A novel role for c-Jun N-terminal kinase and phosphoinositide 3-kinase in the liver X receptor-mediated induction of macrophage gene expression

Etimad A. Huwait [1], Kirsty R. Greenow [1], Nishi N. Singh, Dipak P. Ramji *

Cardiff School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3AX, UK

A B S T R A C T

Liver X receptors (LXRs) are ligand-dependent transcription factors that are activated by metabolites of cholesterol, oxysterols, and a number of synthetic agonists. LXRs play potent anti-atherogenic roles in part by stimulating the efflux of cholesterol from macrophage foam cells. The LXR-induced expression of ATP-binding cassette transporter (ABC)-A1 and Apolipoprotein E (ApoE) in macrophages is essential for the stimulation of cholesterol efflux and the prevention of atherosclerotic development. Unfortunately, the signaling pathways underlying such regulation are poorly understood and were therefore investigated in human macrophages. The expression of ApoE and ABCA1 induced by synthetic or natural LXR ligands [TO901317, GW3965, and 22-(R)-hydroxycholesterol (22-(R)-HC), respectively] was attenuated by inhibitors of c-Jun N-terminal kinase (JNK) (curcumin and SP600125) and phosphoinositide 3-kinase (PI3K) (LY294002). Similar results were obtained with ABCG1 and LXR-α, two other LXR target genes. LXR agonists activated several components of the JNK pathway (SEK1, JNK and c-Jun) along with AKT, a downstream target for PI3K. In addition, dominant negative mutants of JNK and PI3K pathways inhibited the LXR-agonists-induced activity of the ABCA1 and LXR-α gene promoters in transfected cells. LXR agonists also induced the binding of activator protein-1 (AP-1), a key transcription factor family regulated by JNK, to recognition sequences present in the regulatory regions of the ApoE and ABCA1 genes. These studies reveal a novel role for JNK and PI3K/AKT signaling in the LXR-regulated expression in macrophages of several key genes implicated in atherosclerosis.

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1. Introduction

Liver X receptors (LXRs) belong to the nuclear receptor family of transcriptional regulators that are emerging as key modulators of lipid metabolism and inflammation [1,2]. They play important roles in diseases associated with perturbations in these processes, such as atherosclerosis, obesity and diabetes [1,2]. There are two LXRs, LXRα and LXRβ, with the latter expressed ubiquitously and the former present at high levels in a restricted set of tissues/cell types, including macrophages [1,2]. Both LXRs are activated by oxidized derivatives of cholesterol [e.g., 22R-hydroxycholesterol (22-(R)-HC), 27-hydroxycholesterol and 24(S), 25-epoxycholesterol] and therefore act as intracellular sensors of cholesterol [1,2]. Ligand-activated LXRs form obligate heterodimers with the retinoid X receptor (RXR) and regulate the transcription of target genes containing LXR response element(s) [1,2]. Such target genes include those implicated in the efflux of cellular cholesterol [e.g., Apolipoprotein E (ApoE), ATP-binding cassette transporter (ABC)-A1] and fatty acid metabolism [e.g., sterol response element binding protein 1c] [1,2]. The expression of the LXRα gene is also subject to autoregulation [3]. In addition, LXR activators inhibit inflammatory gene expression by antagonizing the actions of key transcription factors, such as nuclear factor κB, largely via a mechanism that does not require sequence-specific DNA binding by the LXRs [1,2].

A number of *in vitro* and *in vivo* studies have revealed potent anti-atherogenic roles for the LXRs [1,2]. For example, cholesterol and bile metabolism are impaired in mice lacking LXRα [1,2], and the removal of LXR from the hematopoietic compartment by bone marrow transplantation results in a marked increase in atherosclerotic lesion formation in murine models of this disease [4]. The administration of LXR agonists also leads to a reduction in lesion development in such model systems [5]. Additionally, the overexpression of the LXRs or their ligand-dependent activation stimulates macrophage cholesterol efflux through the induced expression of several key genes implicated in the process, including ApoE and ABCA1 [1,2].

The precise mechanisms by which activated LXRs regulate the transcription of target genes are not fully understood. A putative model for the co-activator/co-repressor recruitment has been derived on the basis of some initial studies on LXR-mediated gene transcription and extensive research on other nuclear receptors [1,2,6]. Intracellular signal transduction pathways are also known to regulate the action of nuclear receptors by the covalent modification of the receptors themselves or other factors required for activation (e.g., co-activators) [7]. For example, the activity of peroxisome proliferator-

activated receptor (PPAR)-γ1 is regulated by mitogen-activated protein kinases [7]. Unfortunately, very little is currently understood regarding such regulation of the LXRs. We have investigated this aspect using ApoE and ABCA1 as model genes. Both these genes are known to have potent anti-atherogenic actions [8,9]. We show for the first time a novel role for JNK and PI3K signaling pathways in the response.

2. Materials and methods

2.1. Materials

The human THP-1, U937 and Hep3B cell lines were from the European Collection of Animal Cell Cultures. The antisera were obtained from Biogenesis (ApoE), Abcam (ABCA1), Sigma (β-actin), Santa-Cruz Biotechnology [c-Jun (H-89), phospho-c-Jun (Ser63; KM-1)] and Cell Signaling Technology [AKT, phospho-AKT (Ser473), SEK1, phospho-SEK1 (Ser257/Thr261), JNK, phospho-JNK (Thr183/Tyr185)]. The non-radioactive AKT and JNK activity kits were from Cell Signaling Technology, the inhibitors were from Merck, and the ligands were from Sigma [22-(R)-HC, 22-(S)-HC, 9cRA], Merck (TO901317) and GlaxoSmithKline (GW3965). The plasmids used were: ABCA1 promoter-luciferase (−928 to +101) from Philippe Costet (INSERM, France); LXR-α promoter (−3027 to +463) from Steven Kliewer (University of Texas Southwestern Medical Centre); and dominant negative LXR-α (Thomas Kocarek, Wayne State University), AKT and p110 (Brian Hemmings, Friedrich Miescher-Institut, Basel), JNK (Eisuke Nishida, Kyoto University), c-Jun (Powel Brown, Bayor College of Medicine, Texas) and SEK1 (Jim Woodgett, Ontario Cancer Institute).

2.2. Cell culture

Human monocyte-derived macrophages (HMDM) were isolated from buffy coats as previously described [10–12]. The cells were maintained in RPMI-1640 (HMDM, THP-1, U937) or DMEM (Hep3B) with Stabilix supplemented with 10% (v/v) heat-inactivated FCS (HI-FCS) (56 °C, 30 min) in the presence of Penicillin (100 U/ml) and Streptomycin (100 μg/ml). Differentiation of THP-1 monocytes into macrophages was carried out by incubation for 24 h with 0.16 μM phorbol 12-myristate 13-acetate. For experiments involving the use of LXR ligands, macrophages were incubated in medium containing dilapidated HI-FCS, prepared as described by Cham and Knowles [13]. For experiments with inhibitors, these were added 1 h before the addition of the ligands (pre-treatment).

2.3. Real-time quantitative PCR (RT-qPCR)

The total cellular RNA was prepared using the RNeasy™ total RNA isolation kit or the RNeasy™ Micro kit (Qiagen) and reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega) and random hexamer primers. For Taqman RT-qPCR, the reactions were carried out in a final volume of 29 μl containing 25 ng of cDNA, 2× Taqman Master mix (Applied Biosystems), 300 nM of both the 5′ and 3′ primers, and 300 ng of probe (see Supplementary Table I for sequences). The mixture was overlaid with 12 μl of mineral oil, and the reactions were cycled on an ABI PRISM 7700 Sequence detector (Applied Biosystems) using the following program: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s for denaturation and at 60 °C for annealing and extension. Each set of reactions also contained a standard curve, where a range of concentration of human liver cDNA was employed (0.05 pg/μl to 30 ng/μl). Reactions were monitored in a real-time mode using the Sequenase Detection Systems version 1.7 software and normalized to β2-microtubulin. RT-qPCR for ApoE in HMDM was carried out using the SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma) and primers against ApoE and 28S rRNA (see Supplementary Table I for the

sequences of primers). PCR was performed in optical 96-well plates using the DNA Engine Opticon 2® real-time PCR detection system (MJ Research), and transcript levels were determined using the comparative C_t method and normalized to 28S rRNA [10–12]. All PCRs were performed in duplicate and cDNAs, cloned into pGEM-T® vector, were used as standards for quantitation and to verify specificity by DNA sequencing.

2.4. Western blot analysis and AKT/JNK activity assays

The Western blot analysis of whole cell extracts was carried out as previously described [14–16], except that samples for ABCA1 were not boiled for 5 min before loading on the gels as this caused degradation of this high molecular weight protein. The AKT and JNK activity assays were performed as described by the manufacturer (Cell Signaling Technology).

2.5. Transfection of cells and Electrophoretic mobility shift assays (EMSA)

Transfection of U937 and Hep3B cells was carried out essentially as described previously [14–16]. The radiolabeling of oligonucleotides, preparation of whole cell and nuclear extracts and EMSA were carried out as before [14–16]. The sequences of the oligonucleotides were: 5′-CGCTTGATGAGTCAG-3′ and 5′-TTCCGGCTGACTCAT-3′ (AP-1 consensus probe); 5′-CGCTTGATGAGTCAGCCGGAA-3′ and 5′-TTCCGGCTGACTCATCAAGCG-3′ (AP-1 consensus competition); 5′-GGGTTCAAGCGATTCTCCTGCCTCAGCCTCCCAA-3′ and 5′-GCTACTTGGGAGGCTGAGGCAGGAGAATCGCTTGA-3′ (AP-1 site from ApoE promoter); 5′-GCTGAGTGACTGAACTACATAAA-3′ and 5′-GGTTTATGTAGTTCAGTCACTCAG-3′ (AP-1site from ABCA1 promoter); 5′-CAGTGTTTCCAGAC-3′ and 5′-TTGGTCTGGAAACA-3′ (C/EBP); and 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 5′-GCCTGGGAAAGTCCCCT-CAACT-3′ (NF-κB).

2.6. Statistical analyses of data

The signals from Western blots were subjected to densitometric analysis using the GeneTools software (GRI). Statistical comparisons between all data were carried out using Student's t test with $p < 0.05$ considered as statistically significant.

3. Results

3.1. The induction of gene expression by LXR agonists is attenuated by inhibitors of JNK and PI3K pathways

The human THP-1 cell line is an extensively used model for the study of macrophage gene expression in relation to nuclear receptors and atherosclerosis with demonstrated conservation of numerous responses in vivo [3,10–12,14,17]. Our initial studies used the LXR ligand 22-(R)-HC and ApoE as an LXR-inducible model gene. 22-(R)-HC, but not the 22-(S)-HC enantiomer, which binds to the LXRs but does not activate it, induced the expression of the ApoE mRNA and protein but not that for the control genes (e.g., GAPDH, β-actin, and PPAR-γ) in THP-1 macrophages (data not shown). RT-qPCR showed that such 22-(R)-HC-mediated induction of ApoE mRNA expression was attenuated by inhibitors of JNK (curcumin and SP600125) or PI3K (LY294002) but not the other two mitogen-activated protein kinase pathways, extracellular signal-regulated kinase and p38 kinase (PD98059 and SB202190, respectively) (Fig. 1A). Further experiments showed that curcumin and SP600125 also inhibited the 22-(R)-HC-induced expression of ABCA1, ABCG1 and LXR-α (Fig. 1B). These studies therefore suggested an important role for the JNK and PI3K pathways in LXR signaling and were therefore investigated further using ApoE and ABCA1 as model genes. Because of functional relevance, the majority of subsequent studies focused on protein expression.

(A)

(B)
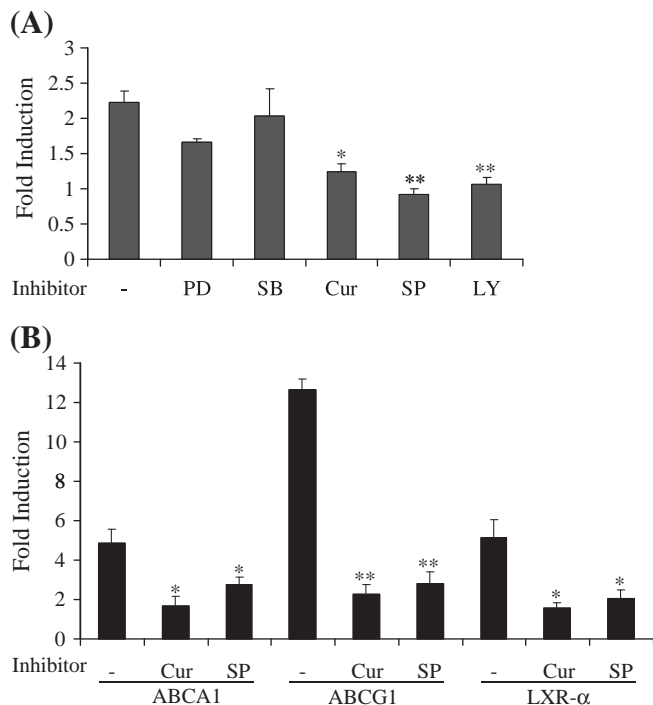
Fig. 1. The 22-(R)-HC-induced gene expression is attenuated by inhibitors of JNK and PI3K. THP-1 macrophages were pre-treated for 1 h with the DMSO vehicle (−) or PD98059 (PD; 50 μM), SB202190 (SB; 10 μM), curcumin (Cur; 25 μM), SP600125 (SP; 50 μM) or LY294002 (LY; 100 μM) as indicated and then exposed for 12 h to vehicle or 22-(R)-HC (2 μg/ml). RT-qPCR was then performed using primers against ApoE, ABCA1, ABCG1, LXR-α or the constitutive control, β2-microtubulin, as shown. For each inhibitor, the gene:β2-microtubulin ratio in vehicle-treated cells has been assigned as 1 (not shown) with that in the presence of the ligand represented relative to this. The data (mean ± SD) are from three independent experiments (*$p < 0.05$ and **$p < 0.005$).

LXRs act as obligate heterodimers with RXRs and 22-(R)-HC is often used in conjunction with 9-cis-retinoic acid (9cRA) as endogenous ligands for the action of this nuclear receptor [18]. Indeed, a marked activation of ABCA1 protein expression was produced when these ligands were present together (Supplementary Fig. IA). The induced expression of ABCA1 was only obtained with 22-(R)-HC but not with 22-(S)-HC. 22-(R)-HC and 9cRA were therefore used for the majority of subsequent studies. In addition, representative experiments were performed using the synthetic ligands GW3965 and/or TO901317, both of which induced ApoE and ABCA1 expression (Supplementary Fig. IB and C; other data not shown). Unless otherwise stated, the concentration of the ligands that produced the maximal expression of ABCA1 expression was used for subsequent studies. In the experiments described above, the cells were incubated with the ligands for 24 h, a period corresponding to the maximal activation of protein expression as judged by time-course experiments using 22-(R)-HC and 9cRA or TO901317 (data not shown). These experiments also showed that a marked induction in expression could be observed within 3 h of the addition of ligands, thereby indicating a rapid kinetics of the response (data not shown).

Further experiments investigated the effects of the inhibitors on such ligand-induced expression of ABCA1 and/or ApoE. Initial studies using a single concentration of these inhibitors showed that the induction of both ABCA1 and ApoE expression by 22-(R)-HC plus 9cRA or GW3965 was inhibited in a statistically significant manner by curcumin, SP600125 and LY294002 (Fig. 2). Similar results were obtained with 22-(R)-HC alone or TO901317 (Supplementary Fig. II). Such attenuation in gene expression was also observed with several concentrations of the inhibitors (Supplementary Figs. III and IV).

To confirm that the effect of the inhibitors was not peculiar to THP-1 macrophages, representative experiments were carried out on primary HMDM using ApoE as a model gene. RT-qPCR was used because of technical problems with Western blot analysis using extracts from HMDM. The 22-(R)-HC and 9cRA-induced expression of ApoE mRNA was inhibited in a statistically significant manner by the incubation of the cells with SP600125, curcumin or LY294002 (Fig. 3A).

3.2. The LXR agonists-induced ABCA1 promoter activity was inhibited by the expression of dominant negative forms of the components of the JNK and PI3K pathways

Transfection experiments were performed to further evaluate the role of the JNK and PI3K pathways in the actions of LXR agonists. Because of difficulties with transfecting THP-1 cells at high efficiency, the human monocytic U937 cell line, which can also be differentiated into macrophages using phorbol 12-myristate 13-acetate, was used. Numerous studies have employed this cell line for transfection-based experiments in relation to the regulation of promoter activity in macrophages, including by nuclear receptors [16,19]. Initial experiments showed that the ABCA1 gene promoter [20] was activated markedly by 22-(R)-HC plus 9cRA or TO901317 (data not shown). To evaluate the roles of different signaling pathways, co-transfection assays were carried out using plasmids specifying for dominant negative forms of key components of these pathways. The induction of ABCA1 promoter activity by these ligands in cells transfected with the control pcDNA3 plasmid was attenuated by the expression of dominant negative forms of LXR-α, two components of the JNK pathway (SEK1 and c-Jun) [21] and AKT, a key downstream target for PI3K actions (Fig. 3B). In relation to PI3K, representative experiments were also carried out in the human hepatoma Hep3B cell line using another target, LXR-α itself [4], to investigate whether the findings could be extended to other systems. 22-(R)-HC plus 9cRA induced the LXR-α promoter activity in these cells and this was inhibited by dominant negative forms of AKT and the p110 PI3K catalytic subunit (Supplementary Fig. V). In addition, representative experiments showed that the expression of dominant negative JNK or SEK1 inhibited the induction of endogenous ApoE expression by 22-(R)-HC alone (Supplementary Fig. VI).

3.3. LXR agonists activate key components of the JNK and PI3K pathways and this was attenuated by the corresponding inhibitors

The activation of key components of the JNK and PI3K pathways can be monitored by Western blot analysis using phospho-specific antibodies and/or *in vitro* kinase assays using immunoprecipitated proteins. Initial time course analysis showed that 22-(R)-HC plus 9cRA induced the levels of phospho-AKT and several components of the JNK pathway with rapid kinetics, with the activation of the JNK pathway being more prolonged than AKT (data not shown). The action of the inhibitors on such activation was then determined. 22-(R)-HC and 9cRA induced the levels of phospho-JNK, -SEK1 and -c-Jun, without affecting the total level of the proteins, and such an activation was inhibited by SP600125 (Fig. 4A). Because curcumin has been shown to affect several components of the pathway, its action on JNK activity was analyzed, where the ability of immunoprecipitated kinase to phosphorylate c-Jun fusion protein is monitored, with Western blot analysis using the β-actin antibody used to verify equal amounts of proteins in extracts. 22-(R)-HC and 9cRA induced JNK activity and this was inhibited by curcumin (Fig. 4B). Representative experiments also showed that 22-(R)-HC alone induced JNK activity or phospho-c-Jun levels and this was inhibited by curcumin and SP600125 (data not shown). Similarly, the ligands increased phospho-AKT levels, but not that for the total protein, and this was associated with an increase in enzymatic activity (Fig. 4C–E). Additionally, the ligand-induced levels
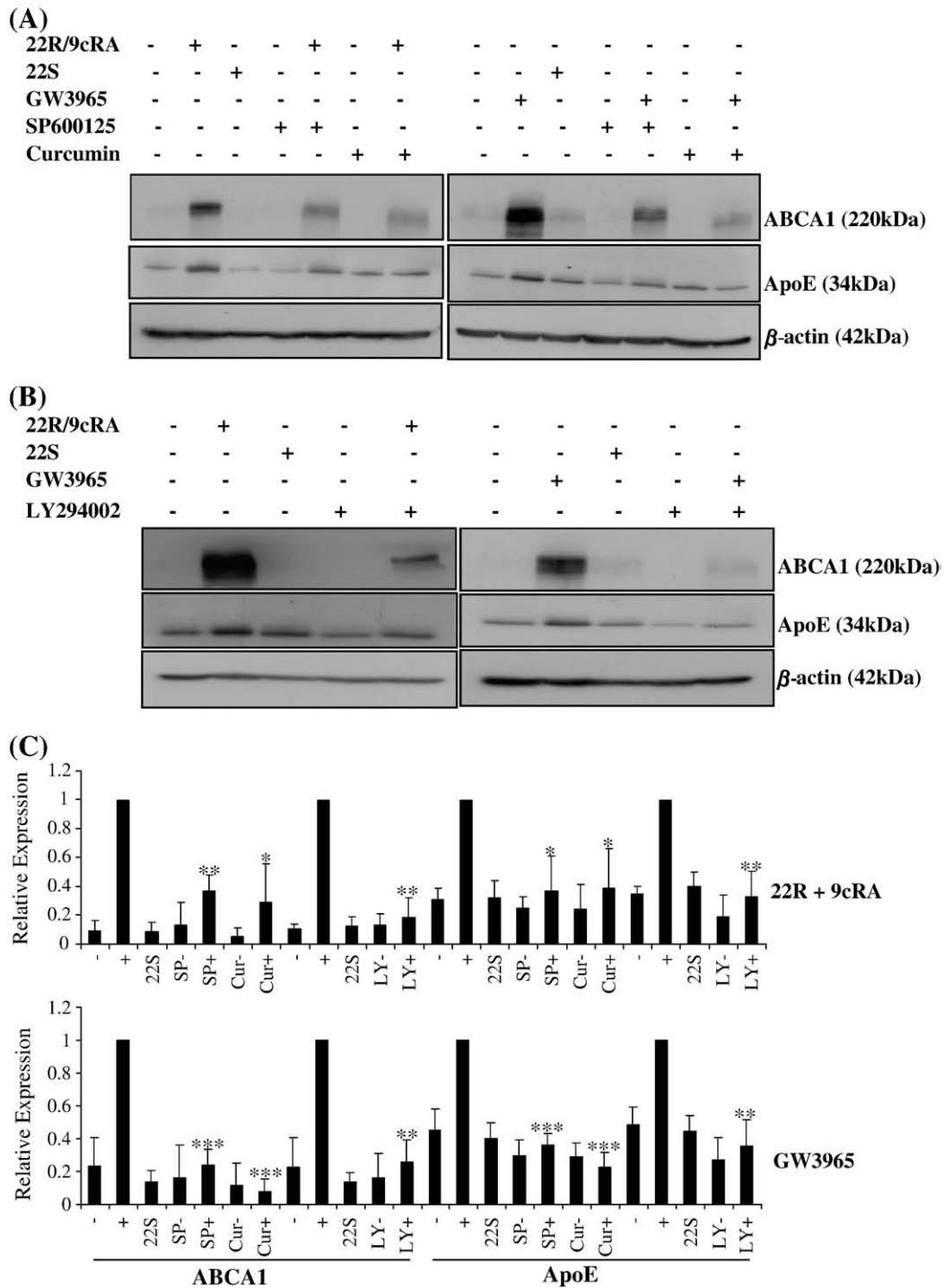
Fig. 2. Induction of ABCA1 and ApoE protein expression by LXR agonists is attenuated by inhibitors of JNK and PI3K. THP-1 macrophages were pre-treated for 1 h with the DMSO vehicle (−) or the inhibitors (25 μM curcumin, 100 μM SP600125 or LY294002) and then incubated for 24 h with the indicated ligands [2 μg/ml 22-(R)-HC plus 10 μM 9cRA (22R/9cRA), 2 μg/ml 22-(S)-HC (22S) or 1 μM GW3965]. Equal amounts of proteins were then subjected to Western blot analysis using antisera against ABCA1, ApoE or β-actin. A representative image is presented in panels A and B and panel C shows the outcome of densitometric analysis of the levels of ABCA1 or ApoE normalized to β-actin (mean ± SD) from three independent experiments (for each inhibitor, the value from cells treated with the ligand in the absence of inhibitor has been arbitrarily assigned as 1) [SP, SP600125; Cur, curcumin; LY, LY294002; +, 22-(R)-HC plus 9cRA; *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$].

of both phospho-AKT and its activity were inhibited by LY294002 (Fig. 4C–E).

3.4. LXR agonists induce activator protein-1 (AP-1) DNA binding

Because c-Jun, which belongs to the AP-1 family of transcription factors, is a key downstream target for JNK [21], the action of the ligands on AP-1 DNA binding was analyzed by EMSA using a probe containing the consensus binding sequence. Either 22-(R)-HC plus 9cRA or TO901317 increased AP-1 binding using whole cell or nuclear extracts (Fig. 5A). The positive action of the ligands was confirmed by monitoring the expression of the ABCA1 protein (data not shown). Competition EMSA using extracts from ligand-treated cells showed that the DNA-protein complexes were markedly competed out with

(A)

(B)
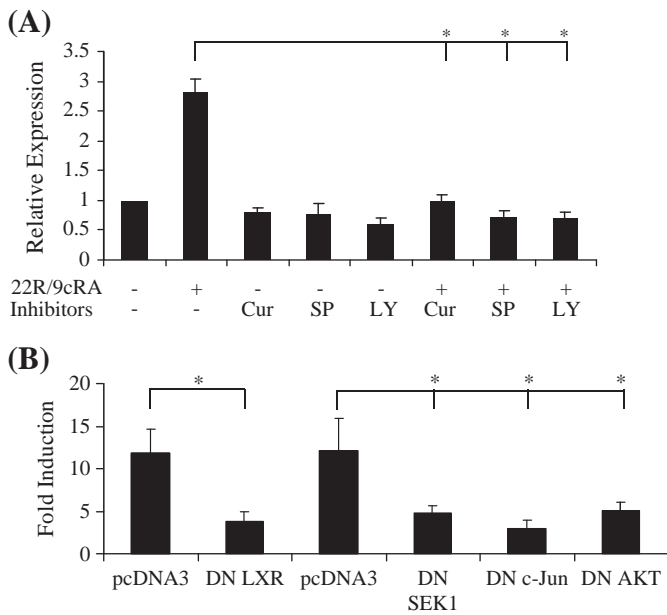
Fig. 3. The 22-(R)-HC/9cRA-induced gene expression is attenuated by the JNK and PI3K inhibitors in HMDM and by dominant negative forms of proteins of these pathways in transfected cells. (A) HMDM were pre-treated for 1 h with 10 μM curcumin (Cur), 50 μM SP600125 (SP), 50 μM LY294002 (LY) or the DMSO vehicle (−) and then incubated for 24 h in medium containing the ligand 22-(R)-HC plus 9cRA (2 μg/ml/10 μM) (+) or the vehicle (−). The total RNA was subjected to RT-qPCR using primers for ApoE or 28S rRNA. The ApoE:28SrRNA ratio from cells treated with vehicle alone has been arbitrarily assigned as 1, with those from the other samples represented to this value (mean ± SD from three independent experiments; *p<0.05). (B) U937 cells were co-transfected with the ABCA1 promoter-luciferase DNA construct along with the pcDNA3 vector or expression plasmids specifying for dominant negative (DN) forms of LXR-α, SEK1, c-Jun and AKT as indicated. The cells were then differentiated for 18 h using 0.16 μM phorbol 12-myristate 13-acetate in the presence of 2 μg/ml 22-(R)-HC plus 10 μM 9cRA or DMSO vehicle. In each case the relative luciferase activity, normalized to protein concentration, from vehicle-treated cells has been arbitrarily assigned as 1 (not shown) with those from cells incubated with the ligands represented to this value (mean ± SD from three independent experiments; *p<0.05).

an excess of oligonucleotides containing the AP-1 binding site but not by containing the unrelated C/EBP or NF-κB binding site (Fig. 5B). Curcumin has previously been shown to inhibit c-Jun/AP-1 binding [22] and this was also found to be the case for the LXR agonist-induced AP-1 activity (Fig. 5C).

The studies so far employed a consensus AP-1 binding site and the findings were therefore extended to natural promoters. An AP-1-like sequence has been identified in the promoter of the ApoE gene [23]. EMSA showed that protein binding to this site was also induced by the treatment of the cells with 22-(R)-HC plus 9cRA or TO901317 (Fig. 6A). The proximal promoter region of the human ABCA1 gene also contains binding sites for several transcription factors, including AP-1 [24]. EMSA using an oligonucleotide containing one of these sites (−131 to −111) again demonstrated increased binding when extracts from ligand-treated cells were used (Fig. 6A). Competition EMSA showed that the formation of DNA-protein complexes was abolished by an oligonucleotide containing the AP-1 binding site but not for NF-κB (Fig. 6B).

4. Discussion

LXR ligands attenuate atherosclerosis by controlling several cellular events, including the stimulation of macrophage cholesterol efflux through the expression of key genes implicated in this process, such as ApoE and ABCA1. The action of nuclear receptor agonists in the regulation of gene transcription is complex with control by intracellular signaling pathways of the receptors themselves, co-factors recruited by them or other transcription factors that are required for the maximal expression of downstream genes [7]. Unfortunately, the impact of intracellular signaling on the actions of LXR agonists in the regulation of gene expression is poorly understood. Using a combination of inhibitors, biochemical analysis and use of plasmids specifying for dominant negative proteins, we show for the first time an important role for the JNK and PI3K pathways in the LXR agonists-induced expression of these genes in macrophages.

Previous studies have suggested potential roles for protein kinase A (PKA), protein kinase C (PKC) and casein kinase 2 (CK2) in the regulation of LXR activity although none of these has been in human macrophages in relation to genes associated with cholesterol efflux. For example, Huang et al. [25] showed a synergistic activation of LXR-α by 22-(R)-HC and agents triggering PKA and PKC signaling in CHOK1 cells. In contrast to this, PKA was found to suppress the expression in hepatocytes of sterol regulatory element binding protein 1c, a key downstream target of LXR actions, through the phosphorylation of LXR-α [26]. The role of PKA therefore appears to be gene- and/or cell-type-specific. LXR-α has also been found to be phosphorylated on serine 198 (S198) under basal conditions and this was enhanced by TO901317 and reduced by CK2 inhibitors or 9cRA [27]. The action of this phosphorylation was gene-specific as the expression of only some LXR targets was increased in cells expressing the S198A phosphorylation-deficient mutant compared to those for the wild-type receptor [27].

The exact mechanism through which LXR agonists activate JNK and PI3K and ultimately how these modulate the actions of this nuclear receptor remains to be determined. In relation to the activation of the signaling pathways, studies on other nuclear receptors have shown that these often represent the so called non-genomic effects [28]. This involves a rapid, transient activation of intracellular signaling cascades often mediated by a subpopulation of the receptor at the cytoplasmic side of the membrane [28]. Although such cytoplasmic receptors exist for a number of nuclear receptors, including the PPARs, to our knowledge this has not yet been identified for the LXRs. In relation to the potential mechanisms, agonists don't affect LXR DNA binding so steps subsequent to these are likely to be affected, such as the phosphorylation of the LXRs leading to promotion of co-activator recruitment at target gene promoters and/or the release of co-repressors and/or direct modulation of the activity of such co-factors by phosphorylating them in a ligand-dependent manner. For example, the PKA-mediated phosphorylation of LXRα has been shown to reduce the recruitment of the SRC-1 coactivator and enhance the recruitment of the corepressor NCoR1 [26].

LXR agonists induced the binding of AP-1, a key factor regulated by JNK, to its recognition sequences in several gene promoters. Interestingly, LXR agonists have also been found to activate AP-1 in keratinocytes and this has been linked to the regulation of expression of key genes implicated in epidermal differentiation [29]. It is thus
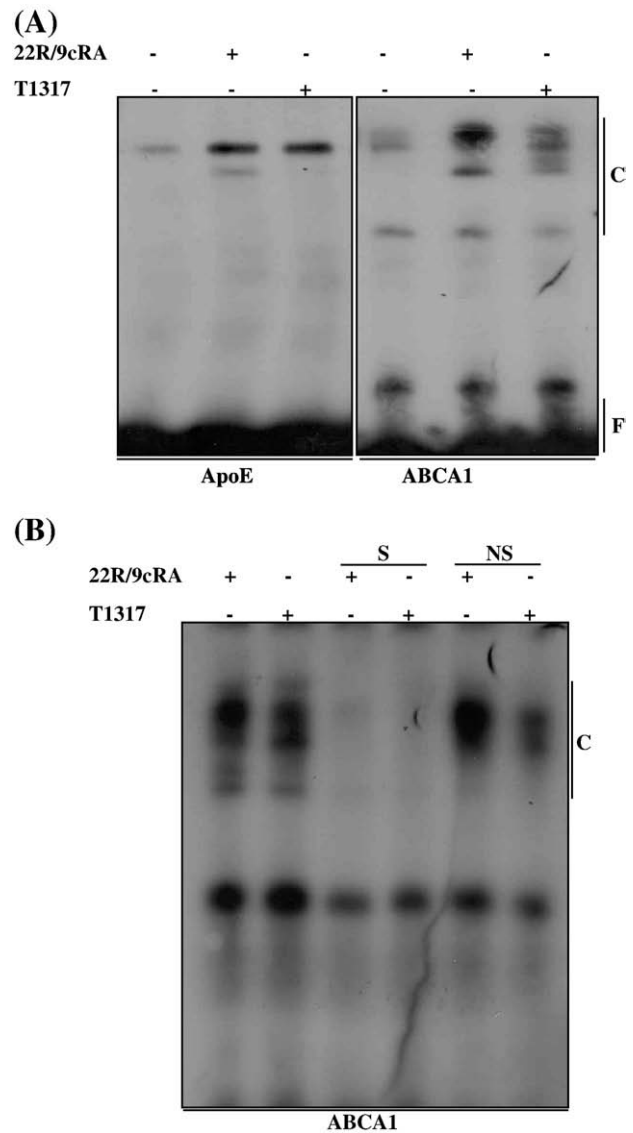
Fig. 4. 22-(R)-HC plus 9cRA activate the JNK and PI3K pathways and this is attenuated by the corresponding inhibitors. THP-1 macrophages were pre-treated for 1 h with vehicle (−) or the indicated concentrations of SP600125, 30 μM curcumin (Cur) or 100 μM LY294002. The cells were then incubated for 24 h (panels A and B) or 1 h (panels C and D) with 2 μg/ml 22-(R)-HC (22R) plus 10 μM 9cRA (DMSO was used as a vehicle control; −). Whole cell extracts were subjected to Western blot analysis using antisera that recognize phosphorylated and total levels (phos/p and Tot/t, respectively) of JNK, SEK1, c-Jun or AKT (panels A and C) or in vitro kinase assay (panels B and D). In the latter, the ability of immunoprecipitated proteins to phosphorylate its downstream fusion protein (FP) substrate is monitored by Western blot analysis (c-Jun for JNK in panel B and GSK-3β for AKT in panel D). The data shown are representative of two (panel B along with JNK and c-Jun in panel A) and three (SEK1 in panel A along with panels C and D) experiments. (E) densitometric analysis was carried out on the data shown in panels C and D and the p-AKT:t-AKT or p-GSK-3α/β:β-actin ratios were determined. The ratios from cells treated with the vehicle alone or in the presence of the ligands have been arbitrarily assigned as 1, as indicated, with the others represented to this (mean ± SD from three independent experiments) (*p<0.05 and **p<0.01).

(A)

(B)

(C)

(D)

(E)
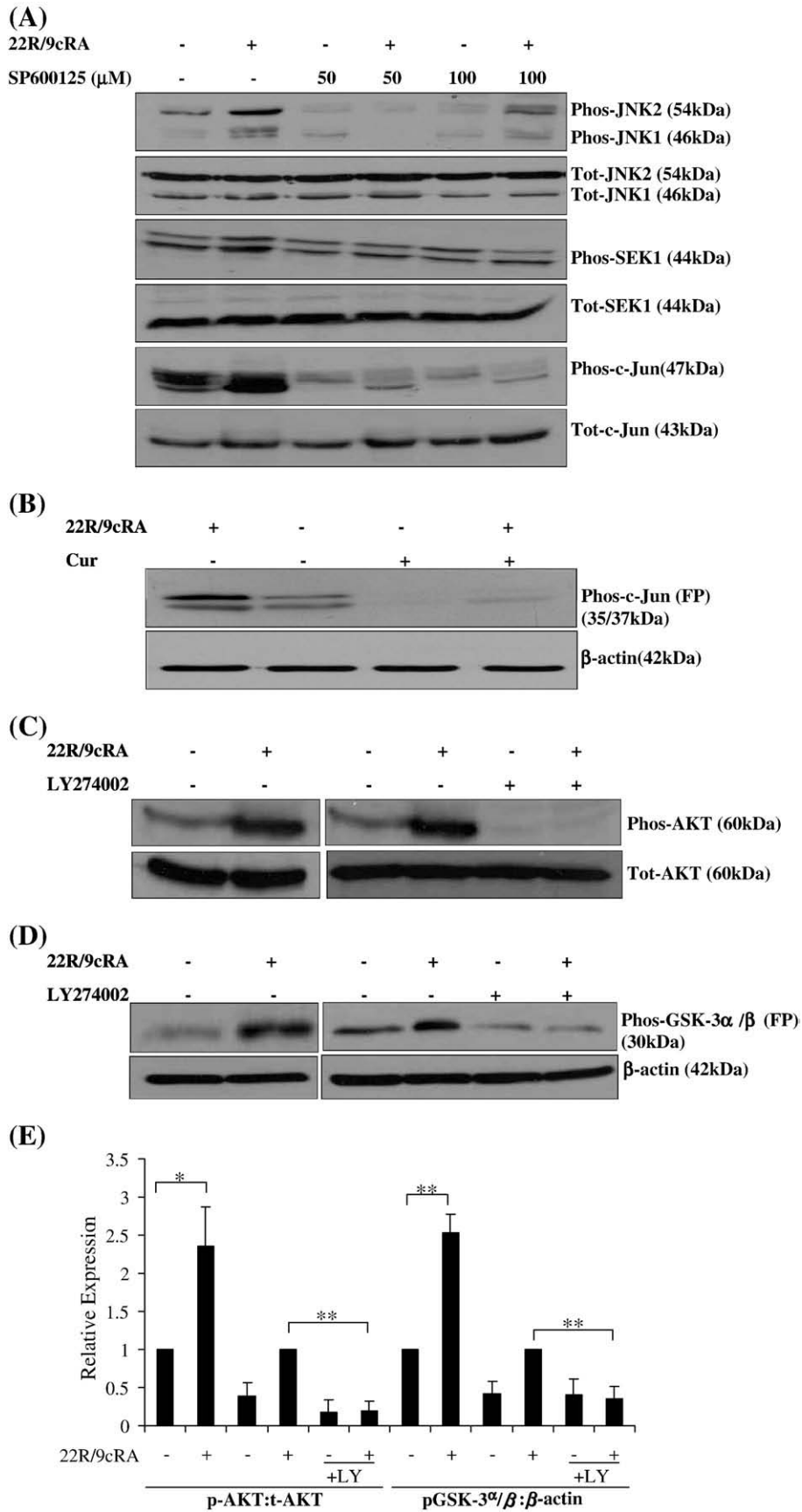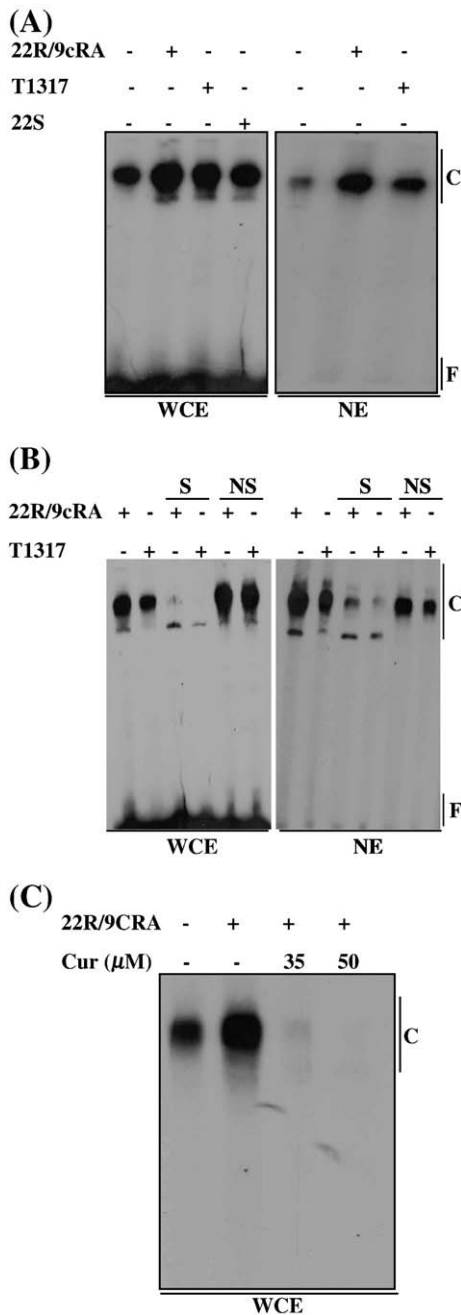
Fig. 5. LXR activators induce AP-1 DNA binding. THP-1 macrophages were incubated for 24 h with 2 µg/ml 22-(R)-HC (22R) plus 10 µM 9cRA, 10 µM TO901317 (T1317) or 2 µg/ml 22-(S)-HC (22S) (panels A and B). For panel C, the cells were pre-treated for 1 h with the vehicle or the indicated concentration of curcumin (Cur) before the addition of the ligands. Whole cell extracts (WCE) or nuclear extracts (NE) were prepared and used for EMSA using a consensus AP-1 binding site probe. The competition assays in panel B employed a 200-fold molar excess of unlabelled specific AP-1 sequence (S) or for the unrelated C/EBP or NF-κB binding sites (NS for WCE and NE, respectively). The AP-1:DNA complex and free probe are indicated by vertical lines labelled C and F, respectively (the free probe has migrated off the gel for NE in panels A and B and in panel C). The data shown are representative of two independent experiments.

Fig. 6. LXR activators induce AP-1 binding to its recognition sequences in the ApoE and ABCA1 gene promoters. THP-1 macrophages were incubated for 24 h with 2 µg/ml 22-(R)-HC (22R) plus 10 µM 9cRA or 10 µM TO901317 (T1317) as indicated. Whole cell extracts were prepared and used for EMSA using radiolabelled probe containing an AP-1 binding site from the ApoE gene promoter (ApoE) or a putative recognition sequence in the regulatory region of the ABCA1 gene (ABCA1). The competition assays in panel B employed a 200-fold molar excess of unlabelled specific AP-1 sequence (S) or for the unrelated NF-κB binding site (NS). The major DNA:protein complexes are shown by a vertical line labelled C (the free probe has migrated off the gel in panel B). The data shown are representative of two to three independent experiments.

possible that such activation of AP-1 might also play important roles in the actions of LXRs in macrophages such as the maximal expression of downstream genes such as ApoE and ABCA1, the promoter regions of which contain AP-1 binding sites [23,24], or regulating the expression of key components involved in LXR-mediated gene transcription.

In conclusion, we have identified a key role for JNK/c-Jun/AP-1 in the LXR agonists-mediated induction of several key genes implicated in the control of macrophage cholesterol homeostasis. These signaling components are likely to play an important role in atherosclerosis, given that LXR agonists act in an anti-atherogenic manner. Future studies should seek to delineate how these agonists activate the identified signaling pathways and modulate LXR actions on downstream genes.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.cellsig.2010.11.002.

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