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Keywords
pparγ, cyp27a1, hydroxylase, 27, sterol, regulation, expression, ligands, rxr, role, macrophages, human

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Expression and regulation of sterol 27-hydroxylase (CYP27A1) in human macrophages: a role for RXR and PPARγ ligands

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CYP27A1 (sterol 27-hydroxylase) catalyses an important sterol elimination pathway in the human macrophage, and consequently may protect against atherosclerosis. We studied the expression and regulation of CYP27A1 in a human macrophage-like cell-line, THP-1, and primary HMDMs (human monocyte-derived macrophages). In both macrophage cell types, we found that CYP27A1 expression is independent of cellular cholesterol levels and of LXR (liver X receptor)-dependent control of transcription. However, the RXR (retinoid X receptor) ligand, 9-*cis*-retinoic acid, up-regulates CYP27A1 expression. Of the RXR heterodimeric partners tested, PPAR (peroxisome-proliferator-activated receptor) γ ligands significantly increased CYP27A1 mRNA levels. Its reversal by a PPARγ antagonist demonstrated the specificity of this effect. Interestingly, HMDMs express markedly higher levels of CYP27A1 than THP-1 macrophages, and this difference was reflected in both protein levels and enzyme activities between the two cell types. In conclusion, stimulation of CYP27A1 by PPARγ may represent a key previously unrecognized mechanism by which PPARγ protects against atherosclerosis.

Key words: atherosclerosis, cholesterol, liver X receptor (LXR), macrophage, peroxisome-proliferator-activated receptor γ (PPARγ), sterol 27-hydroxylase (CYP27A1).

INTRODUCTION

Accumulation of excess cholesterol within intimal macrophages to generate lipid-laden foam cells is a key early event in the development of atherosclerosis. Processes that contribute to the elimination of cholesterol from macrophages are therefore potentially protective against atherosclerosis. These mechanisms involve both export of cholesterol to extracellular acceptors, such as apoA-1 (apolipoprotein A-1), and catabolism of cholesterol to more polar sterols that can be exported more readily than cholesterol, largely independent of such acceptors.

Efflux of cholesterol from macrophages to apoA-1 is dependent on a membrane transporter, ABCA1 (ATP-binding cassette transporter protein A1) [1]. In addition, macrophages produce and secrete apoE (apolipoprotein E), an endogenously generated cholesterol acceptor that may contribute to cholesterol removal [2]. Expression of both ABCA1 and apoE is regulated by cell sterol content, mediated through the nuclear receptors, LXR (liver X receptor) α and PPAR (peroxisome-proliferator-activated receptor) γ [3]. An alternative export route, involving catabolism of cholesterol to more polar sterols, is catalysed by CYP27A1 (sterol 27-hydroxylase). This mitochondrial cytochrome P450 enzyme was first discovered in the liver, where it catalyses multiple oxidation reactions in bile acid synthesis [4]. Subsequent studies have revealed expression of CYP27A1 in a wide range of extrahepatic tissues and cells, including endothelial cells and macrophages [5–7]. The human macrophage exhibits a particularly high expression of CYP27A1 and capacity to convert cholesterol into 27-hydroxycholesterol, 3β-hydroxy-5-cholestenoic acid [8] and downstream water-soluble products [9].

Several lines of evidence suggest that CYP27A1 may be an important defence mechanism against cholesterol accumulation in the macrophage. First, a functional deficiency in this enzyme in humans leading to a rare disorder, CTX (cerebrotendinous xanthomatosis), is associated with sterol deposition in tissue macrophages and an increased risk of developing premature atherosclerosis, despite normal circulating cholesterol levels [8]. Secondly, it is estimated that there is a significant daily flux of CYP27A1-derived sterol products from extrahepatic sources to the liver, where they can be catabolized further and eventually excreted as bile acids [10], indicating that this enzyme contributes to removal of peripheral tissue cholesterol. In addition, CYP27A1 is substantially up-regulated in atherosclerotic lesions, co-localizing mainly with macrophages in advanced lesions [7,11].

Studies on the regulation of CYP27A1 have largely examined hepatic expression of this enzyme. In the liver, expression is increased by glucocorticoids [12], growth hormone and insulin-like growth hormone-1 [12,13], and is decreased by cyclosporin, bile acids and fibrates [13], consistent with its role as an alternative route for hepatic bile acid synthesis. Much less is known about the control of CYP27A1 levels in macrophages. Its expression increases during monocyte-to-macrophage differentiation [14] and is suppressed by some inflammatory mediators (interferon-γ or immune complexes) [15]. While ABCA1 and apoE are up-regulated by cholesterol loading in macrophages [16], little is known of the effects of lipid status on CYP27A1 expression and activity.

Abbreviations used: ABCA1, ATP-binding cassette protein A1; ABCG1, ATP-binding cassette protein G1; AcLDL, acetylated low-density lipoprotein; apoA-1, apolipoprotein A-1; apoE, apolipoprotein E; BCA, bicinchoninic acid; CTX, cerebrotendinous xanthomatosis; CYP27A1, sterol 27-hydroxylase; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; FXR, farnesoid X receptor; LXR, liver X receptor; HMDM, human monocyte-derived macrophage; LDL, low-density lipoprotein; LPDS, lipoprotein-deficient human serum; PBGD, porphobilinogen deaminase; PPAR, peroxisome-proliferator-activated receptor; PPRE, PPAR-response element; PXR, pregnane X receptor; QRT-PCR, quantitative reverse transcription PCR; RT-PCR, reverse transcription PCR; RXR, retinoid X receptor; SREBP, sterol-regulatory-element-binding protein.

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In addition, CYP27A1 products, 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid, have been implicated as weak endogenous ligands for LXRα in some studies [17–19], although this is not always the case [20,21]. Thus CYP27A1 expression might promote cholesterol elimination both directly (by metabolism of cholesterol) and indirectly (by formation of products that stimulate ABCA1 and apoE expression). In addition, overexpression of CYP27A1 is associated with increased efflux of cholesterol to apoA-1, independent of ABCA1 expression [21], by as yet undefined mechanisms. In the present study, we examined whether control of CYP27A1 expression and activity in human macrophages was affected by sterol loading and/or ligands for lipid-dependent nuclear receptors.

EXPERIMENTAL

Reagents

All solvents were of HPLC grade. The following chemicals and reagents were used, with the suppliers indicated: BSA fraction V (Sigma), [1α,2β(n)-^{3}H]cholesterol (specific radioactivity, 49 Ci/mmol; Amersham Biosciences), [1,2,6-^{3}H]7-oxocholesterol (5-cholesten-3β-ol-7-one) (specific radioactivity, 50 Ci/mmol; American Radiolabeled Chemicals), RPMI 1640 (Trace Biosciences), DMEM (Dulbecco's modified Eagle's medium) (Trace Biosciences), FCS (foetal calf serum) (Gibco BRL), PMA (Sigma), Tri Reagent (Sigma), 22(*R*)-hydroxycholesterol (Sigma), 9-*cis*-retinoic acid (Sigma), oligo(dT) (Invitrogen); deoxynucleotides (Sigma), M-MLV Reverse Transcriptase (Invitrogen), RNasin (Promega), Red Hot DNA Polymerase (ABGene), iQ SyBr Green Supermix (Bio-Rad), and ECL® (enhanced chemiluminescence) assay kit (Amersham Biosciences). Oligonucleotides were synthesized by Sigma-Genosys. GW273297x, GW3965, GW1929, GW6777 (pioglitazone), GW9662, GW0742, GW9578, GW7647 and rosiglitazone were gifts from GlaxoSmithKline. TO-901317 was from Cayman Chemicals. AcLDL (acetylated low-density lipoprotein) and LPDS (lipoprotein-deficient human serum) were prepared from normolipidaemic human peripheral blood as described previously [22]. White cell concentrates and human serum were kindly supplied by the Red Cross Blood Bank (Sydney, NSW, Australia).

Cell culture

Cells were cultured (37 °C, 5 % CO_2) in medium supplemented with L-glutamine (2 mM) and penicillin (100 units)/streptomycin (100 μg/ml). THP-1 cells, obtained from the A.T.C.C. (Manassas, VA, U.S.A.), were plated (1.2×10^6 cells/ml) and grown in 10 % (v/v) FCS in RPMI 1640 (Trace Biosciences) in the presence of PMA (50 ng/ml, 72 h) to promote differentiation into macrophages. HepG2 cells, also obtained from the A.T.C.C., were plated (1.2×10^6 cells/ml) and grown in 10 % (v/v) FCS in DMEM (Trace Biosciences). HMDMs (human monocyte-derived macrophages) were prepared from white cell buffy coat concentrates from healthy donors as described [23]. Purified monocytes (> 95 % purity by non-specific esterase staining) were differentiated into macrophages by plating (2×10^6 cells/ml) in RPMI 1640 with 10 % (v/v) heat-inactivated whole human serum for 9 days.

For cholesterol loading, differentiated THP-1 cells and HMDMs were incubated with RPMI 1640 containing LPDS (10 %, v/v) and AcLDL at final concentrations of 150 μg/ml (THP-1) or 50 μg/ml (HMDMs) for 4 days. These conditions give approximately the same degree of loading between the two cell types [24,25].

For other treatments, differentiated THP-1 cells and HMDMs were incubated for 24 h with RPMI 1640 containing 1 % (v/v) FCS (THP-1) or 10 % (v/v) LPDS (HMDMs) and 10 μM (final concentration) of nuclear receptor ligands, except for rosiglitazone and pioglitazone, when 50–100 μM was used. Cells were washed twice in PBS at room temperature (22 °C) before harvesting with Tri Reagent for total RNA or in homogenization buffer for mitochondrial lysates (see below). For activity assays, medium was retained.

CYP27A1 activity

CYP27A1 activity assays were performed by measuring CYP27A1-dependent product formation in whole cells, as described previously [9], using ^{3}H-labelled sterol substrates. The initial products of CYP27A1 activity are 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid [8], but, as we have shown previously, macrophages metabolize the majority of these to more polar products [9]. While not fully characterized, their generation is entirely dependent on initial CYP27A1 action, shown by comparison between macrophages from normal and CTX subjects or by measurement in normal cells in the presence and absence of the specific CYP27A1 inhibitor, GW273297x [9]. We also showed previously that CYP27A1 acts more readily on 7-oxocholesterol than on cholesterol in HMDMs. For routine assay, activity was assessed by determination of total aqueous soluble products $\pm$GW273297x. Cells were incubated for 24 h with RPMI 1640 containing BSA (1 mg/ml) plus [^{3}H]cholesterol or [^{3}H]7-oxocholesterol, both at 1 μCi/culture, delivered in ethanol (final concentration 0.1 %, w/v). Control cultures included GW273297x (1 μM). Media were removed and centrifuged at 5000 ***g*** for 5 min to remove any detached cells. Media were extracted with chloroform/methanol (2:1, v/v), and the aqueous-soluble counts were measured in a Packard Tri-Carb 2100 TR Liquid Scintillation Analyzer. Cell monolayers were washed twice in PBS and lysed in 0.2 M NaOH (15 min, 4 °C) for determination of total cell protein. In some cases, 27-hydroxycholesterol (lipid phase) was determined by TLC.

CYP27A1 protein expression

Mitochondrial fractions were prepared to measure CYP27A1 expression [26]. Briefly, 1×10^7 cells were washed, then scraped into PBS on ice and centrifuged at 800 ***g*** for 5 min. The pellet was resuspended in homogenization buffer (200 mM sucrose, 100 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA and 1 mM dithiothreitol), sonicated on ice (MSE sonicator) and centrifuged (500 ***g***, 5 min, 4 °C). The pellet was resuspended and centrifuged again under the same conditions, then lysed in 0.1 % (v/v) Triton X-100. Some aliquots were retained for determination of protein concentration by the BCA (bicinchoninic acid) assay (Pierce), and others were stored in SDS/PAGE loading buffer [100 mM Tris/HCl, pH 6.8, 200 mM dithiothreitol, 4 % (w/v) SDS, 0.2 % (w/v) Bromophenol Blue and 20 % (v/v) glycerol] at -20 °C for subsequent electrophoresis. Samples (20 μg per lane) were run on a SDS/10 % PAGE gel. Protein was transferred on to nitrocellulose (Hybond C), which was blocked for 1 h in blocking solution [5 % (w/v) non-fat dried milk and 0.1 % (v/v) Tween 20 in PBS]. The primary antibody was an affinity-purified rabbit polyclonal antipeptide antibody raised against residues 15–28 of the human CYP27A1 protein (a gift from Dr David Russell, University of Texas Southwestern Medical Centre, Dallas, TX, U.S.A.) [27]. This was used at a dilution of 1:300 in blocking solution, incubated overnight at 4 °C. After washing, the blots were incubated with a 1:5000 dilution of donkey

Table 1 Primer sequences for gene expression analysis

Gene	Accession number	Direction	Primer sequence	Predicted size (bp)	Reference
CYP27A1	NM_000784	Forward	5′-TGC GCC AGG CTC TGA ACC AG-3′	311	[6]
		Reverse	5′-TCC ACT TGG GGA GGA AGG TG-3′		
ABCA1	NM_005502	Forward	5′-GCA CTG AGG AAG ATG CTG AAA-3′	205	[28]
		Reverse	5′-AGT TCC TGG AAG GTC TTG TTC AC-3′		
GAPDH	NM_002046	Forward	5′-TGA AGG TCG GAG TCA ACG GAT TTG GT-3′	981	Present study
		Reverse	5′-CAT GTG GGC CAT GAG GTC CAC CAC-3′		
PBGD	X04217	Forward	5′-GAG TGA TTC GCG TGG GTA CC-3′	201	[28]
		Reverse	5′-GGC TCC GAT GGT GAA GCC-3′		

anti-rabbit IgG conjugated to horseradish peroxidase (Jackson Laboratories) for 1–2 h. Bound antibodies were visualized by ECL® and exposed to film at room temperature.

RNA extraction and RT-PCR (reverse-transcription PCR)

Cells were harvested for total RNA using Tri Reagent, according to the manufacturer's instructions. Concentrations of total RNA were measured by UV spectrophotometry at 260 nm ('DNA Calculator'; Amersham Biosciences). For each reverse-transcription reaction, 5 μg of total RNA was reverse-transcribed using oligo(dT) primers (Gibco) and M-MLV (Moloney murine leukaemia virus) reverse transcriptase (Gibco).

Semi-quantitative RT-PCR was performed using CYP27A1-specific primers [6] (Table 1). These span a 311 bp sequence of the CYP27A1 cDNA, crossing an intron of approx. 100 bp allowing for distinction between amplification from contaminating genomic DNA and that of the reverse-transcribed mRNA. Other primers used were for ABCA1 and the housekeeping genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and PBGD (porphobilinogen deaminase) [28] (Table 1). PCR products were verified by sequencing.

Relative 'real time' QRT-PCR (quantitative RT-PCR) was performed using an ABI 7700 Sequence Detector (PE Biosystems) and analysed using ABI Prism Sequence Detector Software version 1.6.3 (PE Biosystems). iQ SyBr Green Supermix (Bio-Rad) was used as the amplification system. The same primer sets were used for CYP27A1, and the housekeeping gene used was PBGD. Melting curve analysis was performed to confirm production of a single product in these reactions.

Protein determination

The protein contents of cell lysates, mitochondrial lysates and LDL (low-density lipoprotein) preparations were measured using the BCA assay using BSA as a standard.

Statistical analysis

Statistical analyses used one-way ANOVA followed by Tukey's post-hoc test, unless otherwise stated.

RESULTS

Effect of cholesterol loading and LXR ligands on THP-1 human macrophage expression of CYP27A1

The expression of several proteins implicated in cholesterol export from macrophages is up-regulated by cholesterol loading. As CYP27A1 contributes to sterol elimination from human macrophages [29] and macrophages in cholesterol-rich human atherosclerotic lesions strongly express CYP27A1, we examined the effect of macrophage cholesterol loading on CYP27A1 expression.

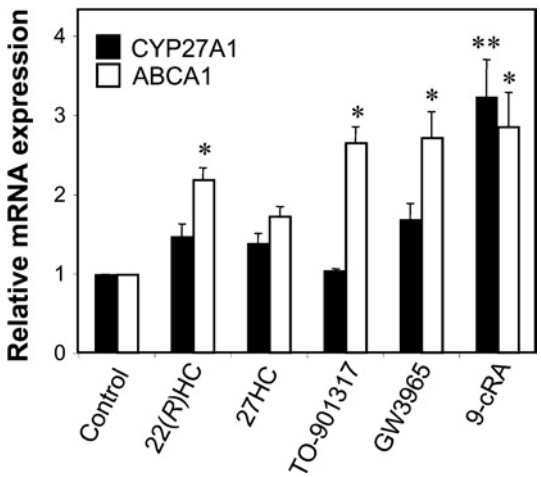

Figure 1 RXR, but not LXR, ligands stimulate CYP27A1 expression in THP-1 human macrophages

Ligands for LXR or RXR were incubated with PMA-differentiated THP-1 cells for 24 h. Final concentrations were 10 μM, except for TO-901317 which was 1 μM. LXR ligands: 22(*R*)HC, 22(*R*)-hydroxycholesterol; 27HC, 27-hydroxycholesterol; TO-901317; GW3965. RXR ligand: 9-cRA, 9-*cis*-retinoic acid. CYP27A1, ABCA1 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA levels were measured using RT-PCR as described in the Experimental section. Pooled data derived from densitometric scans of three separate experiments are presented relative to vehicle-treated controls (means + S.E.M.). *Significantly different ($P < 0.05$) compared with ABCA1 control. **Only 9-*cis*-retinoic acid produced a significant increase in CYP27A1 expression compared with control (ANOVA followed by Tukey's post-hoc test).

We have shown previously that incubation with AcLDL under the conditions used in the present study increases total cell cholesterol 3-fold, of which approx. 50 % is deposited as cytoplasmic lipid droplets [24,30], and is associated with approx. 2-fold increases in ABCA1 mRNA and apoE protein expression [25,31,32]. In contrast, we found that cholesterol loading had no effect on CYP27A1 mRNA levels in THP-1 macrophages. Results from RT-PCR, performed on RNA extracts from replicate cultures of AcLDL-loaded THP-1 cells, showed that CYP27A1 mRNA expression was 1.2-fold relative to non-loaded control cells [$\pm$0.12 (S.E.M.); $P > 0.05$; $n = 10$].

Cholesterol regulation of ABCA1 and apoE expression in macrophages is thought to be by endogenous formation of activating ligands for the transcriptional regulator, LXRα. Consistent with the responses to cholesterol loading, human macrophage ABCA1 expression is stimulated by direct addition of LXR agonists 22(*R*)-hydroxycholesterol, GW3965 and T0901317 [33], while, under the same conditions, CYP27A1 expression was unaffected (Figure 1). Interestingly, 27-hydroxycholesterol, which acts as a weak LXR ligand in some circumstances [19], did not significantly increase expression of ABCA1 or CYP27A1 in these experiments. Overall, THP-1 macrophage CYP27A1 expression

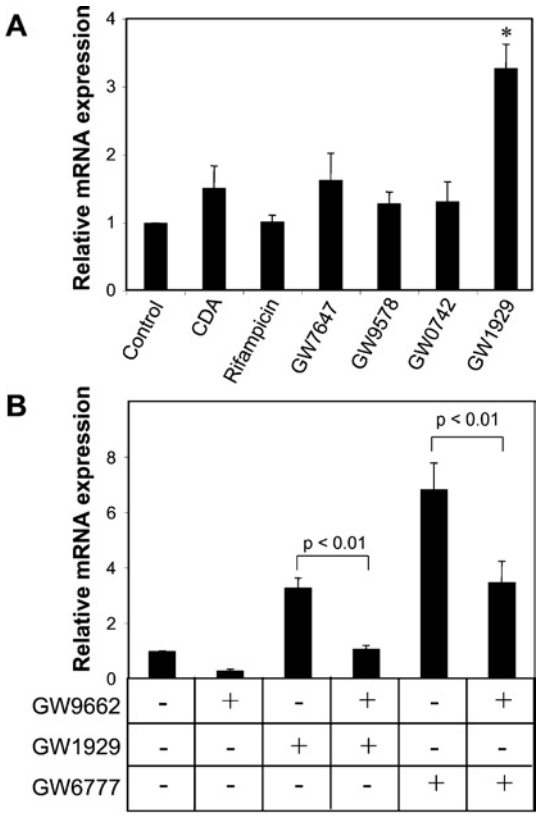

Figure 2 Control of CYP27A1 expression by ligands for RXR heterodimer partners in THP-1 human macrophages

(**A**) Ligands for nuclear receptors, chenodeoxycholic acid (FXR), rifampicin (PXR), GW7647 and GW9578 (PPARα), GW0742 (PPARδ) and GW1929 (PPARγ), were incubated (10 μM) with PMA-differentiated THP-1 macrophages for 24 h. CYP27A1 mRNA levels were measured using QRT-PCR, normalized to PBGD mRNA levels. Data are presented relative to vehicle-treated controls and are means + S.E.M. (n = at least 3). *Significantly different ($P < 0.01$) compared with control. (**B**) Ligands for PPARγ (GW1929 and GW6777/pioglitazone) and a PPARγ antagonist (GW9662) were incubated (10 μM, and 50 μM for GW6777) with PMA-differentiated THP-1 macrophages for 24 h. CYP27A1 mRNA levels were measured as in (**A**). Data are means + S.E.M. (n = at least 3). Significant differences ($P < 0.01$) are indicated.

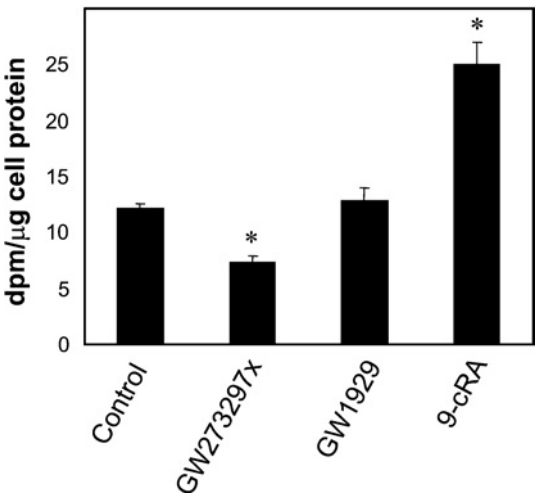

Figure 3 Effects of RXR and PPARγ ligands on CYP27A1 activity in THP-1 human macrophages

PMA-differentiated THP-1 cells were treated with PPARγ ligand (GW1929), RXR ligand (9-*cis*-retinoic acid, 9-cRA), all at 10 μM, for 24 h. CYP27A1 enzyme activity was measured using [^{3}H]7-oxocholesterol as substrate. Activity is expressed as aqueous-soluble counts generated in the presence or absence of the specific CYP27A1 inhibitor (GW273297x, 1 μM) and is normalized for total cell protein. Data are presented relative to vehicle-treated controls and are means + S.E.M. (n = 6). *Significantly different ($P < 0.001$) from control.

was unresponsive to cholesterol loading or to synthetic LXR ligands.

RXR (retinoid X receptor) and PPARγ ligands stimulate CYP27A1 expression in THP-1 human macrophages

In contrast with the unresponsiveness of CYP27A1 expression to cholesterol loading and LXR ligands, 9-*cis*-retinoic acid, a ligand for RXRα, consistently increased CYP27A1 mRNA levels in PMA-differentiated THP-1 cells (Figure 1). RXR is the heterodimeric partner for LXR and several other lipid-activated nuclear receptors. Some of these partners are implicated in the regulation of other cytochrome P450 enzymes [FXR (farnesoid X receptor), PXR (pregnane X receptor)] and/or lipid homoeostasis (PPARα, γ, δ). Their involvement in CYP27A1 gene expression was investigated following treatment of PMA-differentiated THP-1 cells with a range of specific agonists. Agonists for FXR (chenodeoxycholic acid), PXR (rifampicin), PPARα (GW9578 [34]; GW7647 [35]) and PPARδ (GW0742 [36]) had little or no effect (Figure 2A). While these cells do not express detectable FXR, both PPARα and PPARδ levels increase during differentiation [37] and are functional in transcriptional control. To our knowledge, macrophage PXR levels have not been measured. In any case, there was no evidence that activating ligands for any of these transcription factors affected CYP27A1 expression. On the other hand, a striking and consistent increase in CYP27A1 mRNA expression was induced by PPARγ-specific agonists GW1929 and pioglitazone (GW6777). A third PPARγ ligand, rosiglitazone, had a similar effect, although higher doses (100 μM) were required (results not shown). Moreover, the PPARγ-specific antagonist GW9662 [38] significantly reduced CYP27A1 mRNA relative to control levels, and abolished the stimulatory effects of the PPARγ ligands (Figure 2B).

We next determined if CYP27A1 activity was also increased in THP-1 human macrophages treated with RXR and PPARγ ligands. Previously, we demonstrated in HMDMs that CYP27A1 can use both cholesterol and 7-oxocholesterol as substrate [9]. As with HMDMs [9], the vast majority of the products of the CYP27A1 reaction in THP-1 cells was found in the aqueous-soluble fraction. Approx. 10 times more labelled cholesterol was converted into aqueous products than could be recovered as 27-hydroxycholesterol in cells or medium when THP-1 cells were incubated with [^{3}H]cholesterol for 24 h. Figure 3 shows the aqueous-soluble counts after THP-1 cells were incubated with [^{3}H]7-oxocholesterol for 24 h. The specificity of the assay was confirmed using a selective inhibitor of CYP27A1 (GW273297x [9]). The RXR ligand, 9-*cis*-retinoic acid, but not the PPARγ ligand, GW1929, significantly increased CYP27A1 activity.

Primary HMDMs express significantly higher levels of CYP27A1 than THP-1 human macrophages

Up to this point, our studies were performed in THP-1 cells. The human THP-1 cell line can be differentiated to a macrophage-like phenotype by exposure to PMA [24], and this is frequently used as a convenient experimental model for primary human macrophages. However, CYP27A1 activity in control THP-1 cells was at the limits of detection, and was at least an order of magnitude lower than we had observed previously in HMDMs [9]. In a direct

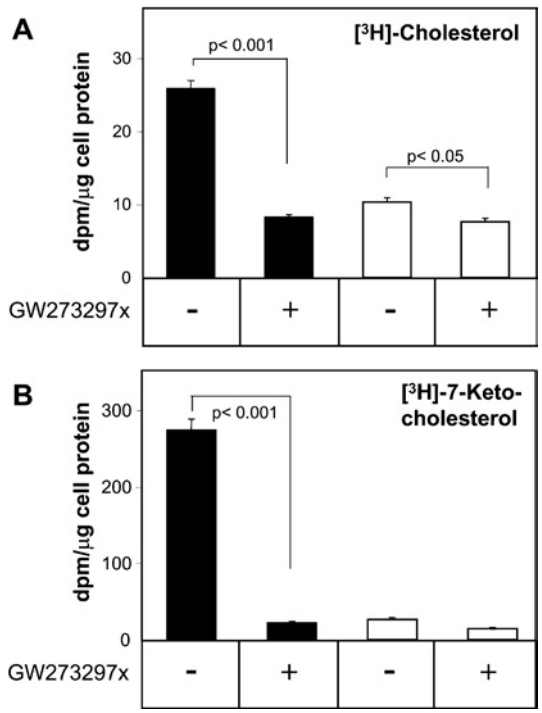

Figure 4 Comparison of CYP27A1 activity between THP-1 and primary human macrophages

CYP27A1 enzyme activity was measured in terminally differentiated THP-1 cells (open bars) and in HMDMs (closed bars), using either [^{3}H]cholesterol (**A**) or [^{3}H]7-oxocholesterol ([^{3}H]-7-ketocholesterol) (**B**) as substrate. Activity is expressed as aqueous-soluble counts generated in the presence or absence of the specific CYP27A1 inhibitor (GW273297x, 1 μM) and is normalized for total cell protein. Data are presented relative to vehicle-treated controls and are means + S.E.M. ($n = 3$) for all cell types. Significant differences ($P < 0.001$ or $P < 0.05$) are indicated.

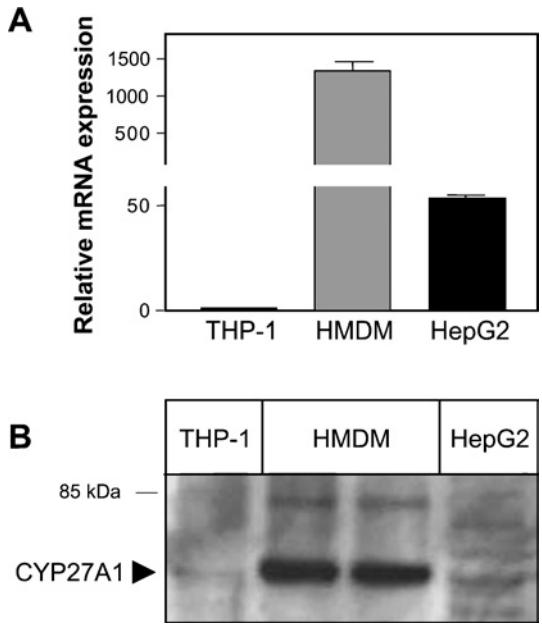

Figure 5 Comparison of CYP27A1 mRNA and protein expression between THP-1, primary human macrophages and HepG2 cells

(**A**) CYP27A1 mRNA levels in PMA-differentiated THP-1, HMDM and HepG2 cells were compared directly using QRT-PCR, normalized to PBGD mRNA and then expressed relative to THP-1 levels. (**B**) Western blots of mitochondrial fractions isolated from terminally differentiated THP-1, HepG2 (both 20 μg per lane) and HMDMs (12 μg per lane in duplicate), after separation on SDS/10 % PAGE and blotting with polyclonal anti-human CYP27A1.

comparison, we confirmed that activity against either [^{3}H]cholesterol (Figure 4A) or [^{3}H]7-oxocholesterol (Figure 4B) was much higher in HMDMs than in THP-1 macrophages. We then used QRT-PCR to quantify CYP27A1 mRNA levels in THP-1 cells, HMDMs and a liver cell line (HepG2) (Figure 5A). mRNA levels were substantially lower (up to 1000-fold) in differentiated THP-1 macrophages than in primary human macrophages. The mRNA expression of the housekeeping gene PBGD was similar between the cell types in that the threshold for all three cell types was 18–19 cycles. The higher expression of CYP27A1 mRNA in primary HMDMs was also reflected in relative protein levels between the two cell types, measured by Western blot. A strong band of CYP27A1 (approx. 60 kDa) was detected in HMDM mitochondria (duplicate samples), but was detected only weakly in THP-1 cells, even when more protein was loaded for the latter (Figure 5B). Interestingly, CYP27A1 mRNA and protein expression was also higher in HMDMs than in the liver cell line, HepG2 (Figures 5A and 5B).

THP-1 monocytes are induced to undergo differentiation by a 3-day exposure to PMA. PMA is a potent activator of protein-kinase-C-mediated protein phosphorylation, which can cause substantial alterations to protein structure and function. We considered that PMA treatment, besides inducing THP-1 differentiation, might repress CYP27A1 expression selectively, as has been reported previously in HepG2 cells [12]. Therefore the effect of PMA on CYP27A1 expression and activity in HMDMs was measured. PMA did induce a significant decrease in CYP27A1 mRNA (approx. 60 %; $P < 0.05$; $n = 3$) in HMDMs and a corresponding significant, though lesser, decline in activity (approx. 25 %; $P = 0.01$; $n = 5$), although CYP27A1 levels were still much higher than in PMA-differentiated THP-1 cells. The mRNA expression of the housekeeping gene PBGD remained unchanged in HMDMs after PMA treatment. Also, when differentiation was induced by an alternative method independent of PMA [39], the level of CYP27A1 expression in THP-1 macrophages was similar to PMA-differentiated cells (results not shown). Therefore it seems likely that, while PMA may contribute, other factors are also important in the relatively low expression of CYP27A1 in THP-1 macrophages.

CYP27A1 is also regulated through PPARγ/RXR in primary human macrophages

To determine if our findings in THP-1 cells hold in primary macrophages, we repeated key experiments in HMDMs. AcLDL-loading of HMDMs increased mRNA expression levels of the LXR target gene, ABCA1 [2.7 ± 0.3 (mean $\pm$ S.E.M.), when the level in non-loaded cells was set as 1]. As observed in THP-1 cells, AcLDL-loading of HMDMs did not alter CYP27A1 mRNA levels (1.24 ± 0.15). Message levels and the enzyme activity of CYP27A1 are also significantly increased when HMDMs were treated either with the RXR ligand, 9-*cis*-retinoic acid, or with the PPARγ ligand, GW1929, but not with the LXR ligand, 22(*R*)-hydroxycholesterol (Figure 6).

DISCUSSION

The wide tissue distribution of CYP27A1 suggests a metabolic role beyond hepatic bile acid synthesis. In particular, its relatively high expression in tissue macrophages has led to the suggestion

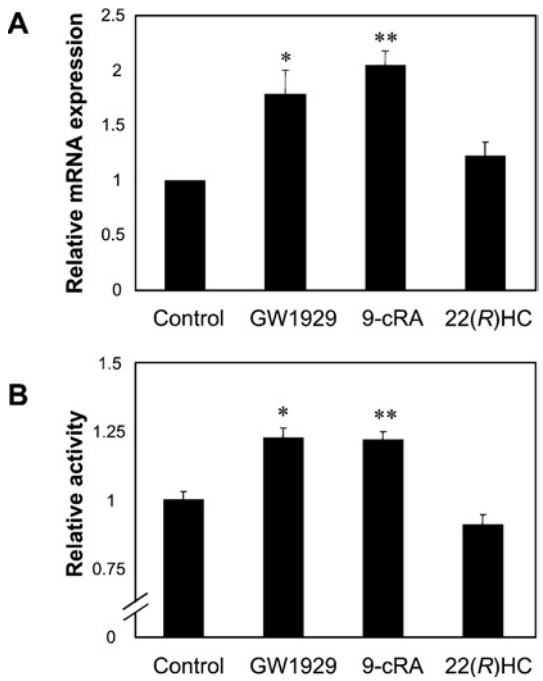

Figure 6 CYP27A1 expression is regulated by PPARγ and RXR ligands in primary human macrophages

(**A**) HMDMs were treated with PPARγ ligand (GW1929), RXR ligand (9-*cis*-retinoic acid, 9-cRA) or LXR ligand [22(*R*)-hydroxycholesterol, 22(*R*)HC], all at 10 μM, for 24 h. CYP27A1 mRNA levels were measured using QRT-PCR, normalized against PBGD mRNA levels. (**B**) Cells were treated for 24 h with GW1929, 9-cRA or 22(*R*)HC (10 μM) in medium containing [^{3}H]7-oxocholesterol, and activity was measured after 24 h as described in the Experimental section. Data are presented relative to vehicle-treated controls and are means + S.E.M. (n = at least 3). Significantly different from control: $^*P < 0.01$, $^{**}P < 0.001$.

that the enzyme is important in cholesterol metabolism and elimination from these cells. Interest in a role for CYP27A1 in cholesterol elimination from macrophages was stimulated by observation of tendon xanthomas containing cholesterol-loaded macrophages in CTX subjects and the increased risk of atherosclerosis in these individuals. In non-CTX subjects, CYP27A1 is expressed in cholesterol-loaded macrophages in atherosclerotic lesions [7,11] and in tendon xanthomas [40]. Many other proteins involved in control of cholesterol metabolism [ABCA1, ABCG1 (ATP-binding cassette protein G1), apoE, SREBP (sterol-regulatory-element-binding protein) 1c, HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase, LDL receptor, etc.] are regulated by cellular cholesterol status, through either LXR- or SREBP-dependent transcriptional control [41,42]. However, we found that neither cholesterol loading nor direct addition of LXR ligands altered CYP27A1 expression in human macrophages.

We excluded the possibility that the level of LXR expression could be limiting the responsiveness of CYP27A1 transcription, because another LXR-dependent gene (ABCA1) was stimulated by either cholesterol loading or LXR ligands in the same cells. This demonstrates that CYP27A1 expression is independent of cell cholesterol levels in general and of LXR-dependent control of transcription. The reason for strong expression of CYP27A1 in cholesterol-loaded macrophages *in vivo* therefore remains to be determined. Other potential contributing factors are the stimulatory effects of monocyte/macrophage differentiation, which is associated with increased CYP27A1 expression *in vitro* [14], and increased PPARγ expression in lesion macrophages (see below).

While unaffected by cellular cholesterol levels, expression of CYP27A1 was responsive to lipid ligands for other nuclear receptors. Thus 9-*cis*-retinoic acid (an RXRα ligand) consistently and strongly stimulated CYP27A1 expression and activity. However, of the panel of ligands for possible RXRα partners tested, only those for PPARγ substantially increased CYP27A1 expression. Its reversal by a PPARγ antagonist demonstrated the specificity of this effect.

Recent studies have indicated that activation of macrophage PPARγ is generally anti-atherogenic and anti-inflammatory [43]. Despite stimulating expression of macrophage CD36, a putative receptor for oxidized LDL, PPARγ agonists reduced atherosclerosis in mice [44]. This was probably, at least partly, due to stimulation of macrophage cholesterol export, through increased expression of ABCA1, ABCG1 and apoE. The present study raises the possibility that up-regulation of CYP27A1 may also contribute to the enhanced sterol efflux caused by PPARγ-agonists.

Expression of both PPARγ [45] and CYP27A1 [14] is up-regulated during monocyte differentiation to macrophages. These proteins are both highly expressed in human lesion macrophage foam cells [7,11,45,46], which is consistent with a functional role for PPARγ in the control of CYP27A1 expression. It has been reported that the pattern of PPARγ protein expression is highly correlated with oxidation-specific epitopes in human lesions [46]. If CYP27A1 similarly co-localizes with oxidation-specific epitopes, it could have a role in metabolism of potentially atherogenic oxysterols in macrophage foam cells [9].

It is possible that the PPARγ ligands could be acting directly on the CYP27A1 promoter by binding to a PPRE (PPAR-response element), a direct hexanucleotide repeat separated by one nucleotide (also known as a DR1 element). We have performed *in silico* analysis of the proximal 2 kb of CYP27A1 5′ flanking sequence for potential PPREs and, although there are several partial matches to a consensus sequence, there are no prime candidates. A detailed promoter analysis would be necessary to identify if any of the partial matches act as functional PPREs. It could also be the case that a PPRE may lie further upstream or that a PPARγ ligand is up-regulating CYP27A1 gene expression via an indirect mechanism.

While their relative responses to nuclear receptor ligands were very similar, there were large differences in CYP27A1 levels between primary HMDMs and THP-1 macrophages. One contributory factor to the difference may be the suppressive effect of PMA on CYP27A1 expression, as PMA did suppress expression when added to HMDMs. This could be mediated through activation of protein kinase C by PMA, leading to cytokine release, as interferon-γ decreases CYP27A1 expression in human arterial endothelium and macrophages [15]. However, this could not completely account for the very large difference between primary human macrophages and the cell line, and highlights the caution that should be exercised in applying results obtained from continuous cell lines to the *in vivo* condition.

While both 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid are generated by macrophages during CYP27A1-dependent metabolism [8], it is important to note that the majority of the products are much more polar and partition into the aqueous phase during Folch extraction of cells or tissues [9]. Normal assay protocols, designed to extract and measure the primary products, 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid, discard this fraction and so significantly underestimate macrophage CYP27A1 activity. We have not determined the identity of these products, but they are likely to include bile acid-like molecules. Their functional importance in macrophage biology is not known and merits further study.

In summary, we have provided evidence to show that expression of CYP27A1 is independent of cellular cholesterol status, but is controlled through PPARγ/RXR in human macrophages, and suggest that this may explain its high expression in human

atherosclerotic foam cells. Our finding that the important macrophage sterol elimination pathway catalysed by CYP27A1 is up-regulated by PPARγ may represent a key previously unrecognized mechanism by which PPARγ protects against atherosclerosis.

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