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Journal of Cellular Biochemistry 116:2032–2038 (2015)

Journal of Cellular Biochemistry

Protein Kinase C Is Involved in the Induction of ATP-Binding Cassette Transporter A1 Expression by Liver X Receptor/ Retinoid X Receptor Agonist in Human Macrophages

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ABSTRACT

The transcription of the ATP-binding cassette transporter A1 (ABCA1) gene, which plays a key anti-atherogenic role, is known to be induced by agonists of liver X receptors (LXRs). LXRs form obligate heterodimers with retinoid X receptors (RXRs) and interact with their recognition sequences in the regulatory regions of key genes implicated in the control of cholesterol, fatty acid and glucose homeostasis. We have previously shown a novel role for c-Jun N-terminal kinase (JNK) and phosphoinositide 3-kinase (PI3K) in the LXRs-mediated induction of macrophage gene expression. Protein kinase C (PKC) is often found to regulate the action of nuclear receptors and cross talk between this kinase family and JNK and/or PI3K has been shown in several settings. We have, therefore, investigated a potential role for PKC in the action of LXR/RXR agonist 22-(*R*)-hydroxycholesterol (22-(*R*)-HC)/9-cis-retinoic acid (9cRA) in THP-1 macrophages, including the induction of ABCA1 expression. The pan PKC inhibitor bisindoylmaleimide was found to attenuate the induction of ABCA1 protein expression, the activation of the JNK signaling pathway and the stimulation of activator protein-1 (AP-1) DNA binding activity in macrophages treated with 22-(*R*)-HC and 9cRA. The role of PKC in the action of these ligands was confirmed further by the use of more isotype-specific inhibitors. These studies, therefore, reveal a potentially important role for PKC in the action of 22-(*R*)-HC and 9cRA in human macrophages. J. Cell. Biochem. 116: 2032–2038, 2015.

KEY WORDS: ATHEROSCLEROSIS; CELL SIGNALING; GENE EXPRESSION; LIVER X RECEPTORS; MACROPHAGES; NUCLEAR RECEPTORS

ATP-binding cassette transporter A1 (ABCA1) belongs to the ATP-binding cassette transporter family and mediates the efflux of cholesterol (and phospholipids) to lipid-poor donors such as apolipoprotein (Apo)-E and -A1 [Fitzgerald et al., 2010]. Tangier disease arises due to mutations in the ABCA1 gene and is associated with the accumulation of foam cells in several tissues and increased risk of cardiovascular disease [Fitzgerald et al., 2010]. The expression of ABCA1 is induced by several factors, including agonists of liver X receptors (LXRs) [Fitzgerald et al., 2010; McLaren et al., 2011; Michael et al., 2012a; Hong and Tontonoz, 2014].

LXRs are members of the nuclear hormone receptor subfamily of ligand-activated transcription factors that have potent anti-atherogenic actions [Michael et al., 2012a; Hong and Tontonoz, 2014]. There are two LXR isoforms; α, which is expressed in a restricted set of tissues/cell types, including macrophages, and β, which displays a ubiquitous expression pattern [Michael et al., 2012a; Hong and Tontonoz, 2014]. LXRs are activated by oxidized metabolites of cholesterol and therefore act as intracellular sensors of cholesterol [Michael et al., 2012a; Hong and Tontonoz, 2014]. LXRs form obligate heterodimers with retinoid X receptors (RXR) and regulate gene transcription by binding to LXR response elements (LXR-REs) in the promoter regions of target genes. These include several genes implicated in the control of macrophage lipid homeostasis, such as ABCA1 and ApoE [Michael et al., 2012a; Hong and Tontonoz, 2014]. LXRs also attenuate the transcription of many genes associated with inflammation via a mechanism that is

Abbreviations used: 9cRA, 9-cis-retinoic acid; 22-(*R*)-HC, 22-(*R*)-hydroxycholesterol; ABCA1, ATP-binding cassette transporter A1; ApoE, apolipoprotein E; JNK, c-Jun N-terminal kinase; LXR, liver X receptor; LXR-REs, LXR response element; RXR, retinoid X receptor; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C.

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Grant sponsor: King Abdulaziz University (PhD Scholarship to Etimad A. Huwait).

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Manuscript Received: 3 July 2014; Manuscript Accepted: 3 March 2015

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 9 March 2015

DOI 10.1002/jcb.25157 • © 2015 Wiley Periodicals, Inc.

independent of sequence specific binding to LXR-REs [Ghisletti et al., 2007; Michael et al., 2012a; Hong and Tontonoz, 2014]. Consistent with all these actions of the LXRs, deficiency of this nuclear receptor potentiates atherosclerosis in mouse models of this disease [Michael et al., 2012a; Hong and Tontonoz, 2014]. On the other hand, agonists of LXRs inhibit atherosclerosis in such model systems [Michael et al., 2012a; Hong and Tontonoz, 2014].

Nuclear receptors are also subject to regulation by intracellular signal transduction pathways [Luconi et al., 2010; Berrabah et al., 2011]. However, the impact of such signaling pathways on the action of LXRs is poorly understood. Our recent studies showed that the induction of several genes in macrophages by LXR agonists, including ABCA1, was attenuated by pharmacological inhibitors of c-Jun N-terminal kinase (JNK) and phosphoinositide 3-kinase (PI3K) pathways [Huwait et al., 2011]. LXR agonists activated key components of the JNK and PI3K pathways, and the induction of ABCA1 promoter activity in transfected cells by such agonists was attenuated by expression of dominant negative forms of key enzymes in the JNK and PI3K pathways [Huwait et al., 2011]. Cross-talk between protein kinase C (PKC) and JNK and/or PI3K pathways has been found in several settings [De Windt et al., 2000; Martin et al., 2001; Im et al., 2007] and PKC is also known to modulate the activity of many nuclear receptors [Rochette-Egly, 2003; Gray et al., 2005]. However, the role of PKC in the induction of ABCA1 expression by LXR/RXR agonists has not been determined and, therefore, formed the focus of the current study.

MATERIALS AND METHODS

MATERIALS

The human THP-1 cell line was from the European Collection of Animal Cell Cultures whereas the antibodies were from Abcam (ABCA1), Sigma–Aldrich (β-actin) and Cell Signaling Technology (AKT, phospho AKT Ser 473; SEK1, phospho SEK1 Ser 257/Thr 261; JNK, phospho JNK Thr 183/Tyr 185). The pharmacological inhibitors were from Merck Millipore and 22-(*R*)-hydroxycholesterol [22-(*R*)-HC)] and 9-cis-retinoic acid (9cRA) were from Sigma–Aldrich.

CELL CULTURE

THP-1 cells were maintained in RPMI-1640 with Stabilix supplemented with 10% (v/v) heat-inactivated foetal calf serum (56°C, 30 min) in the presence of streptomycin (100 μg/ml) and penicillin (100 U/ml). The cells were differentiated into macrophages by incubation with 0.16 μM phorbol 12-myristate 13-acetate for 24 h. In experiments where 22-(*R*)-HC was employed, delipidated heat-inactivated foetal calf serum, prepared as described by Cham and Knowles (1976), was used. Pharmacological inhibitors were added 1 h before the addition of the ligands (pre-treatment) [Huwait et al., 2011].

WESTERN BLOT ANALYSIS

Cellular extracts were prepared and subjected to Western blot analysis as our previous studies [Ali et al., 2010; Huwait et al., 2011; Michael et al., 2012b] except that the samples were not boiled before loading onto the gels as this caused the degradation of the high molecular weight ABCA1 protein.

ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSA)

The radiolabeling of the oligonucleotides, preparation of whole cell extracts and EMSA were carried out as our previous studies [Hughes et al., 2002; Huwait et al., 2011]. The sequences of the AP-1 consensus site oligonucleotides were 5′-CGCTTGATGAGTCAG-3′ and 5′-TTCCGGCTGACTCAT-3′.

STATISTICAL ANALYSIS

This was carried out using one-way ANOVA with Tukey′s post-hoc analysis and Student′s *t* test with similar conclusions. The *P* values from ANOVA are shown and were considered significant when they were below 0.05.

RESULTS

THE 22-(*R*)-HC AND 9cRA-MEDIATED INDUCTION OF ABCA1 EXPRESSION IS ATTENUATED BY PHARMACOLOGICAL INHIBITORS AGAINST PKC

The human THP-1 cell line is a widely used model system for studies on macrophage gene expression, including the actions of nuclear receptors in relation to atherosclerosis, because of conservation of responses with primary cultures and in vivo [Li et al., 2010; McLaren et al., 2010a,b; Huwait et al., 2011; Michael et al., 2012b and references therein]. Because LXRs regulate gene transcription by forming obligate heterodimers with RXRs, 22-(*R*)-HC can be used in combination with 9cRA as endogenous ligands [Koldamova et al., 2003; Huwait et al., 2011]. Indeed, our previous studies showed that although the induction of ABCA1 expression in macrophages was obtained with 22-(*R*)-HC alone, but not with the inactive enantiomer 22-(*S*)-HC, a marked activation was seen when 22-(*R*)-HC and 9cRA were present together [Huwait et al., 2011]. We, therefore, used combinations of these two ligands to delineate the role of PKC in the activation of ABCA1 expression. As our previous studies, the expression of proteins was investigated because of functional relevance.

We initially used the pan PKC inhibitor bisindoylmaleimide I (BIM) [Toullec et al., 1991] to investigate the role of this kinase in the action of 22-(*R*)-HC and 9cRA. ApoE, another key target of LXR/RXR actions, was included for comparative purposes. As shown in Figure 1, the 22-(*R*)-HC and 9cRA-mediated induction of ABCA1 and ApoE protein expression was attenuated by two different concentrations of BIM (1 and 2 μM).

RELATIONSHIP BETWEEN PKC, JNK AND PI3K/AKT SIGNALING PATHWAYS

AKT is a key downstream target of PI3K signaling [Huwait et al., 2011]. Our previous studies have shown that 22-(*R*)-HC and 9cRA induced the levels of phospho-AKT, but not total AKT, along with AKT activity and this was attenuated by the PI3K inhibitor LY294002 [Huwait at al., 2011]. In addition, the ligands induced the levels of phospho-JNK and key components of this pathway and this was attenuated by the inhibitors SP600125 and curcumin [Huwait et al., 2011]. The potential relationship between PKC and the activation of the PI3K/AKT and JNK pathways by the ligands was analysed using the pan PKC inhibitor BIM. The 22-(*R*)-HC and 9cRA-induced levels of phospho-JNK (both p46 and p54) was attenuated by BIM (Supplementary Fig. 1). In addition, the 22-(*R*)-HC and

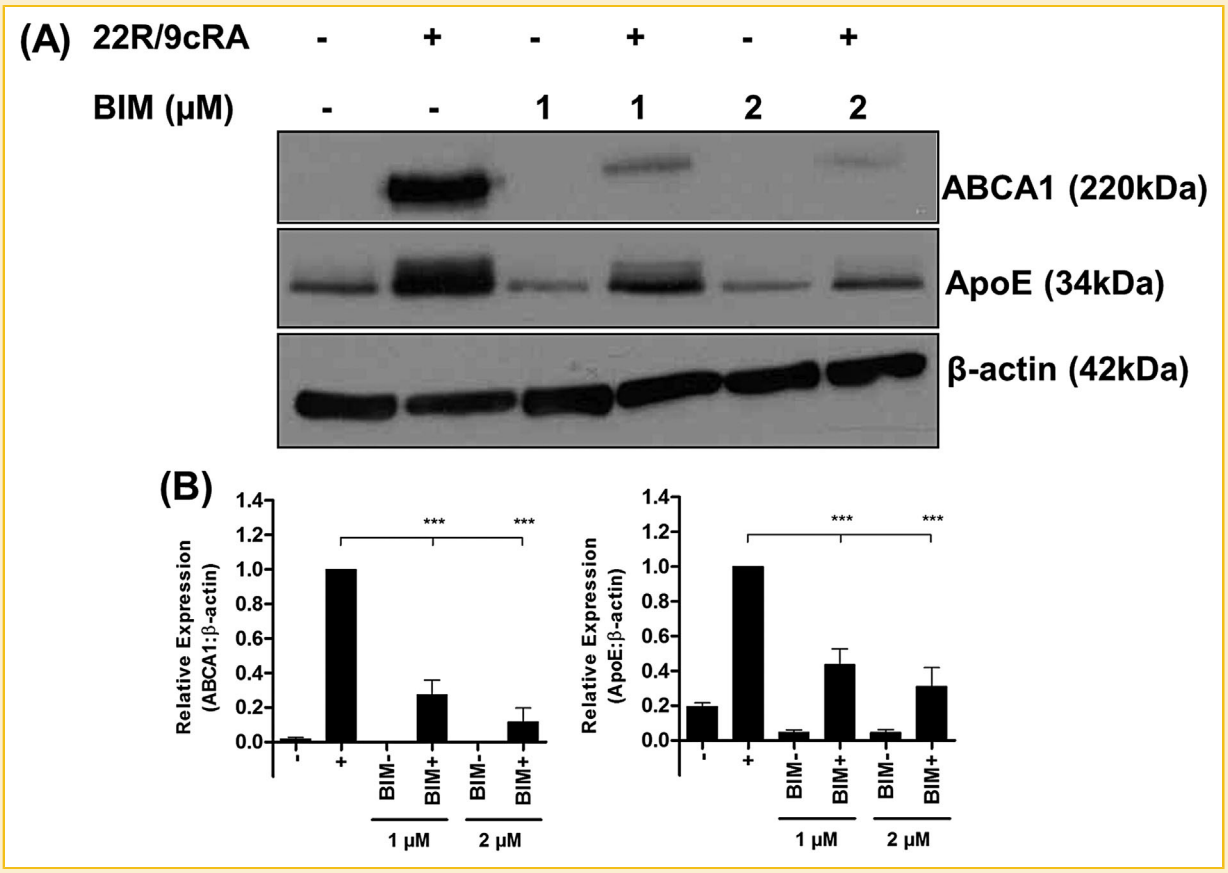

Fig. 1. The 22-(*R*)-HC and 9cRA-mediated induction of ABCA1 and ApoE expression is attenuated by BIM. THP-1 macrophages were treated for 24 h with either the DMSO vehicle (−) or 2 μg/ml 22-(*R*)-HC plus 10 μM 9cRA (22R/9cRA) (+) in the absence or the presence of the indicated concentrations of BIM. The inhibitor was added 1 h before the addition of the ligands (pre-treatment). Western blot analysis on equal amount of proteins was carried out using antisera against ABCA1, ApoE or β-actin. The image shown in panel A is representative of three independent experiments. The histogram in panel B shows mean (± SEM) of the ABCA1:β-actin or ApoE:β-actin ratio, as determined by densitometric analysis, from three independent experiments (the value in ligand treated cells has arbitrarily been assigned as 1) (***$P < 0.001$).

9cRA-induced levels of phospho-SEK1/MKK4, which is upstream of JNK, was also inhibited by BIM (Fig. 2; the two immunoreactive bands for phospho-SEK1 are in accordance with the manufacturer's instructions). These data suggest that PKC is probably upstream of SEK1/JNK in the signaling cascade. In contrast to JNK/SEK1, BIM did not inhibit the 22-(*R*)-HC and 9cRA-induced levels of phospho-AKT (Fig. 3). Interestingly, there was an induction in the levels of activated, phospho-AKT by BIM (Fig. 3). This possibly represents a compensatory mechanism whereby inhibition of PKC, and therefore JNK, leads to the activation of PI3K/AKT.

THE EFFECT OF MORE ISOFORM-SELECTIVE PKC INHIBITORS IN THE ACTIONS OF 22-(*R*)-HC AND 9cRA

There are several PKC isoforms [Mellor and Parker, 1998] and some inhibitors are more selective for certain members. In order to further confirm the role of PKC and also gauge the potential involvement of different isoforms, experiments were carried out with more isoform-specific PKC inhibitors. Gö6983 affects several PKC isoenzymes in a concentration dependent manner with the IC_{50} value of 6 nM for the γ isoform, 7 nM for α and the β isoforms, 10 nM for the δ isoform, 60 nM for the ζ isoform and 20 μM for the μ isoform. We used this inhibitor at concentrations of 10 and 60 nM. A marked attenuation of 22-(*R*)-HC and 9cRA-mediated induction of ABCA1 expression was seen with 60 nM Gö6983 and a slight inhibition at 10 nM (Supplementary Fig. 2), thereby suggesting potentially important roles for PKC-ζ, -δ and possibly -α, -β and -γ but not -μ. Rottlerin inhibits PKCδ with IC_{50} values between 3 and 6 μM compared to 30–42 μM for PKC-α, -β and -γ and 80–100 μM for PKC-ε, -η and -ζ. As shown in Figure 4, rottlerin inhibited the 22-(*R*)-HC and 9cRA-mediated induction of ABCA1 expression. In contrast, inconclusive results were obtained with Gö6976, an inhibitor of the classical Ca^{2+}-dependent PKC-α and -β (data not shown). Overall, these results confirm the importance of PKC identified from studies using BIM, and suggest a potentially important role for PKC-δ and -ζ in the 22-(*R*)-HC and 9cRA-mediated induction of ABCA1 expression by the ligands, and indicate that PKC-α, -β and -μ are probably not involved in this response. However, definite proof will require analysis of the response from macrophages deficient in the expression of each member of this large family.

BIM inhibited the 22-(*R*)-HC and 9cRA-induced levels of phospho-JNK (both p46 and p54) (Supplementary Fig. 1). The analysis was extended to isoform-selective PKC inhibitors to

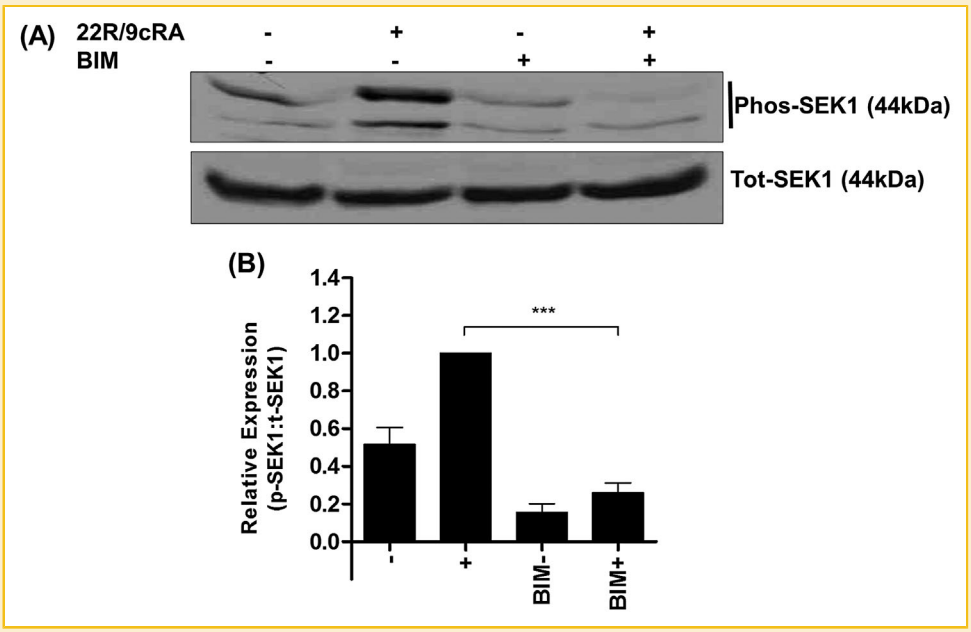

Fig. 2. The 22-(*R*)-HC and 9cRA-mediated activation of SEK1 is attenuated by BIM. THP-1 macrophages were treated for 24 h with either the DMSO vehicle (−) or 2 μg/ml 22-(*R*)-HC plus 10 μM 9cRA (22R/9cRA) (+) in the absence or the presence of 2 μM BIM. The inhibitor was added 1 h before the addition of the ligands (pre-treatment). Western blot analysis on equal amount of proteins was carried out using antisera that recognize phosphorylated and total levels (Phos and Tot, respectively) of SEK1. The image shown in panel A is representative of three independent experiments. The histogram in panel B shows mean (± SEM) of the phospho-SEK1:total-SEK1 ratio, as determined by densitometric analysis, from three independent experiments (the value in ligand treated cells has arbitrarily been assigned as 1) (***$P < 0.001$).

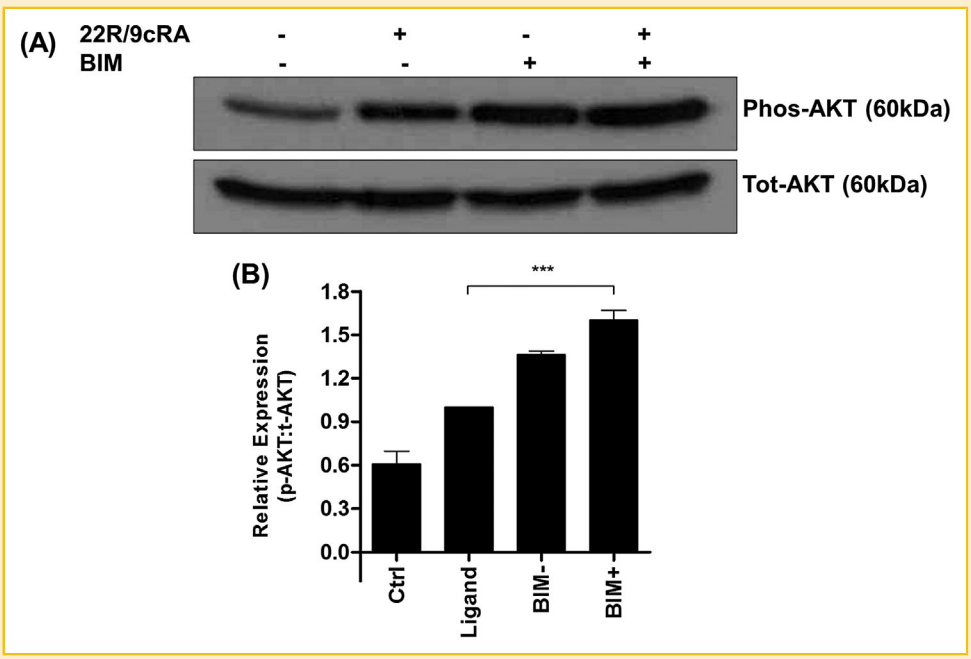

Fig. 3. The effect of BIM on the 22-(*R*)-HC and 9cRA-mediated activation of AKT.THP-1 macrophages were treated for 1 h (maximal time for AKT activation identified from time course experiments—data not shown) with either the DMSO vehicle (−) or 2 μg/ml 22-(*R*)-HC plus 10 μM 9cRA (22R/9cRA) (+) in the absence or the presence of 2 μM BIM. The inhibitor was added 1 h before the addition of the ligands (pre-treatment). Western blot analysis on equal amount of proteins was carried out using antisera that recognize phosphorylated and total levels (Phos and Tot, respectively) of AKT. The image shown in panel A is representative of three independent experiments. The histogram in panel B shows mean (± SEM) of the phospho-AKT:total-AKT ratio, as determined by densitometric analysis, from three independent experiments (the value in ligand treated cells has arbitrarily been assigned as 1) (***$P < 0.001$).

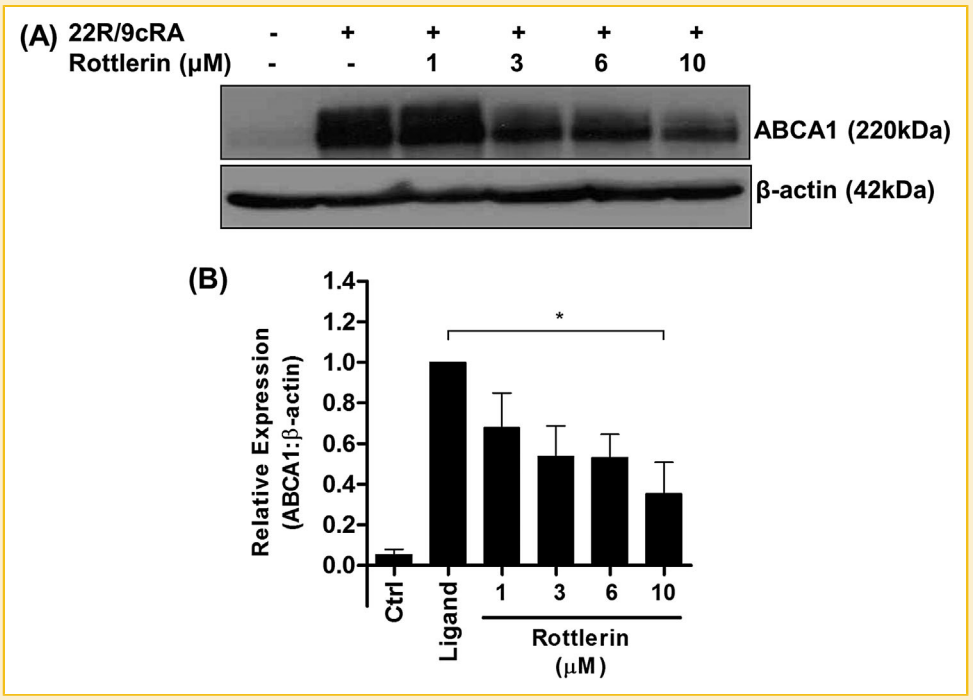

Fig. 4. The effect of rottlerin on the 22-(*R*)-HC and 9cRA-mediated induction of ABCA1 expression. THP-1 macrophages were treated for 24 h with either the DMSO vehicle (−) or 2 μg/ml 22-(*R*)-HC plus 10 μM 9cRA (22R/9cRA) (+) in the absence or the presence of the indicated concentration of rottlerin, which was added 1 h before the addition of the ligands (pre-treatment). Western blot analysis on equal amount of proteins was carried out using antisera against ABCA1 or β-actin. The image shown in panel A is representative of five independent experiments. The histogram in panel B shows mean (± SEM) of the ABCA1:β-actin ratio, as determined by densitometric analysis, from five independent experiments (the value in ligand treated cells has arbitrarily been assigned as 1) (*$P < 0.05$).

delineate whether they also acted via inhibition of JNK activation. As shown in Figure 5, the 22-(*R*)-HC and 9cRA-induced levels of phospho-JNK were attenuated by rottlerin and Gö6976 but not Gö6983. These data suggest that the action of BIM and rottlerin on 22-(*R*)-HC and 9cRA-induced ABCA1 expression was mediated, at least in part, via inhibition of JNK activation whereas an alternative mechanism was utilised in the case of Gö6983.

PKC IS ALSO INVOLVED IN THE 22-(*R*)-HC AND 9cRA-MEDIATED INDUCTION OF AP-1 DNA BINDING ACTIVITY

c-Jun, a member of the AP-1 family of transcription factors, is a one of the key downstream target for JNK action [Singh and Ramji, 2006] and indeed LXR agonists have been found to activate AP-1 in keratinocytes [Schmuth et al., 2004]. We had, therefore, previously studied the effect of 22-(*R*)-HC and 9cRA on the AP-1 DNA binding activity in macrophages by EMSA and found this to be increased by

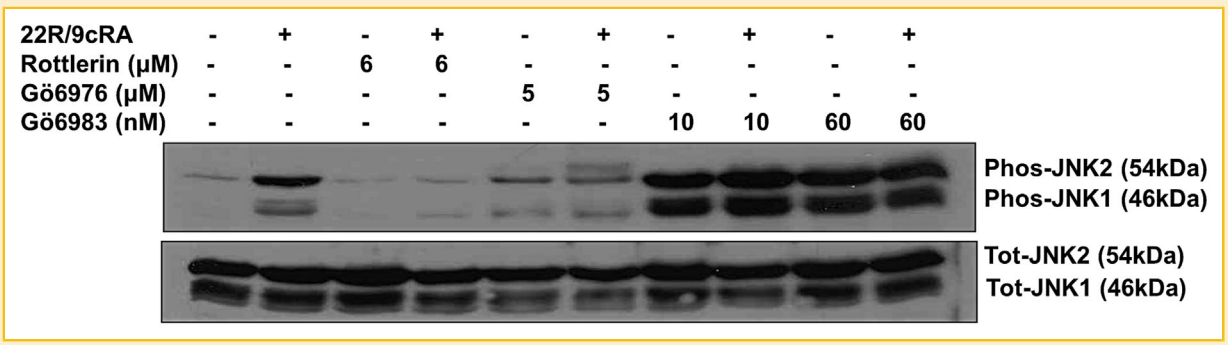

Fig. 5. The effect of different PKC inhibitors on the 22-(*R*)-HC and 9cRA-mediated activation of JNK1/2. THP-1 macrophages were treated for 24 h with either the DMSO vehicle (−) or 2 μg/ml 22-(*R*)-HC plus 10 μM 9cRA (22R/9cRA) (+) in the absence or the presence of the indicated concentrations of the inhibitors shown. The inhibitors were added 1 h before the addition of the ligands (pre-treatment). Western blot analysis on equal amount of proteins was carried out using antisera that recognize phosphorylated and total levels (Phos and Tot, respectively) of JNK1/2. The image shown is representative of two independent experiments.

the ligands and attenuated by inhibitors of the JNK pathway [Huwait et al., 2011]. We investigated here the effect of BIM, Gö6976 and rottlerin, which inhibited the activation of JNK (Supplementary Fig. 1 and Fig. 5), on the 22-(*R*)-HC and 9cRA-induced AP-1 DNA binding. EMSA using consensus AP-1 binding site oligonucleotides showed that the induction of AP-1 DNA binding activity by the ligands was attenuated by all three inhibitors (Fig. 6), thereby further substantiating the role of PKC in the action of 22R-(*R*)-HC and 9cRA.

DISCUSSION

LXRs play a key role in atherosclerosis by regulating the expression of key genes implicated in the control of macrophage cholesterol homeostasis [Michael et al., 2012a; Hong and Tontonoz, 2014]. Intracellular signaling pathways are also known to modulate the activities of nuclear receptors either directly, by for example post-translational modifications, or through the regulation of specific co-factors [Luconi et al., 2010; Berrabah et al., 2011]. The impact of such intracellular signaling pathways on the action of the LXRs is poorly understood. However, we have recently found a novel role for JNK and PI3K in the LXRs-mediated induction of macrophage gene expression. We extend these findings by showing here a role of PKC in the action of 22-(*R*)-HC and 9cRA in THP-1 macrophages. Thus, the 22-(*R*)-HC and 9cRA-mediated induction of ABCA1 expression was attenuated by the pan PKC inhibitor BIM (Fig. 1) and some more isoenzyme-selective inhibitors (Supplementary Fig. 2 and Fig. 4). In addition, the activation of JNK, SEK1 and AP1 DNA binding activity was attenuated by BIM (Supplementary Fig. 1 and Figs. 2, 5 and 6). These studies, therefore, suggest a potentially important role of PKC in the regulation of LXR actions in macrophages during atherosclerosis.

The PKC family is composed of at least ten members; α, βI, βII, γ, δ, ε, η, θ, ζ and λ [Mellor and Parker, 1998]. The precise roles of individual PKC isoforms will clearly require analysis of the effect of 22-(*R*)-HC and 9cRA on the activities of individual members along with the effect of their knockdown or knockout on the various cellular responses. The role of PKCα in the regulation of trans-activation of LXRα has been previously investigated though these studies were not in macrophages [Delvecchio and Capone, 2008]. Thus, transactivation by LXR was decreased by activation of PKC in monkey kidney COS-1 cells, human embryonic kidney HEK293 cell line and human hepatocellular carcinoma HepG2 cell line [Delvecchio and Capone, 2008]. In addition, PKCα was found to phosphorylate LXRα in vitro [Delvecchio and Capone, 2008]. The precise reasons why these findings are different from ours in relation to the role of PKC (i.e., activation vs. inhibition) are currently unclear but could represent cell-specific differences that have been previously observed. For example, LXR ligands attenuate the interferon-γ-induced phosphorylation of the transcription factor signal transducer and activator of transcription-1 in microglia cells but not in astrocytes [Lee at al., 2009]. In addition, opposite actions have been seen on the effects of LXRs on de novo lipogenesis in the adipose tissue and the liver of C57BL/6 female mice [Korach-André et al., 2011]. Apart from JNK, PI3K and PKC, previous studies have also suggested potential roles for protein kinase A (PKA) and casein kinase 2 (CK2) in the action of the LXRs [Huang et al., 1998; Yamamoto et al., 2007; Torra et al., 2008]. The action of PKA also appears to be cell-type-specific [Huang et al., 1998; Yamamoto et al., 2007]. LXR-α has also been found to be phosphorylated on serine 198 under basal conditions and this is enhanced by LXR ligands and inhibited by inhibitors of CK2 [Torra et al., 2008]. The interaction between these different signaling pathways remains to be determined but the inhibition of 22-(*R*)-HC and 9cRA-mediated activation of JNK, SEK1 and AP-1 DNA binding activity by BIM (Figs. 2, 5 and 6; Supplementary Fig. 1) suggests that PKC is likely to be upstream of JNK/SEK1/c-Jun/AP-1 in the signaling cascade.

c-Jun, a member of AP-1 family, is a key downstream target for the action of JNK [Singh and Ramji, 2006]. LXR agonists have been found to activate AP-1 in keratinocytes and this is associated with transcription of several key genes involved in the epidermal differentiation programme [Schmuth et al., 2004]. Similarly, we have previously shown that LXR agonists induce AP-1 DNA binding activity in macrophages [Huwait et al., 2011]. We also show here that the 22-(*R*)-HC and 9cRA-mediated stimulation of AP-1 DNA binding activity is attenuated by several PKC inhibitors (Fig. 6). It is, therefore, possible that AP-1 might also play a key role in LXR signaling in macrophages such as maximal expression of down-stream genes or regulation of expression of key components involved in signaling by this nuclear receptor. Interestingly, chromatin immunoprecipitation coupled with microarray technology revealed that more than 77% of all binding sites for LXR-RXR heterodimer also show the presence of AP-1 motifs at adjacent locations, thereby suggesting coordination of LXR and AP-1 signaling in keratinocytes [Shen et al., 2011]. It is likely that this also extends to macrophages.

In conclusion, we have identified a potentially important role for PKC in the actions of 22-(*R*)-HC and 9cRA in macrophages and this is likely to be upstream of JNK/c-Jun/AP-1. Future studies should seek to identify the role of individual PKC isoforms in the regulation of LXR

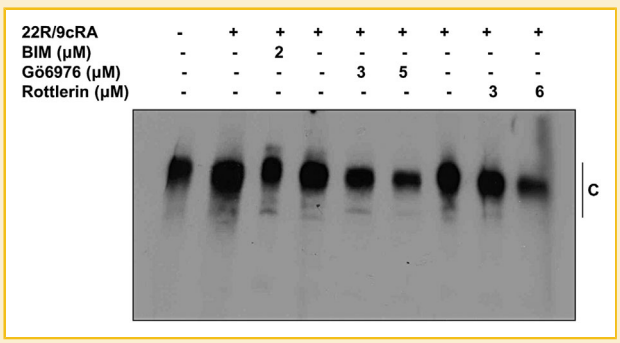

Fig. 6. The effect of different PKC inhibitors on the 22-(*R*)-HC and 9cRA-mediated induction of AP-1 DNA binding activity. THP-1 macrophages were treated for 24 h with either the DMSO vehicle (—) or 2 μg/ml 22-(*R*)-HC plus 10 μM 9cRA (22R/9cRA) (+) in the absence or the presence of the indicated concentrations of the inhibitors (added 1 h before the addition of the ligand pre-treatment). Whole cell extracts were prepared and used for EMSA using a consensus AP-1 binding site probe. The AP-1:DNA complex is shown by a vertical line labelled C (the free probe has migrated off the gel). The data are representative of two independent experiments.

actions in vitro and in vivo, and how the various signaling pathways induced by 22-(*R*)-HC and 9cRA in macrophages converge to modulate lipid metabolism and anti-atherogenic actions of these ligands.

ACKNOWLEDGMENTS

Etimad Huwait was supported by a PhD Scholarship from King Abdulaziz University, Kingdom of Saudi Arabia.

REFERENCES

Ali S, Singh NN, Yildirim H, Ramji DP. 2010. Requirement for nuclear factor kappaB signalling in the interleukin-1-induced expression of the CCAAT/enhancer binding protein-delta gene in hepatocytes. Int J Biochem Cell Biol 42:113–119.

Berrabah W, Aumercier P, Lefebvre P, Staels B. 2011. Control of nuclear receptor activities in metabolism by post-translational modifications. FEBS Lett 585:1640–1650.

Cham BE, Knowles BR. 1976. A solvent system for delipidation of plasma or serum without protein precipitation. J Lipid Res 17:176–181.

De Windt LJ, Lim HW, Haq S, Force T, Molkentin JD. 2000. Calcineurin promotes protein kinase C and c-Jun NH_2-terminal kinase activation in the heart. Cross-talk between cardiac hypertrophic signaling pathways. J Biol Chem 275:13571–13579.

Delvecchio CJ, Capone JP. 2008. Protein kinase C α modulates liver X receptor α transactivation. J Endocrinol 197:121–130.

Fitzgerald ML, Mujawar Z, Tamehiro N. 2010. ABC transporters, atherosclerosis and inflammation. Atherosclerosis 211:361–370.

Ghisletti S, Huang W, Ogawa S, Pascual G, Lin ME, Wilson TM, Rosenfeld MG, Glass CK. 2007. Parallel SUMOylation-dependent pathways mediate gene- and signal-specific transrepression by LXRs and PPARγ. Mol Cell 25:57–70.

Gray JP, Burns KA, Leas TL, Perdew GH, vanden Heuvel JP. 2005. Regulation of peroxisome proliferator-activated receptor alpha by protein kinase C. Biochemistry 44:10313–10321.

Hong C, Tontonoz P. 2014. Liver X receptors in lipid metabolism: Opportunities from drug discovery. Nat Rev Drug Discov 13:433–444.

Huang CJ, Feltkamp D, Nilsson S, Gustafsson JA. 1998. Synergistic activation of RLD-1 by agents triggering PKA and PKC dependent signalling. Biochem Biophys Res Commun 243:657–663.

Hughes TR, Tengku-Muhammad TS, Irvine SA, Ramji DP. 2002. A novel role of Sp1 and Sp3 in the interferon-gamma-mediated suppression of macrophage lipoprotein lipase gene transcription. J Biol Chem 277:11097–11106.

Huwait EA, Greenow KR, Singh NN, Ramji DP. 2011. A novel role for c-Jun N-terminal kinase and phosphoinositide 3-kinase in the liver X receptor-mediated induction of macrophage gene expression. Cell Signal 23:542–549.

Im HJ, Muddasani P, Natarajan V, Schmid TM, Block JA, Davis F, van Wijnen AJ, Loeser RF. 2007. Basic fibroblast growth factor stimulates matrix metalloproteinase-13 via the mitogen-activated protein kinases and protein kinase C delta pathways in human adult articular chondrocytes. J Biol Chem 281:11110–111121.

Koldamova RP, Lefterov IM, Ikonomovic MD, Skoko J, Lefterov PI, Isanski BA, Dekosky ST, Lazo JS. 2003. 22R-hydoxycholesterol and 9-cis-retinoic acid induce ATP-binding casette transporter A1 expression and cholesterol efflux in brain cells and decrease amyloid beta secretion. J Biol Chem 278:13244–13256.

Korach-André M, Archer A, Gabbi C, Barros RP, Pedrelli M, Steffensen KR, Pettersson AT, Laurencikiene J, Parini P, Gustafsson JÅ. 2011. Liver X receptors regulate de novo lipogenesis in a tissue-specific manner in C57BL/6 female mice. Am J Physiol Endocrinol Metab 301:E210–E222.

Lee JH, Park SM, Kim OS, Lee CS, Woo JH, Park SJ, Joe EH, Jou I. 2009. Differential SUMOylation of LXRalpha and LXRbeta mediates transrepression of STAT1 inflammatory signaling in IFN-gamma-stimulated brian astrocytes. Mol Cell 35:806–817.

Li N, McLaren JE, Michael DR, Clement N, Fielding CA, Ramji DP. 2010. ERK is integral to the IFN-γ-mediated activation of STAT1, the expression of key genes implicated in atherosclerosis, and the uptake of modified lipoproteins by human macrophages. J Immunol 85:3041–3048.

Luconi M, Cantini G, Serio M. 2010. Peroxisome proliferator-activated receptor γ (PPARγ): Is the genomic activity the only answer? Steroids 75:585–594.

Martin AG, San-Antonio B, Fresno M. 2001. Regulation of nuclear factor kappaB transactivation. Implications of phosphatidylinositol 3-kinase and protein kinase C zeta in c-Rel activation by tumor necrosis factor alpha. J Biol Chem 276:15840–15890.

McLaren JE, Calder CJ, McSharry BP, Sexton K, Salter RC, Singh NN, Wilkinson GW, Wang EC, Ramji DP. 2010a. The TNF-like protein 1A-death receptor 3 pathway promotes macrophage foam cell formation *in vitro*. J Immunol 184:5827–5834.

McLaren JE, Michael DR, Salter RC, Ashlin TG, Calder CJ, Miller AM, Liew FY, Ramji DP. 2010b. IL-33 reduces macrophage foam cell formation. J Immunol 185:1222–1229.

McLaren JE, Michael DR, Ashlin TG, Ramji DP. 2011. Cytokines, macrophage lipid metabolism and foam cells: Implications for cardiovascular therapy. Prog Lipid Res 30:331–347.

Mellor H, Parker PJ. 1998. The extended protein kinase C superfamily. Biochem J 332:281–292.

Michael DR, Ashlin TG, Buckley ML, Ramji DP. 2012a. Liver X receptors, atherosclerosis and inflammation. Curr Atherocler Rep 14:284–293.

Michael DR, Salter RC, Ramji DP. 2012b. TGF-β inhibits the uptake of modified low density liprotein by human macrophages through a Smad-dependent pathway: A dominant role for Smad-2. Biochim Biophys Acta 1822:1608–1616.

Rochette-Egly C. 2003. Nuclear receptors: Integration of multiple signalling pathways through phosphorylation. Cell Signal 15:355–366.

Schmuth M, Elias PM, Hanley K, Lau P, Moser A, Wilson TM, Bikle DD, Feingold KR. 2004. The effect of LXR activators on AP-1 proteins in keratinocytes. J Invest Dermatol 23:41–48.

Shen Q, Bai Y, Chang KC, Wang Y, Burris TP, Freedman LP, Thompson CC, Nagpal S. 2011. Liver X receptor-retinoid X receptor (LXR-RXR) heterodimer cistrome reveals coordination of LXR and AP1 signaling in keratinocytes. J Biol Chem 286:14554–14563.

Singh NN, Ramji DP. 2006. Transforming growth factor-beta-induced expression of the apolipoprotein E gene requires c-Jun N-terminal kinase, p38 kinase, and casein kinase 2. Arterioscler Thromb Vasc Biol 26:1323–1329.

Torra IP, Ismaili N, Feig JE, Xu CF, Cavasotto C, Pancratov R, Rogatsky I, Neubert TA, Fisher EA, Garabedian MJ. 2008. Phosphorylation of liver X receptor alpha selectively regulates target gene expression in macrophages. Mol Cell Biol 28:2626–2636.

Toullec D, Pianetti P, Coste H, Belleverque P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, Duhamel L, Charon D, Kirilovsky J. 1991. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J Biol Chem 266:15771–15781.

Yamamoto T, Shimano H, Inoue N, Nakagawa Y, Matsuzaka T, Takahashi A, Yahagi N, Sone H, Suzuki H, Toyoshima H, Yamada N. 2007. Protein kinase A suppresses sterol regulatory element-binding protein-1C expression via phosphorylation of liver X receptor in the liver. J Biol Chem 282:11687–11695.

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