efflux through involvement of the LXR regulatory pathway, which may play an important role in the prevention of atherosclerotic lesions.

2. Materials and methods

2.1. Cells, plasmids, transfections, and luciferase assays

Mouse RAW 264.7 macrophages (ATCC, Manassas, VA) were cultured in DMEM/F12 medium (Invitrogen Life Technologies, Grand Island, NY) supplemented with 10% FBS, 1% glutamine, and antibiotics.

The 5-flanking −254/−1bp region of the mouse StAR promoter was synthesized using a PCR based cloning strategy [9,20]. Expression plasmids for HSL, SREBP-1c, pRL-SV40, and pLXREx3 have been described [15,20]. Full-length mouse StAR cDNA [21] was inserted into *Eco*RI and *Xba*I sites of the pCMV5 vector. All plasmids were verified by sequencing.

Macrophages were transfected with HSL, StAR, LXRE, and SREBP-1c expression plasmids using Lipofectamine 2000 reagent (Invitrogen) under optimized conditions [9,15,22]. Transfection efficiency was determined by co-trasfecting 10–20 ng of the pRL-SV40 plasmid. Luciferase activity in cell lysates was determined by the Dual-luciferase reporter assay (Promega) [9,15,20].

2.2. Determination of cholesterol efflux

Cholesterol efflux was determined following the procedures described previously [3,23]. Briefly, macrophages were labeled with ^{3}H-cholesterol (0.5 μCi/ml) for 24 h in DMEM/F12 media containing 0.1% BSA. Macrophages were equilibrated in serum-free medium containing 0.1% BSA, washed with 0.01 M PBS, and then treated with retinoids and/or LXR ligands for 12 h in the absence or presence of apolipoprotein A1 (Apo-A1, 20 μg/ml). Following treatments, medium and cells were collected separately and counted in a liquid scintillation counter (LS6500, Beckman), and cholesterol efflux was calculated as the percentage of radioactivity recovered in the media over total (cells plus media) radioactivity.

2.3. Determination of cholesteryl esters (CEs)

Levels of CE were determined using Amplex Red Cholesterol Assay (Invitrogen) Kit [15]. Briefly, macrophages were extracted with chloroform:isopropanol:NP40 (7:11:1). Following centrifugation, the organic phase was transferred to another tube, dried, and dissolved in 250 μl of cholesterol assay buffer. Extracted samples or cholesterol standard (10–20 μl) were mixed with Amplex Red working solution without (for measuring free cholesterol) or with (for measuring total cholesterol) cholesterol esterase. After incubation at 37 °C for 30 min, fluorescence was measured utilizing excitation at 560 nm and emission at 590 nm. Esterified cholesterol was calculated by subtracting the free cholesterol from the total cholesterol.

2.4. Quantitative RT-PCR

Total RNA from macrophages was extracted using Trizol reagent (GIBCO-BRL, Grand Island, NY). StAR and L19 cDNAs were amplified using one-step RT-PCR procedure described previously [9,24,25]. RT and PCR were run sequentially in the same assay, which included [α^{32}P]-dCTP (Perkin–Elmer) in the dNTP mixture. The molecular sizes of StAR and L19 were determined on 1.2% agarose gels, which were vacuum dried and exposed to X-ray films. Levels of StAR and L19 signals were quantified using a computer-assisted image analyzer (Quantity One Software, Bio-Rad Laboratories).

2.5. Immunoblotting

Immunoblotting studies were carried out using total cellular protein [9,15,26]. Briefly, equal amounts of protein were loaded onto 10–12% SDS-PAGE (Bio-Rad Laboratories, Inc., Hercules, CA). The proteins were electrophoretically transferred onto Immuno-Blot PVDF membranes which were probed with the specific antibodies that recognize StAR [27], HSL (Cell Signaling Technology, Beverly, MA), ABCA1 (Millipore, Temecula, CA), and β-actin (Applied Biosystems/Ambion, Austin, TX). Following overnight incubation with primary antibodies, the membranes were washed and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The immunodetection of different proteins was determined using a Chemiluminescence Imaging Kit (Perkin–Elmer), and the intensity of bands was quantified using an image analyzer (Quantity One Software).

2.6. Statistical analysis

All experiments were repeated at least three times. Statistical analysis was performed by ANOVA using Statview (Abacus Concepts Inc., Berkeley, CA) followed by Fisher's protected least significant differences test. Data presented are the mean ± SE, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Assessment of retinoid signaling in cholesterol efflux in mouse macrophages

The hypothesis that retinoid signaling increases StAR expression, and thereby can influence macrophage cholesterol efflux, was examined. Mouse macrophages treated with atRA and 9-cis RA (10 μM; [9]), for 12 h, demonstrated 2.7 ± 0.4 and 2.4 ± 0.3 fold increases in cholesterol efflux to Apo-A1 (20 μg/ml), respectively, over untreated cells (Fig. 1A). Both atRA and 9-cis RA had no apparent effects on cholesterol efflux in the absence of Apo-A1. A suboptimal dose of a cAMP analog, $(Bu)_2$cAMP (0.1 mM), moderately but consistently, elevated ($p < 0.05$) cholesterol efflux to Apo-A1. Addition of $(Bu)_2$cAMP, to either atRA or 9-cis RA incubation, enhanced macrophage cholesterol efflux by 2–3 fold when compared with their responses individually.

To ascertain the involvement of retinoid signaling in cholesterol efflux, selective analogs with affinities to both RAR (TTNPB) and

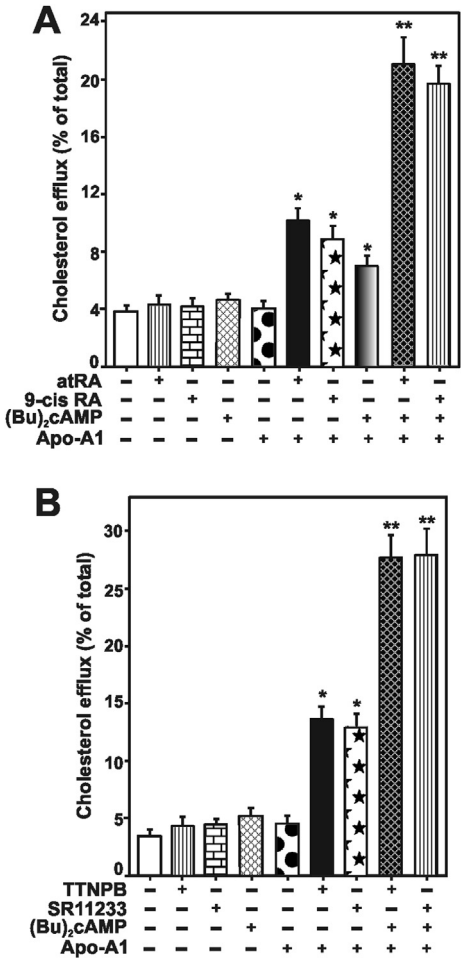

Fig. 1. Effects of retinoids on $(Bu)_2$cAMP stimulated macrophage cholesterol efflux. Mouse macrophages were labeled with ^{3}H-cholesterol for 24 h, and then treated without or with atRA and 9-cis RA (both at 10 μM; A), TTNPB and SR11233 (both at 5 μM; B), and $(Bu)_2$cAMP (0.1 mM; A and B), or their combination, for 12 h, in the absence or presence of Apo-A1 (20 mg/ml). Cholesterol efflux was determined following optimized procedure described under *Materials and Methods*. Data represent the mean ± SE of four independent experiments. *, $p < 0.05$; **, $p < 0.01$; vs. control.

RXR (SR11233) were examined. Macrophages treated with TTNPB (5 μM) and SR11233 (5 μM) resulted in 3.9 ± 0.7 and 3.2 ± 0.5 fold increases in macrophage cholesterol efflux to Apo-A1 when compared with control (Fig. 1B). $(Bu)_2$cAMP further augmented TTNPB- and SR11233-induced cholesterol efflux.

3.2. Overexpression of HSL on CE hydrolysis, StAR and ABCA1 protein levels, and their correlation to cholesterol efflux

Regulation of HSL mediated StAR expression involves LXR signaling [9,15]. Macrophages overexpressing HSL increased HSL protein over basal. HSL appeared as a doublet, with an 83-kDa major and an 81-kDa minor species, respectively [15]. Overexpression of HSL decreased CE content ~50% when compared to mock-transfected (pcDNA3) cells (Fig. 2A), suggesting HSL enhances the hydrolysis of CEs in macrophages.

An increase in HSL levels augmented ($p < 0.05$) both StAR and ABCA1 protein levels. Macrophages treated with atRA and T0901317 (an LXR agonist; T1317; 5 μM, [15]) resulted in increases in StAR (2.4 ± 0.3 and 2.7 ± 0.4 fold) and ABCA1 (3.6 ± 0.5 and 4.1 ± 0.7 fold) protein expression, respectively (Fig. 2B). Overexpression of HSL increased atRA- and T1317-induced StAR and

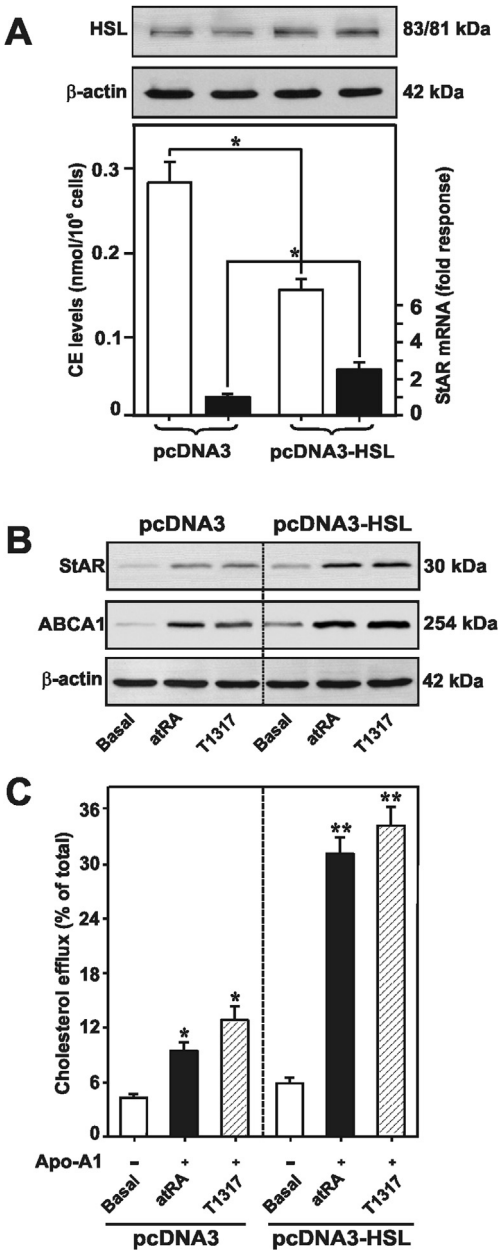

Fig. 2. Overexpression of HSL on CE and StAR mRNA levels, and their correlation to atRA and T1317 induced StAR, ABCA1, and cholesterol efflux. Macrophages were transfected with either pcDNA3 or pcDNA3-HSL expression plasmid, as described under *Materials and Methods*. Following 36 h of transfection, CE content in the cell lysates was assessed using Amplex Red Cholesterol Assay Kit and presented as nmol/10^6 cells (A). Macrophages were also extracted for total RNA for determining StAR mRNA expression by RT-PCR analysis (A). Macrophages were also directly utilized for treatments or labeled with ^{3}H-cholesterol for 24 h and then treated without (Basal) or with atRA (10 μM; B,C) and T1317 (5 μM; B,C), for an additional 12 h. Representative immunoblots (n = 4–5) illustrate expression of HSL, StAR and ABCA1 in different groups using 70–90 μg of total protein (A,B). β-actin expression was assessed as loading controls. C, cholesterol efflux was determined following assay procedure demonstrated in the legend of Fig. 1. Data represent the mean ± SE of four independent experiments. *, $p < 0.05$; **, $p < 0.01$ vs. pcDNA3 or Basal.

ABCA1 protein levels between 2 and 5 fold. Both atRA and T1317, individually, exhibited ~3-fold increase in macrophage cholesterol efflux, an effect was further enhanced ($p < 0.01$) following HSL overexpression. This suggests that HSL enhances the efficacy of RAR and LXR ligands on StAR and ABCA1 expression, which may result in cholesterol clearance from macrophage-derived foam cells.

3.3. Effects of retinoids on StAR promoter activity, and role of StAR in retinoid mediated cholesterol efflux

Macrophages transfected with the −254/−1bp StAR-Luc segment, containing an LXR-RXR/RAR heterodimeric motif, resulted in ~2-fold increase in StAR promoter activity in response to either atRA or 9-cis RA (Fig. 3A). Whereas $(Bu)_2$cAMP displayed no effect in StAR reporter activity, it elevated ($p < 0.01$) atRA/9-cis RA-induced StAR promoter responsiveness.

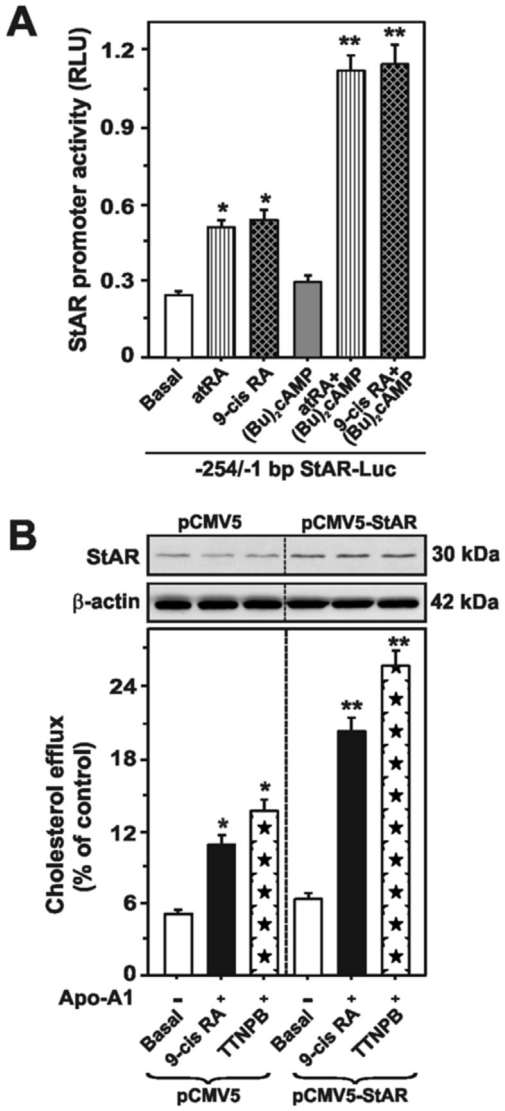

Fig. 3. Effects of atRA and 9-cis RA on $(Bu)_2$cAMP-induced StAR promoter activity, and role of StAR on retinoid mediated cholesterol efflux. Macrophages were transfected with the −254/−1bp StAR luciferase reporter segment (−254/−1bp StAR-Luc) in the presence of pRL-SV40 (A). Following 36 h of transfection, cells were treated without (Basal) or with atRA (10 μM), 9-cis RA (10 μM), $(Bu)_2$cAMP (0.1 mM), or their combination, for an additional 12 h. Luciferase activity in the cell lysates was determined and expressed as StAR promoter activity, (RLU, luciferase/renilla). Macrophages were also transfected with pCMV5-StAR expression plasmid. Representative immunoblots (n = 4) illustrate expression of StAR in different groups. Following transfection, macrophages were labeled with ^{3}H-cholesterol for 24 h and then treated without (Basal) or with 9-cis RA (10 μM) and TTNPB (5 μM), for additional 12 h, in the absence or presence of Apo-A1 (20 mg/ml), as indicated. Cholesterol efflux was determined following assay procedure demonstrated in the legend of Fig. 1. (B, bottom panel). Immunoblots shown are representative of four independent experiments. β-actin expression was assessed as a loading control. Data represent the mean ± SE of four independent experiments. *, $p < 0.05$; **, $p < 0.01$; vs. pCMV5 or Basal.

StAR protein, by mediating the delivery of cholesterol, influences oxysterol formation; thus, it was of interest if an increase in StAR levels is capable of potentiating retinoid mediated cholesterol efflux. Macrophages transfected with the pCMV5-StAR plasmid enhanced ($p < 0.05$) StAR protein expression over mock-transfected (pCMV5) cells (Fig. 3B). Overexpression of StAR resulted in 2–4 fold increases in Apo-A1 mediated cholesterol efflux in response to either 9-cis RA or TTNPB when compared with mock controls (Fig. 3B, bottom).

3.4. Involvement of LXR signaling and its relevance to macrophage cholesterol efflux

Oxysterols act as ligands for LXRs. To gain more insights into the LXR regulatory events, macrophages were transfected with an LXRE luciferase reporter plasmid (pLXREx3-Luc; [28]). As depicted in Fig 4A, pLXREx3-Luc transfected macrophages treated with either atRA or SR11233, resulted in 2.9 ± 0.4 or 3.1 ± 0.5 fold increase in LXR activity over untreated cells. Whereas $(Bu)_2$cAMP had no apparent effect, it markedly enhanced ($p < 0.001$) atRA- and TTNPB-induced LXR activity.

In additional studies, macrophages transfected with the −2.7kb/+1bp SREBP-1c promoter reporter plasmid demonstrated 3.1 ± 0.4 and 3.5 ± 0.7 fold increases in SREBP-1c reporter activity by atRA and SR11233 over untreated cells, respectively (Fig. 4B). These responses were further enhanced in the presence of $(Bu)_2$cAMP. Both atRA and SR11233 demonstrated ~3 fold increases in ABCA1 protein over basal (Fig. 4C). $(Bu)_2$cAMP (0.1 mM) also enhanced ($p < 0.05$) ABCA1 protein. Macrophages co-treated with either atRA or SR11233 in the presence of $(Bu)_2$cAMP markedly elevated ABCA1 protein levels, suggesting retinoid-mediated macrophage cholesterol efflux involves modulation of LXR pathways. Immunocytochemical analyses revealed localization of StAR and ABCA1 in basal, atRA, and atRA plus $(Bu)_2$cAMP treated macrophages (Supplemental Fig. 1), and corroborated the results presented in Figs. 2–4. These data indicate that retinoids activate LXR signaling, which, in turn, enhances plasma membrane cholesterol trafficking and efflux in mouse macrophages.

4. Discussion

Accumulation of excess cholesterol and lipids in the arterial walls, resulting in foam cell formation, is a critical event in the initiation and development of atherosclerosis [1,2,4]. Elimination of excess cholesterol from macrophage foam cells is the key to prevention and/or regression of atherosclerotic lesions. Epidemiological evidences indicate that eating a diet rich in vitamins has numerous health benefits, as well as protective effects on development of diseases [29,30]. Retinoids exert a multifaceted array of effects on development, differentiation, vision, reproduction, skin physiology, and bodily homeostasis [8,9,30]. As such, a number of retinoid and/or its receptors, transporters, and metabolizers have been used as therapeutic targets in numerous complications and diseases, including atherosclerosis [30,31]. The present studies extend previous observations and expand our understanding by elucidating the molecular events in which retinoids act to drive macrophage cholesterol efflux, which is a fundamental process in stabilizing/regressing atherosclerosis.

Our results demonstrate that retinoid signaling effectively enhanced cholesterol efflux in mouse macrophages. The activation of cAMP/PKA signaling markedly augmented retinoid-induced macrophage cholesterol efflux, concomitant with increased LXR activity. These results indicate that the protection of atherosclerotic lesions involves modulation of LXR signaling [15,18]. RAW macrophages express relatively higher level of RARα than that of RXRα

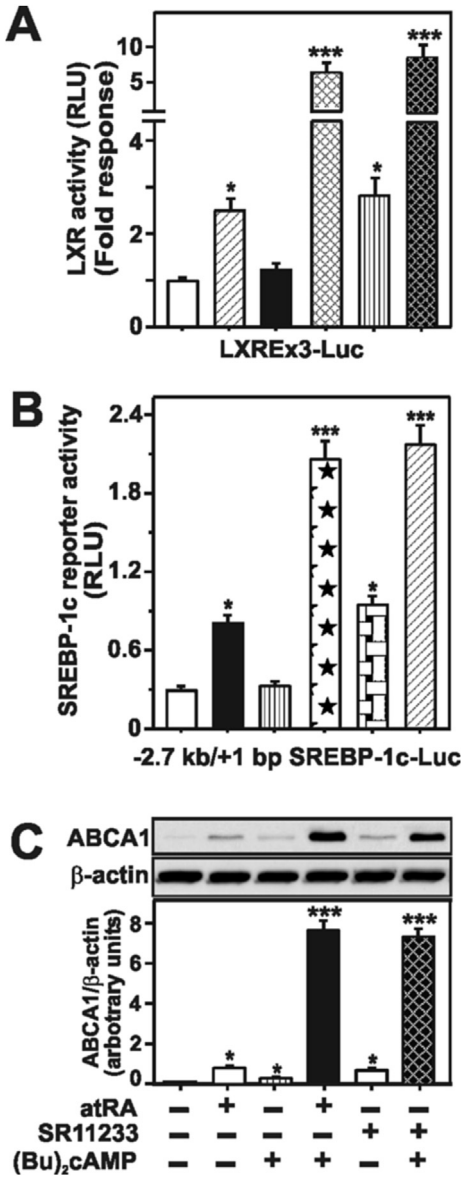

Fig. 4. Involvement of retinoids in activation of LXR signaling. Macrophages were transfected with either pLXREx3-Luc (A) or −2.7kb/+1bp SREBP-1c-Luc (B) plasmid in the presence of pRL-SV40. Following 36 h of transfection, macrophages were treated without or with atRA (10 μM), SR11233 (5 μM), $(Bu)_2$cAMP (0.1 mM), or their combination, for an additional 12 h (A–C). Luciferase activity in the cell lysates was determined and expressed as either fold LXR activity (A) or SREBP-1c (B) reporter activity (RLU, luciferase/renilla). Results represent the mean ± SE of four independent experiments. Representative immunoblot illustrates ABCA1 protein expression using 70–90 μg of total protein (C). β-actin expression was assessed as a loading control. Integrated optical density values of ABCA1 in each band were quantified and normalized with the corresponding β-actin expression, and compiled data (n = 4, ±SE) are presented in bottom panel. *, $p < 0.05$; ***, $p < 0.001$ vs. control.

(data not shown), implicating the majority of retinoid-responsive cholesterol efflux is effected by RARs. Additionally, a low level of PKA activity is required for retinoid mediated cholesterol transport, reinforcing the notion that phosphorylation of StAR is an indispensable event for its optimal cholesterol transferring capacity [9,22,25]. Noteworthy, the increase in retinoid-mediated cholesterol efflux requires the presence of Apo-A1, a cholesterol-binding component of high-density lipoprotein (HDL). The latter is considered to have atheroprotective functions, in contrast to LDL that is atherogenic [1,2]. Consequently, cholesterol efflux has been reported to be regulated by ABCA1 that transports cellular cholesterol to Apo-A1 [32,33]. In the present study, whereas retinoid mediated macrophage cholesterol efflux was tightly linked with enhanced expression of SREBP-1c and ABCA1, involvement of additional LXR-target genes that have been associated with cholesterol trafficking and efflux [4,7], cannot be excluded.

The functional importance of retinoid signaling in macrophage cholesterol efflux was elucidated in connection with HSL that plays an essential role in regulating intracellular cholesterol metabolism [14–16,18]. Our present results demonstrate that macrophages overexpressing HSL increased the hydrolysis of CEs resulting in a depletion of CE content and elevated StAR mRNA expression. Concurrently, the efficacy of RAR and LXR ligands on StAR and ABCA1 protein levels was enhanced. These results imply that an increase in HSL levels promotes oxysterol production, which activates LXR and results in up-regulation of retinoid mediated macrophage cholesterol efflux. We reported that HSL overexpression increases the capability of LXR ligands on StAR expression [15]. Therefore, it is plausible that LXR activation enhances plasma membrane cholesterol trafficking and efflux, and modulates cholesterol esterification, thus contributing to the effects of retinoids in controlling cholesterol balance. In accordance, LXRs and/or their interaction with RXRs have been shown to regulate the expression of SREBP-1c, ABCA1, and StAR for sustaining cholesterol metabolism and homeostasis [12,19].

Oxysterols are regarded as physiological ligands for LXRs and involvement of oxysterols on StAR expression has been reported. Our present data document that the increase in RA-responsive StAR promoter activity was markedly enhanced by cAMP/PKA signaling that phosphorylates StAR. Besides, an increase in StAR levels effectively modulated the efficacy of retinoids in cAMP/PKA mediated macrophage cholesterol efflux [6,7]. As such, elevated expression of StAR (by either retinoids or overexpression), involving efficient cholesterol transport mechanisms triggered by LXR signaling, could be a useful therapeutic approach for the prevention of foam cell formation and atherosclerosis. Of note, there are 15 distinct START (StAR-related lipid transfer) domain proteins (StARD1-StARD15) in humans; thus, it seems likely that additional STARD protein(s) might be involved in lipid/cholesterol transport and metabolism. In support of this, endosomal trafficking protein StARD3 (also known as MLN64, metastatic lymph node 64) has been involved in limiting atherogenesis by influencing cholesterol efflux [34].

An interesting aspect of the present findings is the activation of the LXR regulatory pathway in retinoid mediated macrophage cholesterol efflux. Noteworthy, however, during the transport of cholesterol, StAR protein provides the substrate cholesterol not only to P450scc (that transiently produces 22-hydroxycholesterol) but also to 27-hydroxylase (produces 27-hydroxycholesterol), both enzymes are located at the mitochondrial inner membrane. Concomitantly, involvement of 27-hydroxycholesterol has been reported in cholesterol efflux [7]. It is conceivable that retinoids, by enhancing StAR expression and/or oxysterol production, play an important role in macrophage cholesterol efflux. The identification of oxysterol(s) and their relative contribution on retinoid-mediated macrophage cholesterol efflux is expected to improve understanding on atherosclerosis. Based on our current findings, it is tempting to speculate that regulation of retinoid mediated macrophage cholesterol efflux is influenced by the following linked events: retinoid signaling → increased StAR protein expression → oxysterol production → activation of LXRs and its dimerization with RXRs → increased expression of SREBP-1c and ABCA1 → cholesterol efflux → stabilization and/or regression of atherosclerosis. Further studies, utilizing animal models of atheroma, are necessary to establish the importance of retinoid signaling in limiting/regressing atherosclerotic cardiovascular disease.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.150.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.150.

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