Overexpression of steroidal acute regulatory protein increases macrophage cholesterol efflux to apolipoprotein AI

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Aims
In this study, we investigated the impact of enhancing cholesterol delivery to mitochondrial sterol 27-hydroxylase, via steroidal acute regulatory protein (StAR), on the expression of genes involved in macrophage cholesterol homeostasis and efflux of cholesterol to apolipoprotein (apo) AI.

Methods and results
Stably transfected, murine (RAW 264.7) macrophages were used to investigate the role of StAR in cholesterol homeostasis. Cellular responses were analysed using quantitative PCR, immunoblotting, and an LXRE reporter plasmid; [3H]cholesterol efflux was measured in the presence or absence of apoAI. Macrophage overexpression of mitochondrial cholesterol trafficking protein, StAR, activates and induces expression of liver X receptors (LXRs), and significantly alters expression of genes involved in cholesterol homeostasis, decreasing Fdps, Hmgcr, Mvk, Ldr, and Scap, and markedly increasing Abca1 mRNA and protein. Overexpression of StAR, but not mutated ‘loss-of-function’ (R181L) StAR, enhanced efflux of [3H]cholesterol to apoAI, and this effect was maintained in macrophages pretreated with LDL or acetylated LDL. The effect of StAR overexpression on apoAI-dependent [3H]cholesterol efflux was mimicked by non-sterol agonist, T901317, and 27-hydroxycholesterol, and blocked by LXR inhibitor, geranylgeranyl pyrophosphate, sterol 27-hydroxylase inhibitor, GW273297x, and probucol, inhibitor of ATP binding cassette transporter A1 (ABCA1). Importantly, all observed effects of StAR overexpression were dependent upon cyclic AMP (cAMP analogue, dibutyryl cAMP), which is required for the full activity of the StAR protein to be manifested.

Conclusion
Macrophage overexpression of StAR significantly enhances LXR-dependent apoAI- and ABCA1-dependent cholesterol efflux, by which disposal of excess arterial cholesterol deposits and atheroma regression can be achieved.

Keywords
Atherosclerosis • Liver X receptor • Sterol 27-hydroxylase • Cholesterol efflux • Apolipoprotein • apoAI

1. Introduction
Macrophage ‘foam cells’, laden with cholesterol and cholesteryl esters, are a hallmark of early and developing atherosclerotic lesions, influencing both plaque progression and stability.1 Reversal of macrophage lipid accumulation, by cholesterol efflux and the reverse cholesterol transport pathway mediated by high-density lipoprotein (HDL), remains a key anti-atherogenic strategy.2 Cholesterol efflux to acceptors, such as apolipoprotein (apo) AI, apoE, and HDL, is orchestrated by ATP binding cassette transporters A1, G1, and G4.3–6 Expression of these proteins is controlled, at least in part, by liver X nuclear receptors (LXRx/β),5 which heterodimerize with 9-cis retinoid X receptors (RXRs), and bind endogenous oxysterols, including 27-hydroxycholesterol,6 generated by the mitochondrial sterol 27-hydroxylase encoded by CYP27A1.7 Up-regulation of macrophage CYP27A1 expression, by ligands for the peroxisomal proliferator-activator receptor-γ and RXRs,8,9 results in increased levels of 27-hydroxycholesterol, upregulation of LXR-mediated processes and in elimination of CYP27 metabolites as an alternative means of cholesterol efflux.10,11 High levels of sterol 27-hydroxylase are found in vascular endothelium.12 CYP27A1 is colocalizes with macrophages, in human atherosclerotic lesions,13,14 and 27-hydroxycholesterol is a major...
oxysterol in human atheroma.\textsuperscript{15} The importance of CYP27A1 in regulating macrophage cholesterol homeostasis is highlighted by the rare genetic disorder cerebrotendinous xanthomatosis, associated with macrophage ‘foam cell’ formation and increased risk of premature atherosclerosis, despite normal concentrations of cholesterol in the circulation.\textsuperscript{16} Importantly, the rate-limiting step controlling the activity of sterol 27-hydroxylase is the supply of cholesterol to the enzyme, located on the matrix side of the inner mitochondrial membrane,\textsuperscript{17} which can be mediated by steroidogenic acute regulatory protein (StAR).\textsuperscript{17–25}

In steroidalogenic tissues, StAR is a critical regulator of the synthesis of adrenal and gonadal steroids, delivering cholesterol to the P450 side-chain cleavage enzyme resident on the matrix side of the inner mitochondrial membrane, which converts cholesterol to pregnenolone.\textsuperscript{19–22} StAR is a nuclear encoded, mitochondrially targeted protein, expressed in cytoplasm as a 37 kDa protein, imported into mitochondria and inactivated by cleavage to a 30 kDa protein.\textsuperscript{19–22} Phosphorylation of human StAR on Ser195 by protein kinase A (PKA), combined with rapid induction of StAR biosynthesis, explains the acute regulation of steroidalogenic response in response to trophic hormones.\textsuperscript{23} Mutated StAR is responsible for abrogated steroidalogenesis and abnormalities associated with avoid congenital adrenal hyperplasia;\textsuperscript{24} the same phenotype is reproduced after targeted disruption of the StAR gene in mice.\textsuperscript{25}

Steroidalogenic acute regulatory protein has also been found in the liver, where it increases the activity of CYP27A1, production of oxysterols, and synthesis of bile acids by the ‘alternative’ pathway.\textsuperscript{17,18,26,27} Ma et al.\textsuperscript{23} recently provided evidence for the expression of StAR mRNA and protein in murine macrophages, and aortae of apoE\textsuperscript{-/} mice; our own work found expression of StAR in human monocytes, THP-1 macrophages, and human aortic tissue.\textsuperscript{29} Here, we considered the functional role of StAR in cholesterol homeostasis, demonstrating increases in LXR activity and LXRα gene expression, transient induction of expression of ATP binding cassette transporter A1 (ABCA1) mRNA and protein, and enhanced cholesterol efflux to apoAI, in murine macrophages overexpressing StAR protein.

2. Methods

2.1 Materials

Tissue culture reagents were purchased from Lonza (Wokingham, UK); other sources include: mammalian expression vector, pTK-Hyg (BD Biosciences, Oxford, UK), FuGene6 transfection reagent (Roche), NuPAGE gels, and buffers (InVitrogen), antibodies (AbCAM, Cambridge, UK), T901317 (Tocris), geranylgeranyl pyrophosphate (GGPP) (Sigma), primers/probes (Eurogentec, Belgium). Low-density lipoprotein (LDL) was from Athens Research (USA) and acetylated according to Brown et al.\textsuperscript{20} We are deeply indebted to the following for their very kind gifts: Prof. D. Stocco, University of Texas (murine Stard1 clone), Prof. J. Orly, Hebrew University of Israel (pCMV5 vector), Prof. D.J. Mangelsdorf, South Western Medical Center (LXRα reporter plasmid), Prof. D. Russell, South Western Medical Center (rabbit polyclonal antibody to sterol 27-hydroxylase), Prof. W. Jessup and Dr C. Quinn, University of New South Wales, kindly facilitated by Prof. S.J. Yeaman, University of Newcastle-upon-Tyne (sterol 27-hydroxylase inhibitor, GW2739297x).

2.2 Macrophage cell lines

Murine macrophage cell line, RAW 264.7 (ECACC 91062702), was maintained in DMEM containing UltraGlutamine, supplemented with foetal bovine serum (FBS; 10%, v/v), HEPES (10 mmol L\textsuperscript{-1}), sodium bicarbonate (0.075%, w/v), and penicillin/streptomycin (50 U mL\textsuperscript{-1}), 50 μg mL\textsuperscript{-1}). Subconfluent macrophages (3 x 10\textsuperscript{5}) were co-transfected (48 h) with pTK-Hyg (0.5 μg) and pCMV5 (0.5 μg), the latter containing full-length murine Stard1, or mutated Stard1 (CGA→CTA; Arg181Leu) [site-directed mutagenesis (BioS&T, USA)]. Empty vector (pCMV5) was used as the ‘control’, so that all stably transfected cells were subject to the same procedure. Cells were transfected with pTK-Hyg (0.5 μg) and pCMV5 (0.5 μg) using FuGene6 (6 μL: 1 μg DNA); stably transfected populations were selected and maintained using hygromycin (200 μg mL\textsuperscript{-1}). Transient expression of pCMX.LXR\textsubscript{E} (0.5 μg) in the same way, and luciferase assay (Brlitetile Plus, Perkin Elmer) assessed 24 h later. Lipids were extracted from media\textsuperscript{31} and 27-hydroxycholesterol and free cholesterol identified by co-migration with authentic standards by t.l.c. using petroleum ether:diethyl ether:ethanol glacial acetic acid (90:30:1, v/v/v) as the mobile phase. Efflux of 27-oxygenated sterol was expressed as percentage of dpm in the cholesterol pool (media + cells).

2.3 Analysis of gene and protein expression

Total RNA was isolated (TriZol) from stably transfected macrophages, reverse transcribed to cDNA (BioLine), and polymerase chain reactions (PCR) using primers designed to generate a 355 bp ampiclon from Stard1 (forward: 5’-GGTCTCTGCTAGCTTCAAGC-3’, reverse: 5’-GAACACATGGCCCAACTCT-3’). Levels of mRNA encoding 84 genes implicated in lipoprotein signalling and cholesterol metabolism (SABiosciences) were measured relative to five housekeeping genes, GusB, Hprt1, Hspcb, Gapdh, and Actb. Quantitative PCR (Q-PCR) determined expression levels of Abca1, Abcg1, Abcg4, Lxr, Ldr, Hmgrc, Scap, Mvk, Fdps, Srebf1, Srebf2, Stard3, Olr1, and Soat1 mRNA, and internal ‘housekeeping’ gene, Gapdhs; reactions were performed as previously described\textsuperscript{25} with specific sequences reported in Table 1.

Macrophage cell lysates, prepared in RIPA buffer plus Complete\textsuperscript{34} protease inhibitor cocktail, were separated using NuPAGE gels (10%), transferred to PVDF membrane and probed using rabbit polyclonal anti-StAR antibodies (1:1500); mouse monoclonal antibody to ABCA1 (1:1000), and multi-antibodies (1:2500); rabbit polyclonal antibody to sterol 27-hydroxylase (1:2000), and monoclonal antibody to apoAI (1:2000) to the loading control, GAPDH. Detection was achieved using secondary antibodies and ECL detection system.\textsuperscript{31}

2.4 Macrophage lipid mass and cholesterol efflux

Incorporation of \textsuperscript{14}Cacetic acid (1 μCi mL\textsuperscript{-1}) into fatty acid, phospholipid, cholesterol, cholesteryl ester, and triacylglycerol pools were measured after incubation for 24 h in the presence of cAMP (0.3 mM), in empty vector and StAR-overexpressing macrophages, as previously described.\textsuperscript{29} Macrophage lipids were extracted using hexane: isopropanol (3:2, v/v) and dried under N\textsubscript{2}, before separation by t.l.c., using chloroform, methanol, and water (60:30:5, v/v/v) as the first mobile phase and hexane, diethyl ether, and acetic acid (80:20:1, v/v/v) in the second phase of development; lipids were identified by co-migration with standards.\textsuperscript{29,31} Triacylglycerol mass were determined in lipid extracts, as previously described.\textsuperscript{29,31}

Macrophage efflux of \textsuperscript{3}Hcholesterol to apoAI was determined as previously.\textsuperscript{29,31} Efflux was initiated by the addition of serum-free DMEM containing human apoAI (20 μg mL\textsuperscript{-1}) and in the presence or absence of dibutyryl cAMP (0.3 mM), and terminated after 24 h; addition of cAMP is required both to stimulate PKA-dependent StAR phosphorylation and activity,\textsuperscript{23} and to induce maximal cholesterol efflux in murine RAW 264.7 macrophages.\textsuperscript{2,33} Addition of sterol 27-hydroxylase inhibitor, GW2739297x, LXR agonist, T01317, probucol, N-acetyl-Leu-Leu-norleucine (ALLN), and leupeptin were made using DMSO (<0.01%; v/v) as vehicle; addition of LXR antagonist, geranylgeranyl ammonium pyrophosphate ammonium salt (GGPP), was made in methanol (0.01%; v/v). Results are expressed as % cholesterol efflux, calculated as \textsuperscript{dpm}[media]-\textsuperscript{dpm}[media+cells] x 100%.

All values indicate mean ± SEM, with numbers of independent experiments denoted by n. Significant (P < 0.05) differences were determined.
3. Results

Overall, the strategy was to demonstrate that overexpression, and cAMP-dependent activation, of StAR in macrophages increases oxysterol generation (via Cyp27A1), resulting in agonism of liver X receptors and induction of genes involved in cholesterol efflux, such as ABC transporters, enhancing cholesterol efflux to apoAI. Data on gene and protein expression derived from stably transfected murine macrophages, either expressing StAR or the empty vector, were supplemented with studies utilizing an LXRE reporter plasmid, and pharmacological agents: 27-hydroxycholesterol, LXR agonist (T0901317), and antagonist (GGPP), sterol 27-hydroxylase inhibitor (GW293927x), and inhibitor of ABCA1 function (probucol).

3.1 Overexpression and LXR activating function of StAR in murine macrophages

Expression of StAR mRNA and protein in RAW 264.7 macrophages was increased, as judged by qualitative (Supplementary material online, Figure S1A) and Q-PCR, by 5.2 ± 0.3-fold (P < 0.001) and 2.5 ± 0.66-fold (P < 0.05), respectively, following stable transfection with pCMV.Stard1, compared with empty vector control (Supplementary material online, Figure S1A and B). Moreover, only mature StAR protein (30 kDa) was detected in cell lysates and mitochondria isolated from cells overexpressing StAR, indicating normal mitochondrial processing of this protein19–22 (Supplementary material online, Figure S1B). Increased mitochondrial trafficking of cholesterol to sterol 27-hydroxylase, mediated by overexpression of StAR,17,18,26 resulted in a 45.8% (n = 9; P < 0.05) increase in the flux of [3H]27-oxygenated sterol into the medium, in the presence of cAMP (0.3 mM) and apoAI.

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StAR overexpression was associated with increased activation of an LXRE reporter (luciferase) plasmid (Figure 1A). Importantly, this effect of StAR proved significant only in the presence of a cell permeant cAMP analogue [dibutyryl cAMP (0.3 mM), used throughout, hereafter abbreviated to cAMP]. Cyclic AMP is required to activate the StAR protein,23 indicating normal regulation of the activity of this overexpressed protein (Figure 1A). Addition of LXR agonist, T01317 (10 μM), generated the same degree of LXR activation in both control and StAR-overexpressing macrophages, confirming equivalent transfection efficiencies. Addition of cAMP and T01317 together did not induce any further significant increase in LXR activation in macrophages overexpressing StAR, beyond that seen in the presence of cAMP alone. Activation of LXRE reporter plasmid was attenuated by LXRa antagonist, GGPP (10 μM), although the impact of this inhibitor was reduced in the presence of both T01317 and cAMP in StAR-expressing cells. It is relevant to note that GGPP, a branch point intermediate of isoprenoid biosynthesis, is also involved in post-translational prenylation of several proteins (Ras, Rho, Rac) that modulate a variety of cellular processes including cell signalling, differentiation, and proliferation.

Sterol 27-hydroxylase inhibitor,24 GW2739297x (1 μM), blocked LXR activation in StAR-overexpressing, but not control macrophages (Figure 1B). As LXRa itself possesses an LXRE, steady-state levels of...
mRNA encoding LXRα were measured 12 and 24 h, after the addition of cAMP in both cell lines (Figure 1C). A significant increase (5.1-fold; \( P < 0.001 \)) in LXRα gene expression was observed in StAR-overexpressing macrophages treated with cAMP compared with controls.

### 3.2 Changes in gene expression induced by StAR overexpression in murine macrophages

Overexpression, and activation (cAMP; 0.3 mM), of StAR induced (>2-fold) changes in the expression of 27 of the 84 genes tested using RT² SuperArray (‘Cholesterol and Lipoprotein Signalling’) relative to mRNA levels of housekeeping genes which did not change (Table 2). StAR overexpression increased mRNA levels of Abca1, Cxcl16, and Nsdhl, and decreased the expression of Abca2, Abcg4, Ankra2, Crip, Cxcl16, Ebp, Fdps, Hdlbp, Hmgcr, Ldlr, Mvk, Osbp1, NrOb2, Scap, Stard3, and Srebf1 transcription factors, and of the gene encoding the

### Table 2 Fold changes in the expression of genes implicated in cholesterol metabolism and lipoprotein signalling in StAR-overexpressing macrophages, compared with control cells, as judged by RT² SuperArray profile (SABiosciences) and confirmed for selected genes by Q-PCR in three independent experiments

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>RT² SuperArray profile</th>
<th>Q-PCR (mean ± SEM; n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abca1</td>
<td>41.04</td>
<td>17.54 ± 2.49 (( P &lt; 0.05 ))</td>
</tr>
<tr>
<td>Abca2</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Acaa2</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Abcg4</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>Ankra2</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Crip</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Cxcl16</td>
<td>15.68</td>
<td></td>
</tr>
<tr>
<td>Ebp</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>Fdps</td>
<td>0.42</td>
<td>0.51 ± 0.09 (( P &lt; 0.05 ))</td>
</tr>
<tr>
<td>Hdlbp</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Hmgrc</td>
<td>0.41</td>
<td>0.31 ± 0.15 (( P &lt; 0.01 ))</td>
</tr>
<tr>
<td>Id1</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>Ldr</td>
<td>0.46</td>
<td>0.19 ± 0.04 (( P &lt; 0.01 ))</td>
</tr>
<tr>
<td>Lrp6</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>Mbtps1</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>Mvk</td>
<td>0.46</td>
<td>0.51 ± 0.07 (( P &lt; 0.05 ))</td>
</tr>
<tr>
<td>NrOb2</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Nsdhl</td>
<td>2.19</td>
<td></td>
</tr>
<tr>
<td>Olr1</td>
<td>0.65</td>
<td>1.06 ± 0.44</td>
</tr>
<tr>
<td>Osbp1a</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>Pkao1</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>Pkag2</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>Scap</td>
<td>0.51</td>
<td>0.39 ± 0.10 (( P &lt; 0.01 ))</td>
</tr>
<tr>
<td>Soat1</td>
<td>0.80</td>
<td>1.18 ± 0.25</td>
</tr>
<tr>
<td>Sotr1</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Srebf1</td>
<td>0.36</td>
<td>0.54 ± 0.20</td>
</tr>
<tr>
<td>Srebf2</td>
<td>0.69</td>
<td>0.55 ± 0.20</td>
</tr>
<tr>
<td>Stard3</td>
<td>0.38</td>
<td>0.57 ± 0.25</td>
</tr>
<tr>
<td>Tref1</td>
<td>0.20</td>
<td></td>
</tr>
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</table>

Values quoted are the fold difference in gene expression, where >2-fold changes in mRNA expression are reported; data for Olr1, Soat1, and Srebf2, which changed by <2-fold in the array, are included as references.

Figure 1 Activation of LXRE reporter plasmid in control and StAR-overexpressing macrophages, treated for 24 h + cAMP (0.3 mM) and (A) + T0901317 (10 μM), GGPP (10 μM), or combinations thereof (n = 4) and (B) + GW2739297x (1 μM). Expression of LXRα in control and StAR-overexpressing cells, treated with cAMP (0.3 mM), is shown in Figure 2C. All values are mean ± SEM n = 4–7 experiments; *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \), compared with control macrophages incubated under the same conditions.

StAR increases cholesterol efflux to apoAI
endosomal cholesterol trafficking protein, Stard3 (Table 2). Soat1 and the oxidized LDL receptor (Olr) were confirmed as unaltered by overexpression of StAR protein. The most strongly marked alteration in gene expression associated with StAR overexpression was up-regulation of Abca1 (17-fold; $P < 0.05$), consistent with the observed activation of LXRE in these cells (Figure 2), strongly suggesting a role for StAR in the regulation of cholesterol efflux to apoAI.2,4

3.3 Overexpression of StAR increases macrophage $[^{3}H]$cholesterol efflux to apoAI

Efflux of $[^{3}H]$cholesterol from murine RAW 264.7 macrophages (Figure 2A) is dependent upon the presence of both an acceptor, apoAI, and cAMP analogue:31,32 addition of both apoAI (20 $\mu$g mL$^{-1}$) and cAMP (0.3 mM) significantly ($P < 0.001$) enhanced $[^{3}H]$cholesterol efflux by 2.28-fold in control macrophages. More notably, under the same conditions, % $[^{3}H]$cholesterol efflux was significantly ($P < 0.001$) higher in StAR-overexpressing (3.78-fold) than in control macrophages. Efflux of $[^{3}H]$cholesterol to apoAI (in the presence of cAMP) was inhibited by probucol35 in both control ($P < 0.05$) and StAR-overexpressing ($P < 0.001$) macrophages (Figure 2B), confirming dependence of efflux upon the ABCA1 transporter. Introduction of mutated ‘loss of function’ StAR (Arg181Leu)24 into murine RAW 264.7 macrophages, at equivalent levels to wild-type StAR, did not induce increases in macrophage $[^{3}H]$cholesterol efflux to apoAI, when compared with control macrophages (Figure 2C). Importantly, the effect of overexpression of StAR was mimicked in control cells by the addition of exogenous 27-hydroxycholesterol (5 $\mu$M) added during the labelling period (24 h; Methods) and during the efflux period. Values are the mean ± SEM, $n = 4$ experiments; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with untreated macrophages; ‡$P < 0.05$ for the comparisons indicated.

3.4 Overexpression of StAR in murine macrophages increases expression of Abca1 mRNA and protein during $[^{3}H]$cholesterol efflux to apoAI

Cyclic AMP (0.3 mM) increased the expression of Abca1 by 16.6-fold (12 h, $P < 0.001$; $n = 3$) in StAR-overexpressing macrophages, whereas only non-significant increases were noted in control

![Figure 2](https://academic.oup.com/cardiovascres/article-abstract/86/3/526/317825)
macrophages (Figure 3A and B). In the presence of both cAMP and apoAI, levels of Abca1 mRNA were induced by 32.5-fold and 26.1-fold (P < 0.001; n = 3) after 12 h, compared with controls, in StAR-overexpressing and control macrophages, respectively. This finding is consistent with the previous observations that the promoter region of murine (but not human) Abca1 contains a cAMP response element and that apoAI is required for stabilization of Abca1 mRNA. Protein levels of ABCA1 were substantially increased in StAR-overexpressing macrophages, compared with controls, after 12 h in the presence of cAMP and apoAI together, relative to the GAPDH loading control (Figure 3B). In contrast, after 24 h, protein levels of ABCA1 were significantly lower in StAR-overexpressing macrophages, compared with control macrophages (Figure 3B), and relative to GAPDH, possibly reflecting the higher rate of cholesterol efflux in these cells (Figure 2). As similar levels of ABCA1 mRNA (Figure 3A) were associated with differing levels of ABCA1 protein (Figure 3B), and when compared with cholesterol efflux (Figure 2A), it seemed likely that levels of ABCA1 protein were subject to significant, rapid post-translational regulation. Addition of apoAI appeared to stabilize ABCA1 protein expression, and increase cholesterol efflux, in both control and StAR-overexpressing cells, in good agreement with Arakawa and Yokoyama, however, levels of ABCA1 protein (12 h) were higher in StAR-overexpressing cells than in empty vector controls or in macrophages expressing mutated StAR (R181L) which cannot promote cholesterol efflux (Figure 3C). We also examined levels of ABCA1 protein after treatment with probucol (10 μM) or with thiol protease inhibitors (ALLN 50 μM and leupeptin 100 μM); treatment with thiol protease inhibitors was initiated 4 h after treatment with cAMP and apoAI. In all cases, expression of the housekeeping protein GAPDH remained constant (data not shown for simplicity) and results are representative of three independent experiments.

Figure 3 (A) Expression of Abca1 mRNA 12 h after treatment with cAMP (0.3 mM), apoAI (20 μg mL−1), and both agents together, in control and StAR-overexpressing macrophages. Values are the mean ± SEM of three independent experiments; ***P < 0.001 compared with untreated macrophages. (B) Expression of ABCA1 protein 12 and 24 h after treatment with cAMP (0.3 mM) and cAMP (0.3 mM) and apoAI (20 μg mL−1) together, in control and StAR-overexpressing macrophages, relative to the housekeeping protein GAPDH; the figure shown is representative of three independent experiments. (C) Expression of ABCA1 protein, 12 h after treatment with apoAI (20 μg mL−1), cAMP (0.3 mM), or both together, in control and StAR-overexpressing macrophages, and in macrophages overexpressing mutated R181LStAR (top panel). Levels of ABCA1 protein in control and StAR-overexpressing cells (12 h), following treatment with cAMP (0.3 mM) and apoAI (20 μg mL−1) together, are shown in the middle and bottom panels, and in combination with probucol (10 μM), or protease inhibitors ALLN (50 μM) or leupeptin (100 μM); treatment with thiol protease inhibitors was initiated 4 h after treatment with cAMP and apoAI. In all cases, expression of the housekeeping protein GAPDH remained constant (data not shown for simplicity) and results are representative of three independent experiments.

3.5 Increases in cholesterol efflux caused by StAR overexpression are dependent upon LXR and sterol 27-hydroxylase activities

Expression of CYP27A1 protein did not vary substantively between control or StAR-overexpressing macrophage cell lines (data not shown). Addition of T01317 (10 μM) alone significantly increased apoAI-dependent efflux, in the absence of cAMP, and to a similar degree in both control and StAR-overexpressing macrophages.
Figure 4A), consistent with the LXRE activation data (Figure 1A). Addition of cAMP further enhanced apoAI-dependent $[^{3}H]$cholesterol efflux, and a significantly higher level of efflux in StAR-expressing cells was noted compared with control macrophages (Figure 4A). Notably, the LXR agonist induced a relatively greater increase in $[^{3}H]$cholesterol efflux (62.6%; $P = 0.001$) in control macrophages, compared with StAR-expressing macrophages (24.5%; $P = 0.05$). In contrast, addition of LXR antagonist, GGPP (10 $\mu$M), did not significantly affect $[^{3}H]$cholesterol efflux in control macrophages in the presence of apoAI and cAMP (Figure 4B). However, in StAR-expressing macrophages, the marked increase in cholesterol efflux seen under ‘optimal’ efflux conditions (1.74-fold compared with control macrophages; $P < 0.05$) was effectively negated in the presence of GGPP ($P < 0.05$).

Equally, the specific contribution of CYP27A1 activity to the observed increases in cholesterol efflux was determined using a selective inhibitor of this enzyme, GW2739297x,34 which blocks StAR-dependent activation of LXRE (Figure 1B). GW2739297x did not alter $[^{3}H]$cholesterol efflux under basal conditions (Figure 4C); however, under optimal efflux conditions, GW2739297x significantly reduced ($P < 0.001$) apoAI-dependent $[^{3}H]$cholesterol efflux by 34.2% in control cells and by 43.8% ($P < 0.001$) in StAR-overexpressing macrophages, effectively negating the impact of StAR in these cells.

### 3.6 Overexpression of StAR: impact on macrophage triacylglycerol mass

One negative aspect of non-sterol LXR$\alpha$ agonists is the induction of lipogenesis and accumulation of triacylglycerol, mediated by increased expression of SREBP-1c.29,37 Macrophage triacylglycerol mass (measured in the presence or absence of cAMP, 0.3 mM; 24 h) did not change in StAR-overexpressing cells compared with controls (control $21.6 \pm 2.0$ $\mu$g $\text{mg}^{-1}$ protein vs. cAMP $22.2 \pm 1.8$ $\mu$g $\text{mg}^{-1}$ vs. StAR overexpression $16.9 \pm 0.83$ $\mu$g $\text{mg}^{-1}$ vs. StAR overexpression plus cAMP $24.4 \pm 2.52$ $\mu$g $\text{mg}^{-1}$ protein; mean $\pm$ SEM; n = 3–4).

However, measurement of incorporation of $[^{14}C]$acetate into cellular lipid pools revealed a 2.4-fold increase in cellular lipid biosynthesis, with significant increases in $[^{14}C]$-phospholipid (2.59-fold; $P < 0.01$), $[^{14}C]$-triacylglycerol (4.2-fold; $P < 0.01$), and free $[^{14}C]$cholesterol pools (1.7-fold; $P < 0.05$); no significant changes in $[^{14}C]$-labelled non-esterified fatty acid or cholesteryl ester pools were noted (Figure 5).

Clearly, these data reflect one potential ‘downside’ to the enhanced presence of StAR in vascular cells, an issue which needs to be...
considered carefully when studying the anti-atherogenic impact of StAR in vivo.

4. Discussion

Dysregulated macrophage cholesterol homeostasis lies at the heart of developing atherosclerotic lesions, influencing plaque progression and stability. Evidence is emerging that StAR may play a hitherto unsuspected role in vascular tissues. Our data confirm that StAR is expressed in human monocyte macrophages, and human heart aortae, Ma et al.29 report the presence of StAR in murine macrophages and in aortae of apoE−/− mice, and Ning et al.38 found StAR protein in murine b.End3 endothelium cells. Recently, Ning et al.39 showed that overexpression of StAR in human THP-1 macrophages decreases intracellular lipids and limits secretion of inflammatory factors by these cells. Here, we provide insight into the mechanism(s) by which StAR impacts positively on macrophage lipid phenotype: overexpression of StAR in murine RAW 264.7 macrophages activates and induces LXR, via a pathway which involves sterol 27-hydroxylase (Figure 1). StAR overexpression represses a number of genes involved in cholesterol biosynthesis (Fdps, Hmgcr, Mvk) and uptake (Ldlr) and markedly increases the expression of Abca1 (Table 2), strongly suggesting that StAR increases sterol efflux to apoAI, an effect dependent upon LXR activation, sterol 27-hydroxylase, and ABCA1 activity. Importantly, cAMP was required for the full activity of the StAR protein to be manifested23 and could not be reproduced using a mutated ‘loss-of-function’ StAR protein (R181L).24

It is established that StAR can enhance generation of oxysterols by CYP27A1: overexpression of StAR enhances CYP27A1 activity in transfected COS cells, and in hepatocytes, producing nuclear oxysterols which potently regulate intracellular lipid homeostasis.17,18,26 Here, activation of the LXRE reporter plasmid was enhanced in macrophages overexpressing StAR, but only when StAR was activated by the addition of exogenous cAMP23 and could be blocked by the selective sterol 27-hydroxylase inhibitor, GW2739297x.34 Induction of LXRs mRNA was also observed after cAMP-dependent activation of the StAR protein, in StAR-overexpressing cells, but not in control macrophages (Figure 1).

Activation of LXR appears to be a major factor inducing the expression of Abca1 in macrophages overexpressing StAR. [3H]-cholesterol efflux to apoAI was effectively mimicked by an LXR agonist, and blocked by the inhibition of sterol 27-hydroxylase, and antagonism of LXR (Figure 4). Cyclic AMP up-regulates Abca1 expression in murine macrophages, at the transcriptional level,22,23 via a CAMP response element which acts in conjunction with a nearby STAT3/4 element31 in both control and StAR-overexpressing macrophages the presence of both apoAI and cAMP significantly induced the expression of Abca1 mRNA and protein (Figure 3). However, in macrophages overexpressing StAR, it appears that a sterol 27-hydroxylase-dependent activation of LXR (Figure 2) induces a further up-regulation of Abca1 mRNA and protein (Figure 3), thereby enhancing [3H]-cholesterol to apoAI (Figure 4).

The factor which induces StAR activity in macrophages, via increases in cAMP and activation of PKA, is not known. One hypothesis is that apoAI itself contributes to this process, as the addition of both cAMP and apoAI together enhanced the expression of ABCA1 compared with cAMP alone (Figure 3). ApoAI can activate cellular cAMP signalling through the ABCA1 transporter, triggering serine phosphorylation of ABCA1, and this effect of apoAI is particularly evident in cells expressing high levels of ABCA1.40

Finally, overexpression of StAR alters the expression of other genes implicated in cholesterol metabolism and lipoprotein signalling (Table 2). This profile should be studied more closely as, for example, expression of the pro-atherogenic gene, Cxcl16, appears induced by StAR overexpression. However, the handful of genes investigated here reveals a predominantly anti-atherogenic profile, where StAR overexpression represses transcription of genes implicated in cholesterol biosynthesis (Fdps, Hmgcr, Mvk), and LDL uptake (Ldlr), probably via enhanced oxysterol production, which can suppress SREBP processing by binding to Insig, expression of which declined in this study (Table 2).71,82

In conclusion, StAR appears to regulate macrophage cholesterol homeostasis, highlighting the importance of mitochondrial cholesterol trafficking in regulating LXR activation, downstream regulation of ABCA1 expression and efflux of cholesterol to apoAI.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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References

42. Jaroshovski BA. The hypocholesterolaemic agent Ly295427 upregulates INSIG-1, identifying the INSIG-1 protein as a mediator of cholesterol homeostasis through SREBP. Proc Natl Acad Sci USA 2002;99:12675–12680.