Protein kinase C α modulates liver X receptor α transactivation

Christopher J Delvecchio and John P Capone

Department of Biochemistry and Biomedical Sciences, Faculty of Science, McMaster University, 1280 Main Street West, BSB Room 101, Hamilton, Ontario, L8S 4K1 Canada

(Correspondence should be addressed to J P Capone; Email: caponej@mcmaster.ca)

Abstract

Liver X receptor α (LXR α), an oxysterol-activated nuclear hormone receptor, regulates the expression of genes involved in lipid and cholesterol homeostasis and inflammation. We show here that transactivation by LXR α in monkey kidney COS-1 (Cos-1) cells is decreased by activation of the protein kinase C (PKC) signaling pathway. In transient co-transfection assays, phorbol myristate acetate (PMA) suppressed LXR-dependent transactivation of LXR-responsive reporter genes or the natural promoter of the human ATP-binding cassette (ABC), *ABCA1* gene. The decrease in LXR transactivation after PMA treatment was also observed in human embryonic kidney (HEK) 293 and human hepatocellular carcinoma (HepG2) cells. Moreover, endogenous LXR target genes, *ABCA1* and sterol response

element-binding protein-1c, were also decreased by PMA treatment in HEK293 cells as assessed by real-time PCR. The PMA-mediated decrease of LXR activity was blocked by the PKC inhibitor bisindolylmaleimide and mimicked by constitutively active PKC α . Nuclear extracts treated with PMA show no decrease in LXR α DNA binding as assessed by mobility shift and chromatin immunoprecipitation assays. Additionally, *in vitro* kinase assays demonstrate that PKC α can phosphorylate LXR α . Our findings reveal a mode of regulation of LXR α that may be relevant to disease conditions where aberrant PKC signaling is observed, such as diabetes.

Journal of Endocrinology (2008) **197**, 121–130

Introduction

The liver X receptors (LXRs; α and β subtypes) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors and are activated by oxidized cholesterol metabolites known as oxysterols (Janowski *et al.* 1996). LXR functions as an important regulator of cholesterol homeostasis and also acts as a glucose sensor and modulator of innate immunity and inflammation (Joseph *et al.* 2003, 2004, Mitro *et al.* 2007). The LXRs function by regulating transcription of target genes by binding to LXR response elements (LXREs) as obligate heterodimers with retinoic X receptor α (RXR α ; Willy *et al.* 1995). Since their discovery, multiple LXR target genes that are involved in cholesterol and lipid transport and homeostasis have been identified. These include the ATP-binding cassette (ABC) transporters, *ABCA1*, *ABCG1*, *ABCG5* and *ABCG8*, lipoprotein and lipoprotein-remodeling enzymes such as apolipoprotein E, lipoprotein lipase, cholesterol ester transfer protein as well as lipogenic proteins such as sterol response element-binding protein (SREBP)-1c, and fatty acid synthase (Costet *et al.* 2000, Mak *et al.* 2002a,b, Ulven *et al.* 2004). Despite increasing knowledge of the physiological function and mechanisms of action of LXR, little is known about the mechanisms by which LXR itself is regulated.

The function of nuclear hormone receptors can be regulated at multiple levels, including transcriptional regulation and post-transcriptional modification such as phosphorylation.

The LXR has been shown to exist as a phosphoprotein in human embryonic kidney (HEK) 293 cells; however, the functional consequence of phosphorylation is currently unclear (Chen *et al.* 2006). Protein kinase A (PKA) can directly phosphorylate LXR α in cultured cells and has been reported to both increase and decrease transactivation depending on cell type (Tamura *et al.* 2000, 2004, Yamamoto *et al.* 2007). In primary rat hepatocytes, PKA signaling blocks LXR activation of the SREBP1c promoter (Yamamoto *et al.* 2007). However, Tamura *et al.* (2000) have also demonstrated that PKA signaling can increase LXR transactivation on a consensus LXRE-reporter construct in renal As4.1 mouse cell lines. Therefore, LXR activity appears to be differentially modulated by phosphorylation depending on promoter context and cell type. Indeed, this has been shown to be the case for the functionally related nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPAR γ). The PPAR γ transactivation is repressed by MAPK signaling in adipocytes while the opposite is true in Chinese hamster ovary cells (Hu *et al.* 1996, Juge-Aubry *et al.* 1999).

The PKC family of serine/threonine kinases is composed of at least ten members (α , β I, β II, γ , δ , ϵ , η , θ , ζ , and λ). The multiple isoforms are classified based on the requirements for activation, which depends on the presence or absence of domains that bind to calcium or the lipid diacylglycerol (DAG; Dempsey *et al.* 2000). The PKC signaling pathway is activated by a broad spectrum of extracellular stimuli that

promote lipid hydrolysis and plays a fundamental role in numerous biological facets such as differentiation, proliferation, apoptosis, and neuronal transmitter release (Newton 2003). PKC signaling affects the activity of multiple nuclear receptors including the androgen receptor (AR), glucocorticoid receptor, vitamin D receptor (VDR), PPAR, and the retinoic acid receptor (RAR; Darne *et al.* 1998, Rigas *et al.* 2003). Whereas PKC signaling increases the activity of some nuclear receptors such as AR and PPAR α , it has also been shown to repress VDR and RAR α activity (Hsieh *et al.* 1991, 1993, Delmotte *et al.* 1999).

In order to explore the potential role of PKC signaling on LXR function, we examined the activity of LXR in transient transfection assays in the presence of modulators of PKC signaling pathways. We show here that activation of PKC signaling with phorbol 12-myristate 13-acetate (PMA) repressed LXR-dependent transactivation of LXRE-reporter plasmids as determined by transient transfection assays and activation of endogenous LXR target genes as determined by real-time PCR. The effect of PMA was both dose- and time-dependent and could be mimicked by constitutively active PKC α . The inhibitory effects were abrogated upon co-incubation with the PKC inhibitor bisindolylmaleimide. Finally, PKC α was shown to phosphorylate LXR α *in vitro*. These findings reveal that PKC activation can regulate LXR-mediated gene expression and may have implications in diseases where PKC signaling is altered (Rask-Madsen & King 2005).

Materials and Methods

Reagents and plasmids

22R-hydroxycholesterol (22R-HC), 9-*cis*-retinoic acid (9-*cis*RA), PMA, and GW3965 were purchased from Sigma. Bisindolylmaleimide was purchased from Calbiochem (San Diego, CA, USA). Rabbit antibody to human RXR α was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and mouse antibody to human LXR α was purchased from R&D Systems (Minneapolis, MN, USA). The rabbit LXR α / β antibody (sc-1000) used for chromatin immunoprecipitation (ChIP) was purchased from Santa Cruz Biotechnology. Mammalian expression vectors expressing human LXR α , LXR β , PPAR α , and RXR α (pRC-CMV-LXR α , pRC-CMV-LXR β , pSG5-PPAR α , and pSG5-RXR α respectively) and luciferase reporter plasmids harboring response elements for LXR and PPAR have been described previously (Willy *et al.* 1995, Meertens *et al.* 1998, Miyata *et al.* 1998, Landis *et al.* 2002). PKC7, expressing constitutively active PKC α , was a generous gift from Dr J L Staudinger (University of Kansas, Lawrence, KS, USA). Human ABCA1 promoter (−928/+107) luciferase reporter plasmid was a generous gift from Dr A Tall (Columbia University, New York, NY, USA).

Cell lines

Cos-1, HEK293, and HepG2 cells were obtained from American Type Tissue Collection (ATCC, Manassas, VA, USA). Cos-1 and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v fetal bovine serum, 1% v/v penicillin/streptomycin, and 1% v/v L-glutamine. HepG2 cells were cultured in MEM-F15 supplemented with 10% v/v fetal bovine serum, 1% v/v sodium pyruvate, 1% v/v L-glutamine, and 1% penicillin/streptomycin.

Transfection and reporter gene assays

Transient transfections of cells were carried out using FuGene 6 reagent (Roche) according to manufacturer's instructions. Briefly, cells (3.5×10^5 cells/well in six-well plates) were transfected using 3 μ l Eugene to 0.5 μ g reporter plasmid, 0.25 μ g pRC-CMV-LXR α , 0.25 μ g pSG5-RXR, and 0.1 μ g pCMV-lacZ (encoding β -galactosidase) for normalization of transfection efficiency. The total amount of DNA was kept constant using pRC-CMV and pSG5 empty vectors. Following transfection, the media was aspirated and replaced with complete DMEM supplemented with 10% charcoal-stripped FBS and appropriate ligand as indicated in the figures. LXR agonist 22(R)-hydroxycholesterol was dissolved in 95% ethanol, and 9-*cis*RA and GW3965 were dissolved in Me₂SO (DMSO). Control cells received the equivalent amount of vehicle. Cells were harvested for 24 or 48 h post-transfection as indicated and lysed in reporter lysis buffer (Promega), and luciferase, β -galactosidase and Bradford assays were carried out as described previously (Landis *et al.* 2002).

Electrophoretic mobility shift assays

The Cos-1 cells were transfected with 0.5 μ g LXR α and/or RXR α expression plasmids and incubated overnight, as described previously. The cells were then treated with PMA for an additional 24 h and Cos-1 nuclear extracts were prepared, as described previously (Andrews & Faller 1991). Ten micrograms of nuclear extract were used in gel retardation assays, as previously described (Landis *et al.* 2002). Briefly, double-stranded oligonucleotide probes (sequence 5'-GATCCCAGGTCACAGGAGTCAGAA-3'; 5'-GATCTTCTGACCTCCTGTGACCTGG-3') were annealed and radiolabeled with [³²P] ATP using Klenow enzyme. Binding reactions consisted of 5 μ l Buffer C (20 mM HEPES (pH 7.9); 25% Glycerol; 0.42 M NaCl; 0.2 mM EDTA (pH 8.0); 1.5 mM MgCl₂; 0.5 mM dithiothreitol), 1 μ l BSA (4 mg/ml), 1 μ l PolydI/dC (8 mg/ml), 1 μ l salmon sperm DNA (4 mg/ml), 4 μ l radiolabeled oligo (20 pmol/reaction), 10 μ g nuclear extract, and H₂O to 30 μ l total volume. Reactions were incubated at 30 °C for 1 h and stopped by the addition of 2 μ l loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol). Samples were separated on a 4% polyacrylamide

gel (pre-run for 2 h) in 0.25× Tris-borate-EDTA (TBE) running buffer at 240 V at 4 °C. The gel was dried and probes were detected by autoradiography.

Chromatin immunoprecipitation assay

ChIP assays were performed in HEK293 cells using the ChIP-IT Express Enzymatic Kit according to manufacturer's instructions (Active Motif, Carlsbad, CA, USA). Briefly, HEK293 cells were grown to 85% confluency in 10 cm dishes and treated with GW3965 (2 μ M) and/or PMA (80 nM) for 2 h. Cells were fixed with 1% formaldehyde for 10 min at room temperature. Nuclei were isolated and chromatin was digested enzymatically for 10 min at 37 °C resulting in chromatin fragments of ~200–1500 bps. LXR α / β was immunoprecipitated overnight at 4 °C with 3 μ g antibody and ~8 μ g chromatin, and rabbit pre-immune immunoglobulin served as a negative control. Bound chromatin was washed and the recovered DNA was assayed for enrichment of the human ABCA1 promoter by PCR using the following primers: forward 5'-CCC AAC TCC CTA GAT GTG TC-3'; reverse 5'-CCA CTC ACT CTC GCT CGC A-3'. The PCR contained 5 μ l eluted chromatin, 2.5 μ l PCR buffer, 10 pmol/ μ l primers (forward and reverse), 1 U/reaction platinum *Taq* polymerase (Invitrogen) and H₂O to 25 μ l. The PCR program consisted of 34 cycles of 94 °C for 20 s, 58 °C for 30 s, and 72 °C for 30 s. Reactions were separated on a 1.5% agarose gel and stained with SYBR-green. Gels were imaged on a Typhoon 9200 Variable Mode Imager (Molecular Dynamics, Amersham Biosciences, Baie D'urfee, QC, Canada).

In vitro kinase assay

In vitro kinase assays were performed by mixing 0.4 μ g purified LXR α (ProteinOne, Bethesda, MD, USA) in 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 0.2 mM CaCl₂, 5 μ g/ml phosphatidylserine, 0.5 μ g/ml diolein, 5 μ M ATP, and 5 μ Ci/ μ l [³²P]ATP along with 50 ng purified PKC α (Calbiochem) in a final reaction volume of 20 μ l. The reaction was incubated at 30 °C for 30 min and products were analyzed by sodium dodecyl sulfate PAGE and detected by autoradiography.

Western blot analysis

Western blot analysis was carried out with commercially available kits (Amersham) according to manufacturer's instructions. Briefly, 25 μ g protein isolated from the Cos-1 cells were subjected to PAGE and transferred to a nitrocellulose membrane. Blots were incubated with mouse anti-LXR α (1:1000) or rabbit anti-RXR α (1:200) antibodies for 1 h, followed by 2° goat HRP-conjugated antibody (1:5000) for an additional hour and visualized by enhanced chemiluminescence. Anti- β -actin was used as a loading control.

Real-time PCR

Total RNA was isolated using RNeasy mini kits (Qiagen), and cDNA was prepared from 1 μ g RNA using the quantitect reverse transcription kit (Qiagen) according to the manufacturer's instructions. The real-time PCR was performed using platinum SYBR green supermix-UDG with ROX PCR mix (Invitrogen) according to manufacturer's instructions and as described previously (Bookout & Mangelsdorf 2003). Briefly, 5 μ l SYBR-green Supermix, 2.5 μ l H₂O, 1.25 μ l primer sets (1.25 μ M each forward and reverse primer; specific for human ABCA1, ABCG1, and SREBP1c respectively), and 1.25 μ l cDNA was mixed with a final reaction volume of 10 μ l. The PCR amplification was carried out in 384-well plates in an Applied Biosystems 7900HT real-time PCR machine (Applied Biosystems, Foster City, CA, USA). Values were normalized to β -actin levels and relative abundance of transcripts was calculated using the $\Delta\Delta C_t$ method, as described previously (Bookout & Mangelsdorf 2003).

Statistical analysis

Unpaired *t*-tests were used for comparison of groups. *P* values <0.05 were considered significant. Samples were compared with the corresponding samples treated without PMA.

Results

PMA treatment downregulates LXR α transactivation

To determine whether PKC signaling alters LXR α transactivation, we carried out transient transfection assays with LXR-responsive luciferase reporter genes. As expected, cells co-transfected with expression plasmids for human LXR α and RXR α showed a 12- and 20-fold increase in luciferase activity over basal activity of the reporter gene alone when treated in the absence or presence of the LXR agonist GW3965 respectively (Fig. 1A). Addition of PMA, a PKC activator that mimics DAG in the cellular membrane, inhibited the ligand-independent effect by ~30–50% and completely eliminated the ligand-dependent effect (Fig. 1A). The PMA-mediated decrease in transactivation was not due to increased degradation or decreased expression of LXR α or RXR α as determined by western blot analysis (Fig. 1A). The repressive effect of PMA was blocked by co-treatment with bisindolylmaleimide, a PKC inhibitor (Fig. 1B). Similar effects on LXR transactivation were observed, when 22R-HC was used in place of GW3965 (Fig. 1C). Furthermore, the inactive enantiomer of PMA, 4 α -PMA, did not decrease LXR-mediated transactivation (Fig. 1C).

The PMA-mediated repressive effects on the LXR activity were also observed on a natural LXR target promoter, the human ABCA1 promoter. As shown in Fig. 1D, the PMA treatment inhibited 22R-HC and

Figure 1 PMA modulates LXR transactivation. Cos-1 cells were transfected with luciferase reporter genes containing a consensus LXRE (panels A–C) or (D) an ABCA1 promoter, in the presence or absence of expression vectors for human LXRα and RXRα and treated with GW3965 (1 μM), 22R-hydroxycholesterol (22R-HC; 10 μM), 9-*cis*-retinoic acid (9-*cis*RA; 10 μM), Bisindolylmaleimide (Bis; 50 nM), PMA (1 nM) or 4α-PMA (PMA4α; 1 nM) for 48 h as indicated, and luciferase activity was measured. In panel A, total protein extracts (25 μg) from the corresponding transfections were analyzed by western blot with antibodies specific for human LXRα and RXRα. β-actin levels served as a loading control. (E) Cos-1 cells were transfected with a PPRE-luciferase construct in the presence or absence of expression vectors for human PPARα and RXRα in the presence of WY14,643 (WY; 5 μM) and PMA (100 nM) as indicated, and luciferase activity was measured. (F) Cos-1 cells were transfected with an ABCA1 promoter reporter vector in the presence or absence of expression vectors for human LXRβ and RXRα and treated with GW3965 (1 μM) and/or PMA (1 nM) for 48 h as indicated, and luciferase activity was measured. The values presented represent the average (±s.d.) relative to untreated cells (taken as 1) from three independent transfections carried out in triplicate and normalized for β-galactosidase and protein levels. **P*<0.02 versus corresponding sample minus PMA indicated by †; ***P*<0.02 versus corresponding sample minus PMA indicated by #; In panel B, not significant (N.S.) difference versus sample treated with GW3965 alone indicated by #. In panel C, not significant (N.S.) difference versus corresponding samples minus 4α-PMA indicated by † and #.

9-cisRA mediated transactivation of an ABCA1-linked reporter gene by 50–70%.

We next tested the effects of PMA on PPAR α transactivation to determine whether PKC activation resulted in a general decrease in reporter activity in Cos-1 cells. In contrast to LXR α , transactivation by the related nuclear receptor PPAR α is increased under similar conditions. As shown in Fig. 1E, PPAR α -mediated induction of a PPRE-reporter gene in the presence of the PPAR ligand WY14-643 was increased by co-treatment with PMA in concordance with previous findings (Gray *et al.* 2005).

Finally, we tested the effects of PKC signaling on LXR β -mediated transactivation of the ABCA1 luciferase reporter construct. LXR β activity was also decreased by PMA treatment similar to LXR α (Fig. 1F). Our results indicate that under our experimental conditions, PKC activation represses LXR activity in contrast to the related nuclear hormone receptor PPAR α .

To determine whether the repressive effects of PMA on LXR activity was a general phenomenon of LXR α or specific to Cos-1 cells, we performed similar transfection experiments in HEK293T and HepG2 cells. PMA treatment decreased LXR α transactivation in HEK293T (Fig. 2A) as well as HepG2 cell lines (Fig. 2B).

The repressive effects of PMA on LXR activity was dose-dependent and saturated at 1–2 nM, a concentration that is within the physiological range that activates PKC (Fig. 3A and B). This PMA dose-response profile was similar when LXR agonist 22R-HC was used in place of GW3965 (not shown). Extended treatment of PMA (>24 h) can cause depletion of PKC and, therefore, the response to PMA may not involve signaling via PKC but rather a lack of PKC kinase activity (Ohno *et al.* 1990). To explore this, we examined the time course of PMA-mediated inhibition. As shown in Fig. 3B, the inhibitory effects on LXR activity were observed between 3 and 4 h following PMA addition, implying that PKC depletion is likely not the cause of the decrease in LXR function (Fig. 3B). No decrease in protein expression of LXR or RXR was observed throughout the time trial (Fig. 3C), as determined by western blot analysis.

Constitutively active PKC α mimics the effects of PMA in Cos-1 cells

To further confirm the role of PKC signaling in LXR function, we carried out transfection experiments with a constitutively active PKC α expression vector. Constitutively active PKC α decreased ligand-dependent and ligand-independent LXR transactivation with the LXRE-reporter plasmid (Fig. 4A) and with the ABCA1-luciferase reporter construct (not shown). This finding further confirms that PMA does not act by depleting PKC α . In contrast to the effects observed with LXR, the expression of constitutively active PKC α increased PPAR α ligand-independent transactivation but had minimal effect on the ligand-dependent activation (Fig. 4B).

Figure 2 PMA decreases LXR α transactivation in HEK293T cells and HepG2 cell lines. (A) HEK293T cells or (B) HepG2 cells were transfected with ABCA1-luciferase plasmid in the presence or absence of expression vectors for human LXR α and RXR α and treated as indicated. Following transfection, cells were treated with 22R-hydroxycholesterol (22R-HC; 10 μ M), 9-*cis*-retinoic acid (9-*cis*RA; 10 μ M) or GW3965 (2 μ M) and PMA (1 nM for HEK293 cells; 80 nM for HepG2 cells) for 48 h as indicated and luciferase activity was measured. The values presented represent the average ($\pm$ S.D.) relative to untreated cells (taken as 1) from three independent transfections carried out in triplicate and normalized to β -galactosidase and protein levels. * P <0.02 versus corresponding sample minus PMA indicated by †; ** P <0.02 versus corresponding sample minus PMA indicated by #.

PMA treatment does not decrease LXR α /RXR α DNA binding

PKC-mediated phosphorylation of the VDR causes decreased DNA binding (Hsieh *et al.* 1993) to target sites. To determine whether this is also the case for LXR, Cos-1 cells were transfected with LXR α and RXR α expression plasmids and treated with PMA. Nuclear extracts were prepared and used in gel retardation assays with a radiolabeled LXRE probe. As shown in Fig. 5A, nuclear extracts prepared from cells transfected with LXR α and RXR α treated in the presence or absence of PMA formed a protein/DNA complex of similar intensity, and with migration similar to that formed with *in vitro*-synthesized LXR/RXR translated proteins. We also performed ChIP analysis of endogenous LXR protein bound to the ABCA1 promoter in HEK293 cells. As shown in Fig. 5B, HEK293 cells treated with

PMA+GW3965 show similar enrichment of the ABCA1 promoter following LXR immunoprecipitation when compared with GW3965 alone. These findings indicate that PMA-treated cells do not decrease the binding of LXR/RXR heterodimers to DNA.

PKCα phosphorylates LXRα in vitro

To determine whether PKC could indeed phosphorylate LXR, purified LXRα was added to an *in vitro* kinase assay with PKCα. As shown in Fig. 6A, PKCα was able to phosphorylate LXRα *in vitro* indicating a possible direct modulation of LXR activity *in vivo*.

LXR transactivation of endogenous target genes is repressed by PKC signaling

The above experiments were carried out using transient transfection assays. To determine whether PKC activation downregulates endogenous LXR target genes, we treated HEK293 cells with LXR agonist ± PMA for 24 h and analyzed expression of bona fide LXR target genes by real-time PCR. As shown in Fig. 7A and B, PMA treatment repressed ligand-induced expression of endogenous LXR target genes ABCA1 and SREBP1c. The repressive effects of PMA were blocked by the PKC inhibitor bisindolylmaleimide (Fig. 7A and B). The results indicate that PKC signaling can downregulate LXR activation of endogenous target genes.

Discussion

Post-translational modification of nuclear hormone receptors provides the cell with important modes of regulation and allows for rapid regulation of receptor function in response to intra- and extracellular stimuli. In particular, phosphorylation of nuclear receptors has been shown to affect their sub-cellular localization, stability, ability to bind DNA, affinity for co-activator or co-repressor proteins, and/or alter their

Figure 3 PMA exhibits a dose- and time-dependent effect on LXR transactivation. Cos-1 cells were transfected with (A) an LXRE-luciferase reporter gene or (B) an ABCA1-luciferase reporter gene and human LXRα and RXRα expression plasmids in the presence of GW3965 (1 μM) and increasing levels of PMA (0.25–2 nM) as indicated. Cells were lysed 24 h post-transfection and luciferase activity was measured. (C) Cos-1 cells were transfected with an LXRE-luciferase reporter gene along with expression plasmids for human LXRα and RXRα. GW3965 (1 μM) or both GW3965 and PMA (1 nM) were added to the cells 24 h post-transfection and luciferase activity was measured at the indicated times. The values presented represent the average (±s.d.) relative to untreated cells (taken as 1) from three independent transfections carried out in triplicate and normalized for β-galactosidase and protein levels. (D) Extracts (25 μg) from samples in (C) were analyzed by western blot with antibodies specific for LXRα and RXRα. Antibodies to β-actin served as a loading control. **P*<0.05; ***P*<0.05 versus corresponding sample minus PMA indicated by #.

affinity for ligands (Tahayato *et al.* 1993, Diradourian *et al.* 2005, Xu & Koenig 2005, Khan *et al.* 2006).

The findings reported here show that PKC activation, as shown through activation of endogenous PKC isoforms by PMA as well as by over-expression of constitutively active PKC α and the use of specific PKC inhibitors, downregulates the activity of human LXR α in transient transfection assays. The effect was observed on a consensus LXRE-luciferase construct as well as a luciferase construct harboring the human ABCA1 promoter region that contains the reported LXRE in multiple cell types (Costet *et al.* 2000). Moreover, in contrast to LXR, experiments done under similar conditions with PPAR α and PPRE-reporter plasmids indicate that PKC

signaling does not decrease PPAR α activity, a result reported by others as well (Gray *et al.* 2005).

The mechanisms by which the PKC signaling pathway alters LXR function *in vivo* remains to be determined.

Figure 4 Constitutively active PKC α mimics the effects of PMA. (A) Cos-1 cells were transfected with an LXRE-luciferase reporter plasmid and expression vectors for human LXR α and RXR α and constitutively active PKC α as indicated, in the presence of GW3965 (1 μ M). Cells were lysed 24 h post-transfection and luciferase activity was measured. (B) Cos-1 cells were transfected with a PPRE-luciferase plasmid and expression vectors for PPAR α , RXR α and constitutively active PKC α in the presence of Wy14,643 (WY; 100 μ M) as indicated, and luciferase activity was measured as above. The values presented represent the average ($\pm$ S.D.) relative to untreated cells (taken as 1) from three independent transfections carried out in triplicate and normalized for β -galactosidase and protein levels. * P <0.02 versus corresponding sample minus PKC α indicated by $\dagger$; ** P <0.02 versus corresponding sample minus PKC α indicated by $\#$.

Figure 5 PMA does not block LXR/RXR heterodimer DNA binding. (A) Gel retardation analysis. Cos-1 cells were transfected with expression plasmids for human LXR α and RXR α and treated with PMA (1 nM) for an additional 24 h. Nuclear extracts (NE) were prepared and 10 μ g were incubated with a P^{32} labeled LXRE DNA probe. Complexes were resolved by native PAGE and detected by autoradiography. Arrow indicates the LXR/RXR protein DNA complex. *In vitro* translated (i.v.t.) LXR α and RXR α served as a positive control. (B) Chromatin immunoprecipitation (ChIP) analysis of LXR α / β binding to the ABCA1 promoter following PMA treatment. HEK293 cells were treated with GW3965 (2 μ M) and/or PMA (80 nM) for 2 h prior to ChIP analysis, as described in Materials and Methods. Rabbit pre-immune immunoglobulin G (IgG) or antibodies specific for LXR α / β were used to immunoprecipitate the ABCA1 promoter region. Precipitated DNA was used as template in PCR with primers specific for the human ABCA1 promoter region containing the putative LXRE. SYBR-green-stained bands representing ABCA1 are shown after 36 cycles. Lanes marked 'input' represent DNA isolated prior to immunoprecipitation.

Figure 6 PKC α phosphorylates LXR α *in vitro*. Recombinant PKC α and purified LXR α were incubated alone or in combination in PKC buffer for 30 min as described in experimental procedures in the presence of P³²-labeled ATP. Proteins were separated by SDS-PAGE and visualized by autoradiography.

We demonstrate here that PKC α can phosphorylate LXR α *in vitro*; however, it is not yet clear whether LXR is directly phosphorylated by PKC *in vivo* or whether this in fact correlates with the observed attenuation of receptor function.

Chen *et al.* (2006), using over-expression studies with FLAG-tagged LXR, have recently shown that LXR is constitutively phosphorylated in HEK293 cells and further demonstrated that S198, which is part of a consensus mitogen-activated protein kinase phosphorylation sequence, is the major phosphorylated residue. However, the physiological consequence of this modification is not apparent. Mutation of S198 did not affect the ability of LXR to bind to DNA, its ability to activate target genes, its sub-cellular distribution, nor its response to ligand (Chen *et al.* 2006).

Similarly, while PKA signaling in liver cells has been reported to decrease LXR/RXR heterodimer binding to DNA (Yamamoto *et al.* 2007), we did not observe any changes in LXR/RXR DNA complex formation using nuclear extracts or ChIP analysis from cells stimulated with the PKC activator PMA. Therefore, a potential conformational modification of LXR/RXR heterodimers induced by PKC signaling may alter LXR function. LXR is known to undergo 'heterodimerization-induced activation' when heterodimerized to RXR, a mechanism not reported for other nuclear receptors (Wiebel & Gustafsson 1997, Wiebel *et al.* 1999, Son *et al.* 2007). This response is dependent on the activation domain-2 (AF-2) of LXR and is the result of a conformational change in LXR induced allosterically by RXR (Wiebel & Gustafsson 1997). Indeed, we observe high basal activity when LXR α and RXR α are co-expressed in Cos-1 cells in the absence of ligand (see Fig. 1A). Our findings show that PKC signaling reduces both basal and ligand-induced activation on both a consensus LXRE-reporter plasmid as well as the natural ABCA1-reporter construct, while maintaining the ability of LXR/RXR heterodimers to bind DNA (see Fig. 5). Therefore, PKC activation could potentially prevent the allosteric modulation of LXR induced by RXR or alternatively, mask the transcriptionally active structure via post-translational modification. At present, it cannot be ruled out that RXR itself is post-translationally modified thereby blocking its ability to

Figure 7 PMA downregulates LXR target genes in HEK293 cells. HEK293 cells were treated with GW3965 (2 μ M), PMA (80 nM), and/or bisindolylmaleimide (Bis; 50 nM) as indicated for 24 h and RNA was isolated. One microgram RNA was reverse transcribed and levels of (A) ABCA1 and (B) SREBP1c were determined by real-time PCR, as described in the material and methods. Relative expression was normalized to β -actin levels. Values represent the average of triplicates from two independent experiments $\pm$ s.d. * P < 0.01 versus corresponding sample minus PMA indicated by †; ** P < 0.01 versus corresponding sample minus PMA indicated by #.

allosterically modulate LXR. Ongoing studies to determine the phosphorylation status of LXR and RXR *in vivo*, specific residues phosphorylated by PKC and domains responsible for activation/repression in response to PKC may potentially uncover a direct link between PKC, LXR and RXR phosphorylation and function.

Alternatively, it is also possible that the PKC pathway modulates LXR activity through indirect mechanisms in response to extracellular cues. For instance, it is possible that PKC signaling modulates the recruitment, function, and/or affinity of co-repressor/co-activator complexes to LXR. Altered co-factor recruitment has been reported for the related nuclear receptor PXR in response to PKC activation (Ding & Staudinger 2005), and with LXR in liver cells through PKA activation (Yamamoto *et al.* 2007). At present, it cannot be ruled out that co-activator or co-repressor molecules are themselves post-translationally modified by PKC, thereby blocking LXR-mediated transactivation of

reporter genes (Rochette-Egly 2003). Indeed, co-activators specifically modulating the function of LXR have been identified such as activating signal cointegrator-2 (Lee *et al.* 2001). More recent findings have demonstrated that glucose signaling alters the sub-cellular distribution of LXR, although there is no evidence that this is phosphorylation dependent (Helleboid-Chapman *et al.* 2006).

Tranheim Kase *et al.* (2006) have also demonstrated that the synthetic LXR agonist T0901317 can induce DAG formation in cultured myoblasts. However, it has yet to be tested whether GW3965 induces similar changes in DAG levels and, furthermore, physiological LXR ligands such as 22R-HC did not alter DAG levels. In the studies presented here, we show that PKC activation has similar effects on LXR α transactivation in the presence of GW3965 or 22R-HC, and thus the physiological relevance of DAG formation by T0901317 remains to be determined.

Our findings also show that attenuation of LXR activity by PKC occurs in multiple cells types. In addition to HepG2 cells, PMA decreased LXR α transactivation in Cos-1 cells and HEK293T cells, two kidney-derived cell lines. This points to a possible role for PKC modulation of LXR activity in the kidney. Indeed, LXR plays an important role in regulating cholesterol efflux in the kidney as well as controlling the expression of renin in juxtaglomerular cells (Wu *et al.* 2004, Morello *et al.* 2005, Wang *et al.* 2005, Zhang *et al.* 2005). Interestingly, renin promoter activity is increased by cAMP/PKA signaling, a response dependent on LXR α expression. Consistent with this, we also observed an increase in LXR α activity when Cos-1 cells are treated with 8Br-cAMP, which activates the PKA pathway, on an LXRE-luciferase construct (not shown). Other reports indicate that angiotensin II, a product of renin-induced cleavage in the blood plasma, decreases renin expression via a PKC-dependent pathway (Muller *et al.* 2002). A possible negative feedback loop may therefore exist to regulate blood pressure by modulating LXR α activity. Indeed, LXR has recently been reported to play a role in regulating blood pressure in male Sprague–Dawley rats by modulating the angiotensin-II receptor gene in vasculature (Leik *et al.* 2007).

In summary, we demonstrate that PKC signaling can attenuate LXR α transactivation in Cos-1, HEK293T, and HepG2 cells. The findings reveal a potentially important mechanism of regulation of LXR that warrants further study as abnormal PKC signaling has been observed in diabetes, atherosclerosis, renin expression and glucose metabolism in the liver, all conditions in which LXR is known to play an important role (Collins 2004, Grefhorst *et al.* 2005, Aiello *et al.* 2006, Dey *et al.* 2006).

Acknowledgements

This work was supported by grants from the Heart and Stroke Foundation of Ontario (J P C). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Aiello LP, Clermont A, Arora V, Davis MD, Sheetz MJ & Bursell SE 2006 Inhibition of PKC beta by oral administration of ruboxistaurin is well tolerated and ameliorates diabetes-induced retinal hemodynamic abnormalities in patients. *Investigative Ophthalmology and Visual Science* **47** 86–92.
- Andrews NC & Faller DV 1991 A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Research* **19** 2499.
- Bookout AL & Mangelsdorf DJ 2003 Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. *Nuclear Receptor Signaling* **1** e012.
- Chen M, Bradley MN, Beaven SW & Tontonoz P 2006 Phosphorylation of the liver X receptors. *FEBS Letters* **580** 4835–4841.
- Collins JL 2004 Therapeutic opportunities for liver X receptor modulators. *Current Opinion in Drug Discovery and Development* **7** 692–702.
- Costet P, Luo Y, Wang N & Tall AR 2000 Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. *Journal of Biological Chemistry* **275** 28240–28245.
- Darne C, Veyssiere G & Jean C 1998 Phorbol ester causes ligand-independent activation of the androgen receptor. *European Journal of Biochemistry* **256** 541–549.
- Delmotte MH, Tahayato A, Formstecher P & Lefebvre P 1999 Serine 157, a retinoic acid receptor alpha residue phosphorylated by protein kinase C *in vitro*, is involved in RXR.RARalpha heterodimerization and transcriptional activity. *Journal of Biological Chemistry* **274** 38225–38231.
- Dempsey EC, Newton AC, Mochly-Rosen D, Fields AP, Reyland ME, Insel PA & Messing RO 2000 Protein kinase C isozymes and the regulation of diverse cell responses. *American Journal of Physiology. Lung Cellular and Molecular Physiology* **279** L429–L438.
- Dey D, Basu D, Roy SS, Bandyopadhyay A & Bhattacharya S 2006 Involvement of novel PKC isoforms in FFA induced defects in insulin signaling. *Molecular and Cellular Endocrinology* **246** 60–64.
- Ding X & Staudinger JL 2005 Repression of PXR-mediated induction of hepatic CYP3A gene expression by protein kinase C. *Biochemical Pharmacology* **69** 867–873.
- Diradourian C, Girard J & Pegorier JP 2005 Phosphorylation of PPARs: from molecular characterization to physiological relevance. *Biochimie* **87** 33–38.
- 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.
- Grefhorst A, van Dijk TH, Hammer A, van der Sluijs FH, Havinga R, Havekes LM, Romijn JA, Groot PH, Reijngoud DJ & Kuipers F 2005 Differential effects of pharmacological liver X receptor activation on hepatic and peripheral insulin sensitivity in lean and ob/ob mice. *American Journal of Physiology. Endocrinology and Metabolism* **289** E829–E838.
- Helleboid-Chapman A, Helleboid S, Jakel H, Timmerman C, Sergheraert C, Pattou F, Fruchart-Najib J & Fruchart JC 2006 Glucose regulates LXRalpha subcellular localization and function in rat pancreatic beta-cells. *Cell Research* **16** 661–670.
- Hsieh JC, Jurutka PW, Galligan MA, Terpening CM, Haussler CA, Samuels DS, Shimizu Y, Shimizu N & Haussler MR 1991 Human vitamin D receptor is selectively phosphorylated by protein kinase C on serine 51, a residue crucial to its trans-activation function. *PNAS* **88** 9315–9319.
- Hsieh JC, Jurutka PW, Nakajima S, Galligan MA, Haussler CA, Shimizu Y, Shimizu N, Whitfield GK & Haussler MR 1993 Phosphorylation of the human vitamin D receptor by protein kinase C. Biochemical and functional evaluation of the serine 51 recognition site. *Journal of Biological Chemistry* **268** 15118–15126.
- Hu E, Kim JB, Sarraf P & Spiegelman BM 1996 Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma. *Science* **274** 2100–2103.
- Janowski BA, Willy PJ, Devi TR, Falck JR & Mangelsdorf DJ 1996 An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature* **383** 728–731.

- Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ & Tontonoz P 2003 Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nature Medicine* **9** 213–219.
- Joseph SB, Bradley MN, Castrillo A, Bruhn KW, Mak PA, Pei L, Hogenesch J, O'Connell RM, Cheng G, Saez E *et al.* 2004 LXR-dependent gene expression is important for macrophage survival and the innate immune response. *Cell* **119** 299–309.
- Juge-Aubry CE, Hammar E, Siegrist-Kaiser C, Pernin A, Takeshita A, Chin WW, Burger AG & Meier CA 1999 Regulation of the transcriptional activity of the peroxisome proliferator-activated receptor alpha by phosphorylation of a ligand-independent trans-activating domain. *Journal of Biological Chemistry* **274** 10505–10510.
- Khan SA, Park SW, Huq MD & Wei LN 2006 Ligand-independent orphan receptor TR2 activation by phosphorylation at the DNA-binding domain. *Proteomics* **6** 123–130.
- Landis MS, Patel HV & Capone JP 2002 Oxysterol activators of liver X receptor and 9-*cis*-retinoic acid promote sequential steps in the synthesis and secretion of tumor necrosis factor-alpha from human monocytes. *Journal of Biological Chemistry* **277** 4713–4721.
- Lee SK, Jung SY, Kim YS, Na SY, Lee YC & Lee JW 2001 Two distinct nuclear receptor-interaction domains and CREB-binding protein-dependent transactivation function of activating signal cointegrator-2. *Molecular Endocrinology* **15** 241–254.
- Leik CE, Carson NL, Hennan JK, Basso MD, Liu QY, Crandall DL & Nambi P 2007 GW3965, a synthetic liver X receptor (LXR) agonist, reduces angiotensin II-mediated pressor responses in Sprague–Dawley rats. *British Journal of Pharmacology* **151** 450–456.
- Mak PA, Kast-Woelbern HR, Anisfeld AM & Edwards PA 2002a Identification of PLTP as an LXR target gene and apoE as an FXR target gene reveals overlapping targets for the two nuclear receptors. *Journal of Lipid Research* **43** 2037–2041.
- Mak PA, Laffitte BA, Desrumaux C, Joseph SB, Curtiss LK, Mangelsdorf DJ, Tontonoz P & Edwards PA 2002b Regulated expression of the apolipoprotein E/C-I/C-IV/C-II gene cluster in murine and human macrophages. A critical role for nuclear liver X receptors alpha and beta. *Journal of Biological Chemistry* **277** 31900–31908.
- Meertens LM, Miyata KS, Cechetto JD, Rachubinski RA & Capone JP 1998 A mitochondrial ketogenic enzyme regulates its gene expression by association with the nuclear hormone receptor PPARalpha. *EMBO Journal* **17** 6972–6978.
- Mitro N, Mak PA, Vargas L, Godio C, Hampton E, Molteni V, Kreusch A & Saez E 2007 The nuclear receptor LXR is a glucose sensor. *Nature* **445** 219–223.
- Miyata KS, McCaw SE, Meertens LM, Patel HV, Rachubinski RA & Capone JP 1998 Receptor-interacting protein 140 interacts with and inhibits transactivation by, peroxisome proliferator-activated receptor alpha and liver-X-receptor alpha. *Molecular and Cellular Endocrinology* **146** 69–76.
- Morello F, de Boer RA, Steffensen KR, Gnecci M, Chisholm JW, Boomsma F, Anderson LM, Lawn RM, Gustafsson JK, Lopez-Illasaca M *et al.* 2005 Liver X receptors alpha and beta regulate renin expression *in vivo*. *Journal of Clinical Investigation* **115** 1913–1922.
- Muller MW, Todorov V, Kramer BK & Kurtz A 2002 Angiotensin II inhibits renin gene transcription via the protein kinase C pathway. *Pflugers Archiv* **444** 499–505.
- Newton AC 2003 Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. *Biochemical Journal* **370** 361–371.
- Ohno S, Konno Y, Akita Y, Yano A & Suzuki K 1990 A point mutation at the putative ATP-binding site of protein kinase C alpha abolishes the kinase activity and renders it down-regulation-insensitive. A molecular link between autophosphorylation and down-regulation. *Journal of Biological Chemistry* **265** 6296–6300.
- Rask-Madsen C & King GL 2005 Proatherosclerotic mechanisms involving protein kinase C in diabetes and insulin resistance. *Arteriosclerosis, Thrombosis, and Vascular Biology* **25** 487–496.
- Rigas AC, Ozanne DM, Neal DE & Robson CN 2003 The scaffolding protein RACK1 interacts with androgen receptor and promotes cross-talk through a protein kinase C signaling pathway. *Journal of Biological Chemistry* **278** 46087–46093.
- Rochette-Egly C 2003 Nuclear receptors: integration of multiple signalling pathways through phosphorylation. *Cellular Signalling* **15** 355–366.
- Son YL, Park OG, Kim GS, Lee JW & Lee YC 2007 RXR heterodimerization allosterically activates LXR binding to the second NR box of activating signal cointegrator-2. *Biochemical Journal* (In Press).
- Tahayato A, Lefebvre P, Formstecher P & Dautrevaux M 1993 A protein kinase C-dependent activity modulates retinoic acid-induced transcription. *Molecular Endocrinology* **7** 1642–1653.
- Tamura K, Chen YE, Horiuchi M, Chen Q, Daviet L, Yang Z, Lopez-Illasaca M, Mu H, Pratt RE & Dzau VJ 2000 LXRalpha functions as a cAMP-responsive transcriptional regulator of gene expression. *PNAS* **97** 8513–8518.
- Tamura K, Chen YE, Tanaka Y, Sakai M, Tsurumi Y, Koide Y, Kihara M, Pratt RE, Horiuchi M, Umemura S *et al.* 2004 Nuclear receptor LXRalpha is involved in cAMP-mediated human renin gene expression. *Molecular and Cellular Endocrinology* **224** 11–20.
- Tranheim Kase E, Andersen B, Nebb HI, Rustan AC & Thoresen GH 2006 22-Hydroxycholesterols regulate lipid metabolism differently than T0901317 in human myotubes. *Biochimica et Biophysica Acta* **1761** 1515–1522.
- Ulven SM, Dalen KT, Gustafsson JA & Nebb HI 2004 Tissue-specific autoregulation of the LXRalpha gene facilitates induction of apoE in mouse adipose tissue. *Journal of Lipid Research* **45** 2052–2062.
- Wang Y, Moser AH, Shigenaga JK, Grunfeld C & Feingold KR 2005 Downregulation of liver X receptor-alpha in mouse kidney and HK-2 proximal tubular cells by LPS and cytokines. *Journal of Lipid Research* **46** 2377–2387.
- Wiel FF & Gustafsson JA 1997 Heterodimeric interaction between retinoid X receptor alpha and orphan nuclear receptor OR1 reveals dimerization-induced activation as a novel mechanism of nuclear receptor activation. *Molecular and Cellular Biology* **17** 3977–3986.
- Wiel FF, Steffensen KR, Treuter E, Feltkamp D & Gustafsson JA 1999 Ligand-independent coregulator recruitment by the triply activatable OR1/retinoid X receptor-alpha nuclear receptor heterodimer. *Molecular Endocrinology* **13** 1105–1118.
- Willy PJ, Umesono K, Ong ES, Evans RM, Heyman RA & Mangelsdorf DJ 1995 LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes and Development* **9** 1033–1045.
- Wu J, Zhang Y, Wang N, Davis L, Yang G, Wang X, Zhu Y, Breyer MD & Guan Y 2004 Liver X receptor-alpha mediates cholesterol efflux in glomerular mesangial cells. *American Journal of Physiology. Renal Physiology* **287** F886–F895.
- Xu B & Koenig RJ 2005 Regulation of thyroid hormone receptor alpha2 RNA binding and subcellular localization by phosphorylation. *Molecular and Cellular Endocrinology* **245** 147–157.
- Yamamoto T, Shimano H, Inoue N, Nakagawa Y, Matsuzaka T, Takahashi A, Yahagi N, Sone H, Suzuki H, Toyoshima H *et al.* 2007 Protein kinase A suppresses sterol regulatory element-binding protein-1C expression via phosphorylation of Liver X receptor in the liver. *Journal of Biological Chemistry* **282** 11687–11695.
- Zhang Y, Zhang X, Chen L, Wu J, Lu WJ, Hwang MT, Yang G, Li S, Wei M, Davis L *et al.* 2005 Liver X receptor (LXR) agonist T0901317 upregulates SCD1 expression in renal proximal straight tubule. *American Journal of Physiology. Renal Physiology* **290** F1065–F1073.

Received in final form 20 December 2007

Accepted 7 January 2008

Made available online as an Accepted Preprint
7 January 2008