Inhibition of lipopolysaccharide-induced gene expression by liver X receptor ligands in macrophages involves interference with early growth response factor 1

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Abstract

Liver X receptors (LXRs) are nuclear receptors that act as ligand-dependent transcription factors forming permissive heterodimers with retinoid X receptors (RXRs). In this study we aimed to assess the effect of LXR/RXR activation on the transcriptional induction of pro-inflammatory genes including cyclooxygenase-2 (COX-2) and microsomal prostaglandin E2 synthase-1 (mPGES-1) in activated macrophages. Our study shows that LXR ligands such as oxysterols, GW3965 or TO901317, as well as RXR ligands like 9cis retinoic acid or SR11237, decreased LPS-induced expression of COX-2 and mPGES-1. Consequently, LPS-dependent PGE2 production was substantially reduced in macrophages treated with LXR/RXR ligands. The inhibitory effects of LXR/RXR activation on LPS-induced expression of COX-2 and mPGES-1 in macrophages, occurred by a mechanism involving interference with transcriptional activation of these genes. LXR/RXR activation interfered with the activity of transcription factors essential in the up-regulation of the expression of pro-inflammatory genes in these cells, such as NFKB, but also Egr-1, which had not been previously associated with LXR-mediated gene repression. As this transcription factor is involved in the regulation of a variety of genes involved in inflammatory processes, LXR and RXR-mediated interference with Egr-1 signaling could represent an important event mediating the anti-inflammatory effects of these receptors in macrophages.

1. Introduction

Liver X receptors (LXRs) belongs to the nuclear receptor family of ligand-activated transcription factors. There are two isoforms of LXRs known as LXRα and LXRβ, which share high sequence homology but differ in localization. While the β isoform is expressed broadly in most tissues, LXRα is more abundant in some tissues such as liver, adipose tissue, kidney or macrophages [1]. These nuclear receptors modulate different metabolic processes by regulating transcription of a variety of genes. These receptors exert their actions by forming permissive heterodimers with retinoid X receptors (RXRs). LXR/RXR heterodimers can be activated by either LXR agonists, as oxysterols or synthetic ligands such as TO901317 (TO) or GW3965 (GW), or by RXR ligands as 9-cis retinoic acid (9cisRA) [1–4].

LXRs regulate gene expression by two different mechanisms of action. The best-characterized one is the transactivation mechanism, through which these nuclear receptors are involved in lipid metabolism regulation by inducing the expression of target genes like ABC transporters (ABCA1 and ABCG1) and transcription factors as SREBP-1c, among others [5–7]. LXRs bind to DNA at specific sequences defined as LXR response elements (LXRE) along with corepressor molecules. Upon activation by ligand binding, the LXR/RXR heterodimers suffer a conformational change that allows clearance of corepressors and binding of coactivators such as SRC-1 (steroid receptor co-activator) and p300 [1,8,9]. In this way, the expression of LXR target genes is induced, having a variety of effects at different levels of cholesterol homeostasis in different tissues: induction of reverse cholesterol transport in macrophages, increase of lipogenesis or cholesterol excretion in liver and reduction of intestinal cholesterol absorption [7,10–13].

Research on the effects of the activation of these nuclear receptors has gained great relevance since their recent characterization as integrators of both cholesterol metabolism and inflammatory responses [14–16]. These findings supported LXR involvement in cardiovascular pathologies with macrophage activation and alterations in cholesterol homeostasis such as atherosclerosis [17–19].
Activated LXRs can exert anti-inflammatory effects by inducing the expression of arginase II, which competes for substrate with the inflammatory protein iNOS [20]; Mer, involved in phagocytosis of apoptotic cells [21]; or AIM (apoptotic inhibitor of macrophages) [22]. These receptors can also act as transcriptional repressors, inhibiting the expression of a variety of pro-inflammatory genes such as COX-2, interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1), inducible nitric oxide synthase (iNOS) or matrix metalloproteinase-9 (MMP-9) [14,23–26]. Different studies have reported that LXR-mediated transrepression involves interference with the activity of transcription factors, as nuclear factor kappa B (NFκB) [14,23,27], which plays an essential role in inflammatory signaling [28,29].

Among the inflammatory mediators involved in immune responses, prostanooids, which include prostaglandins (PGs), prostacyclin (PGI2) and thromboxane (TXA2), are determinant for the progress but also for the resolution of inflammation. Their synthesis takes place from arachidonic acid metabolism through the action of cyclooxygenases, mainly COX-2, which is induced by proinflammatory stimuli, and terminal synthases as mPGES-1 as case of prostaglandin E2 (PG_E2) production. The regulation of the expression of these enzymes is essential for the final balance of prostaglandin production and therefore, for the outcome of inflammatory responses and function of immune cells such as macrophages [30,31]. Transcription factors as NFκB and Early growth response factor-1 (Egr-1) are involved in the expression of both COX-2 and mPGES-1 [32–35]. NFκB proteins comprise a family of transcription factors that are involved in the control of different biological processes, including immune and inflammatory responses. Activation of this transcription factor plays an essential role in a number of diseases, including cancer, arthritis, chronic inflammation, asthma, neurodegenerative diseases, and heart disease [28,29]. Egr-1 is also crucial for inflammatory signaling, playing a determinant role in the development of inflammatory pathologies by regulating the expression of a variety of genes [36,37].

Several aspects of LXR-mediated transrepression are still to be elucidated in order to fully understand how activation of LXR/RXR heterodimers can affect inflammation under different experimental or pathological conditions. Here we have focused on the repressive effects of LXR and RXR agonists on the LPS-induced expression of COX-2 and mPGES-1 in macrophages. We demonstrate that both LXR and RXR ligands are able to reduce the induction of both enzymes by a mechanism that involves interference with transcription factors such as NFκB, but also Egr-1. We provide new insights into the studies on the LXR-mediated transrepression mechanisms by demonstrating the interference of LXR and RXR agonists with the LPS-induced expression and transcriptional activity of Egr-1. Our results point out to LXR/RXR mediated inhibition of Egr-1 dependent signaling as an important event contributing to the anti-inflammatory effects of these receptors in macrophages.

2. Material and methods

2.1. Cell culture and reagents

The mouse macrophage RAW 264.7 and the human THP-1 monocyte cell lines were cultured in RPMI 1640 medium (Invitrogen) supplemented with 5% fetal calf serum (FCS, BioWhittaker-Lonza), 100 U/ml penicillin, 100 μg/ml streptomycin, 1000 U/ml gentamycin, 2 mM L-glutamine and 0.1 mM non-essential amino acids. Peritoneal macrophages were obtained from C57Bl/6j mice (Harlan Laboratories) injected with 1 ml thiglycollate (10% w/v, DIFCO) in the peritoneal cavity as previously described [38]. Briefly, four days post-injection, cells were obtained by peritoneal lavage with cold PBS, centrifuged and seeded in culture plates. After 4 h, non-adherent cells were removed by gentle aspiration and washing with PBS, and macrophage-adherent cells were maintained in culture with complete RPMI medium supplemented with 5% FCS. The population of adherent cells was consistently composed of > 80% F4-80+ CD11b+ macrophages, as determined by flow cytometry analysis. Cells were stimulated with bacterial lipopolysaccharide (LPS) from Escherichia coli 026: B6 (2 μg/ml, Sigma-Aldrich) in complete RPMI medium supplemented with 2% FCS. Treatment of cell cultures with the LXR ligands T0901317 (0.1 to 2.5 μM, Cayman Chemical), GW3965 (0.1 to 2.5 μM, Sigma-Aldrich) and 25-hydroxycholesterol (1 to 15 μM, Sigma-Aldrich); the RXR ligands 9cisRA (0.1 to 2.5 μM, Sigma-Aldrich) and SR11237 (0.1 to 2.5 μM, Tocris bioscience), was performed 1 h before LPS-stimulation.

2.2. Plasmid constructs

COX-2 promoter luciferase construct (COX-2-luc, p2–1900) contains the –1796 to +104 region of the human COX-2 gene cloned in pXP2 plasmid [39]. Luciferase construct mPGES-1-luc containing the –631 to –1 region of the human mPGES-1 gene cloned into a pGL3 basic plasmid was kindly provided by Sabine Grösch (Institute of Clinical Pharmacology, Frankfurt, Germany) [40]. Egr1-luc (Egr1-Pro36-luc) containing two binding sites for Egr-1 inserted upstream of a prolactin minimal promoter was generously provided by Dr. Ana Pérez-Castillo (Instituto de Investigaciones Biomédicas, Madrid, Spain) [41]. NFκB-luc (pNF3ConA-luc) contains three copies of the consensus kB sequence from the immunoglobulin κ chain upstream of a conalbumin minimal promoter and was kindly provided by Dr. F. Arenzana-Seisdedos (Institute Pasteur, Paris, France) [42]. LXRE-luc (pGTK-3×LXRE-luc) contains three consensus binding sites for LXR in upstream of a thyminde kinase promoter and was kindly provided by Dr. A. Castrillo (Instituto de Investigaciones Biomédicas, Madrid, Spain) [20]. RXRE-luc reporter (pTK-CRBPII-luc) vector including 5 copies of the RXRE of the CRBP II gene as well as human LXRα (pCMX-hLXRα) and mouse RXRα (pCMX-mRXRα) expression vectors were a kind gift from Dr. D. J. Mangelsdorf (Howard Hughes Medical Institute, California, US) [43].

2.3. Transfection and luciferase assays

Transcriptional activity of different luciferase reporter constructs was analyzed by luciferase reporter gene assays. Briefly, THP-1 cells were transiently transfected with 0.7 to 1 μg of the different constructs using 2.25 μl of the Jetprime reagent (Polyplus Transfection) diluted in RPMI without antibiotics and supplemented with 0.5% FCS. After 4 h of transfection, cultures were treated with the indicated stimuli. Then, cells were harvested and lysed, and luciferase activity was determined by using a luciferase assay kit (Promega) in a luminometer Monolight 2010 (Analytical Luminescence Laboratory, San Diego, CA). Transfection experiments were performed in triplicate and normalized by mg of protein. Results are expressed as fold induction ± SD (RLUs per mg of protein in the experimental samples/RLUs per mg of protein in the experimental controls).

2.4. mRNA analysis

Total RNA was isolated using the “Absolutely RNA miniprep kit” (Stratagene) and reverse transcribed into cDNA by the “RNA PCR core kit” (Perkin-Elmer). For standard RT-PCR, cDNA was used for PCR amplification with specific primers for human ABCA1, sense 5'-CTCAGGTGTTGGCTGTGAC-3' and antisense 5'-GTATTCACACCCATACGCAA-3'; ABCG1 sense 5'-GGCATCTACGTGGACGAG-3' and antisense 5'-CAAGAAAGGGGTCACTCG-3'; and GAPDH, sense 5'-CCACCCATGGCAAATTTCCATGGCA-3' and antisense 5'-TCTAGACCGAGTCTAGCTCACC-3'. PCR reactions were amplified by 25 to 30 cycles of denaturation at 94 °C for 45 s, annealing at 55 or 60 °C for 45 s, and extension at 72 °C for 45 s. Amplified cDNAs were separated by agarose gel electrophoresis and bands visualized by
ethidium bromide staining. Data shown correspond to a number of cycles where the amount of amplified product is proportional to the abundance of starting material.

For quantitative real-time RT-PCR analysis, total RNA was reversed transcribed using the components of the “High Capacity cDNA Archive Kit” (Applied Biosystems). Amplification of the cDNAs was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems) on an ABI PRISM 7900HT instrument (Applied Biosystems) for 40 cycles with specific primers and Taqman probes (Applied Biosystems). All samples were run in triplicate and normalized by the expression of the endogenous 18S gene. Quantification of gene expression by real-time RT-PCR was calculated by the comparative threshold cycle (ΔΔCT).

**Fig. 1.** LXR ligands induce LXRE-dependent transactivation in macrophages. (A) RAW264.7 and THP-1 cells transfected with the reporter vector LXRE-luc. (B) RAW264.7 cells co-transfected with the plasmid LXRE-luc and expression vectors for LXRα, RXRα or an empty vector (pcDNA3). After transfection, cells were treated with LXR ligands TO901317 (TO) or GW3965 (GW) at 1 μM or 25-hydroxycholesterol (25HC) at 10 μM and/or the RXR ligand 9cisRA (1 μM) for 18 h and then luciferase activity was measured in cellular extracts. Results of a representative experiment of the four performed are shown as fold induction compared to untreated cells (sample RLUs per mg of protein/ control RLUs per mg of protein) ± SD. *p<0.05, **p<0.01, ***p<0.001.
method following the manufacturer’s instructions. Results are shown as Fold induction compared to the reference sample ± SD.

2.5. Western blot

Protein extracts were obtained by lysis in Igepal buffer (50 mM Tris–HCl pH 8, 10 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Igepal) with protease inhibitors (aprotinin, leupeptin and pepstatin at 10 μg/ml), and PMSF (phenyl-methylsulphonyl fluoride, 0.5 mM). Protein concentration was determined by the BCA method (Thermo Scientific). Cell lysates were subjected to Western blot analysis using conventional SDS-PAGE gel electrophoresis and protein transfer to nitrocellulose filters. Membranes were incubated with the indicated antibodies and developed by the enhanced chemiluminescence system (Thermo Scientific). COX-2 and mPGES-1 protein expression was detected using monoclonal antibodies (BD Transduction Laboratories and Cayman Chemical). Antibodies anti-LXRα, LXRβ and Egr-1 were purchased from Santa Cruz Biotechnology. As a loading control, β-actin levels were determined with a specific antibody (Santa Cruz Biotechnology).

Fig. 2. LXR agonists induce transcriptional activation of target genes in macrophages. Cells were treated with LXR ligands TO901317 (TO) or GW3965 (GW) during 18 h (1 μM in RAW264.7 and THP-1 cells; 0.5 μM in peritoneal macrophages) and mRNA levels of ABCA1 and ABCG1 were analyzed by standard or quantitative RT-PCR. For standard RT-PCR, an aliquot of the amplified DNA was separated on an agarose gel and stained with ethidium bromide for qualitative comparison. GAPDH mRNA levels are shown as a control of loading. mRNA levels analyzed by qRT-PCR are normalized to the expression of 18S rRNA. Results are shown as the mean of fold induction over the control group ± SD of three independent experiments performed in triplicate. ***p<0.001.

Fig. 3. Inhibition of LPS-induced expression of pro-inflammatory genes by LXR ligands in macrophages. RAW264.7 cells were incubated with the LXR ligand GW3965 (GW, 1 μM) prior LPS stimulation (2 μg/ml) during 18 h. Expression of IL-6, IL-1β, MCP-1, COX-2 and mPGES-1 was analyzed by quantitative RT-PCR. Results were normalized to the expression of 18S rRNA and shown as the mean of fold induction over the control group ± SD of three independent experiments performed in triplicate. **p<0.01, ***p<0.001 compared to LPS-treated cells in the absence of ligand.
2.6. Prostanoid determination

PGE$_2$ and PGF$_{2\alpha}$ levels were measured in culture supernatants of RAW264.7 cells after the indicated treatments by competitive immunoassay EIA kits (Cayman Chemical). Samples were analyzed in triplicate following manufacturer's instructions.

2.7. Statistical analysis

Results are shown as the mean ± SD of triplicate determinations from at least two independent experiments. When a representative experiment is shown, data are mean ± SD of triplicate determinations of this particular experiment. In these cases, equivalent results were obtained in two or three independent experiments performed in triplicate, as indicated in the figure legends. Statistical analysis and calculation of $P$-values were performed by ANOVA followed by Bonferroni's post-hoc test, using GraphPad Prism 5 software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. LXRE-dependent transcriptional activation by LXR and RXR ligands in macrophages

LXRE-dependent transcriptional activation depends on the binding of specific ligands to LXR/RXR heterodimers [1,4]. In order to study LXR-dependent transcriptional activation in macrophages, murine RAW264.7 and human THP-1 cell lines were transiently transfected with the reporter vector LXRE-LUC, containing three consensus binding sites for LXR upstream of a thymidine kinase promoter [20]. As shown in Fig. 1A, LXR ligands TO901317 (TO) and GW3965 (GW) efficiently induced transcription of the LXRE-luc reporter in both cell lines. Transcriptional activation mediated by TO and, to a minor extent, by the natural LXR ligand 25 hydroxycholesterol (25HC), increased in RAW264.7 cells that were co-transfected with a LXR$\alpha$ expression plasmid (Fig. 1B). Treatment with the RXR ligand 9cis-retinoic acid (9cisRA) also induced...
LXRE-dependent transcriptional activity in the absence and in the presence of overexpressed RXRα. Maximal induction of LXRE-dependent transactivation was observed upon combined treatment with LXRxα and RXRxα ligands (Fig. 1B).

We next evaluated the effect of LXR ligands on the expression of LXR target genes as the ATP-binding cassette transporter proteins ABCA1 and ABCG1, by RT-PCR, in RAW264.7 and THP-1 cell lines as well as in peritoneal macrophages. As shown in Fig. 2, mRNA levels of ABCA1 and ABCG1 genes increased significantly upon treatment with the LXR ligands GW or TO in these cells.

### 3.2. LXR agonists reduce LPS-induced expression of COX-2 and mPGES-1

In addition to promote transcriptional induction of genes involved in lipid homeostasis, LXR activation has been shown to display anti-inflammatory effects by repressing the expression of pro-inflammatory genes [14,23–26]. Thus, we next analyzed the effect of LXR activation on the expression of genes involved in inflammation such as the cytokines IL-6 and IL-1β, the chemokine MCP-1, and the enzymes COX-2 and mPGES-1. As shown in Fig. 3, LXR activation reduced substantially LPS-induced expression of these genes in RAW264.7 macrophages.

Once assessed the potential anti-inflammatory effects of LXR agonists in macrophages, we focused our studies on their effects on the regulation of the expression of COX-2 and mPGES-1, enzymes that play an essential role in the increase of prostanoid synthesis upon macrophage activation. LXR natural ligands such as 25HC, and synthetic ones such as TO or GW, were able to reduce LPS-induced expression of COX-2 at the protein and mRNA levels in RAW264.7 cells in a dose-dependent manner (Fig. 4A and B).

These inhibitory effects of LXR ligands on COX-2 expression were also observed in THP-1 cells and peritoneal macrophages (Fig. 4C). As shown in Fig. 5A and B, LXR ligands were also able to reduce LPS-mediated increase in mPGES-1 expression in macrophage/monocyte-like cell lines and primary murine macrophages.

Both COX-2 and the terminal synthases involved in prostanoid synthesis as mPGES-1 are tightly regulated, in such a way that induction or inhibition of their expression influences the final balance of prostanoid production by macrophages, with a key role in the inflammatory response [30,31]. In accordance with their effect on diminishing LPS-induced COX-2 and mPGES-1 expression, LXR agonists severely impaired prostaglandin production by activated RAW264.7 cells. As shown in Fig. 6, LXR activation highly decreased the production of the COX-2/mPGES-1-dependent production of prostaglandin PGE2. Increased production of PGF2α in response to LPS was also reduced by LXR ligands.

### 3.3. Inhibition of LPS-induced COX-2 and mPGES-1 expression by RXR agonists

LXRs form heterodimers with the obligate partner RXR, in such a way that the LXR/RXR heterodimer can be activated by either LXR or RXR agonists [1,4]. As shown in Fig. 1B, the RXR ligand 9cisRA was able to induce LXRE-dependent luciferase activity both cooperating with LXR ligands as well as on its own, in RAW264.7 cells.

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**Fig. 5.** Inhibition of LPS-induced expression of mPGES-1 by LXR ligands in macrophages. mPGES-1 mRNA expression was analyzed by quantitative RT-PCR in LPS-stimulated (2 μg/ml) and LXR ligand-treated macrophages. (A) RAW264.7 cells were treated with GW3965 (GW) at the indicated concentration (0.1 to 1 μM). THP-1 cells were treated with GW or TO at 2.5 μM. Peritoneal macrophages treated with LXR ligands GW, TO or 25 hydroxycholesterol (25HC) (0.5 μM). Results are normalized to expression of 18S rRNA and shown as mean of fold induction ± SD of two independent experiments performed in triplicate. **p < 0.01, ***p < 0.001 compared to LPS-treated cells in the absence of ligand. (B) mPGES-1 protein levels were analyzed by Western blot in RAW264.7 cells treated with ligands GW, TO or 9cisRA (1 μM) prior to LPS stimulation. Protein levels of β-actin are shown as loading control.
RXR ligands 9cisRA and SR11237 (SR) (Fig. 7). We next examined RXRE-dependent transcription that was efficiently induced by the RXR ligands 9cisRA and SR11237 (SR) (Fig. 7). We next examined the role of RXR activation on the LPS-induced expression of COX-2 and mPGES-1. Treatment with RXR ligands 9cisRA or SR led to a significant reduction in the activation of COX-2 expression in LPS-treated RAW264.7 cells at the protein and mRNA levels, in a dose-dependent manner (Fig. 8A and B). In primary macrophages stimulated with LPS, RXR ligands inhibited COX-2 expression to a similar extent as LXR ligands (Fig. 8B). Treatment of macrophages with the RXR ligands 9cisRA or SR also produced a substantial reduction in the LPS-induced expression of mPGES-1 in a dose-dependent manner (Fig. 8C). Combination of 9cisRA with LXR ligands promoted a mild but significant increase in the inhibitory effect on the induction COX-2 expression but not in the case of mPGES-1. Maximal inhibitory effect in mPGES-1 mRNA levels occurred in the presence of the RXR ligand 9cisRA (Fig. 8D).

3.4. LXR/RXR activation diminishes COX-2 and mPGES-1 promoter activity

To further characterize the LXR/RXR mediated repression of COX-2 and mPGES-1, transfection assays were carried out in THP-1 cells using reporter plasmids including the promoter region of both genes. LPS treatment induced luciferase activity of both promoters and, as shown in Fig. 9, treatment with LXR or RXR agonists produced a significant decrease in the induction of COX-2 and mPGES-1 promoter activity. These results suggested that the inhibitory effect of LXR/RXR ligands occurred through interference at the transcriptional level.

3.5. LXR/RXR activation interferes with the activity of NFκB and Egr-1 transcription factors

Inhibition of the expression of pro-inflammatory genes by LXR/RXR heterodimers has been proposed to take place by interference with the activity of transcription factors. In this sense, different studies have focused on the effects of LXR activation on NFκB activity [14,23,27]. To test the effects of LXR/RXR ligands on NFκB activity, THP-1 cells were transiently transfected with an NFκB-luc reporter plasmid containing three copies of a consensus κB sequence, along with expression vectors for LXRα or RXRα. Upon transfection, cells were activated with LPS, in the presence or absence of LXR or RXR ligands. As shown in Fig. 10A, LXR/RXR activation promoted a significant reduction in the induction of NFκB-luc activity by LPS.

In addition to NFκB, several transcription factors have been shown to be involved in the LPS-mediated induction of these enzymes in macrophages [34,44–46]. In this sense, we have described the cooperation of Egr-1 and NFκB as determinant for PGE2 synthesis by macrophages in inflammatory processes, through the coordinated regulation of COX-2 and mPGES-1 [33]. The effect of LXR/RXR ligands on Egr-1 activity was analyzed in THP-1 cells transfected with a construct containing two Egr-1 consensus-binding sites inserted upstream of a prolactin minimal promoter (Egr1-luc). LPS stimulation of RAW264.7 cells induced Egr1-luc mediated luciferase activity. This induction was significantly reduced upon treatment with LXR or RXR ligands (Fig. 10B).

3.6. LXR/RXR activation interferes with LPS-induced expression of Egr-1 in macrophages

To further analyze the interference of LXR/RXR ligands with Egr-1 activation, we next evaluated the expression of this transcription factor in macrophages stimulated with LPS and treated with increasing doses of the nuclear receptor agonists TO and 9cisRA. Western Blot analysis showed an increase in Egr-1 protein levels in RAW264.7 cells treated for 18 h with LPS. Treatment with increasing doses of TO or 9cisRA resulted in a dose dependent reduction of Egr-1 protein levels (Fig. 11A). Similarly, induction of Egr-1 mRNA expression by LPS, was decreased in RAW264.7 cells treated with the RXR ligands 9cisRA or SR (Fig. 11B).

As Egr-1 expression is rapidly induced upon LPS treatment in macrophages, showing an increase that reaches the highest levels after 1 h of treatment, we also assessed the effect of LXR/RXR
Fig. 8. Inhibition of LPS-induced expression of COX-2 and mPGES-1 by RXR ligands. Cells were incubated with RXR ligands 9cis-retinoic acid (9cisRA) and SR11237 (SR) or LXR ligands (TO901317 (TO) or GW3965 (GW) before LPS stimulation (2 μg/ml) during 18 h. (A) Analysis of COX-2 mRNA levels by quantitative RT-PCR in RAW264.7 cells incubated with 9cisRA and SR11237 at the indicated concentration (0.1 to 2.5 μM) before LPS treatment. (B) Analysis of COX-2 protein levels by Western blot in RAW264.7 cells treated with 9cisRA and SR at different doses (0.1 to 2.5 μM). Murine peritoneal macrophages were treated with 1 μM of TO, 9cisRA or SR11237 prior to LPS stimulation. β-actin protein levels are shown as a control. (C) mPGES-1 mRNA expression analysis by quantitative RT-PCR in LPS-stimulated (2 μg/ml) and RXR ligand-treated RAW264.7 cells (at the indicated concentration). (D) COX-2 and mPGES-1 mRNA levels were determined by quantitative RT-PCR in RAW264.7 cells treated with either the RXR ligand 9cisRA or the LXR ligands TO or GW, alone or in combination, before LPS stimulation. Results from representative experiments of at least two performed in triplicate are normalized to 18S rRNA expression and shown as percentage of expression ± SD, considering 100% that obtained in the LPS stimulated samples. Results from quantitative RT-PCRs are normalized by 18S rRNA levels and shown as mean of fold induction over untreated cells ± SD. *p<0.05, **p<0.01, ***p<0.001, (ns) not significant.
ligands on EGR-1 expression by Western blot and RT-PCR after short incubations with LPS (up to 2 h). As shown in Fig. 12A and B, early induction of Egr-1 protein and mRNA levels was severely impaired after treatment with LXR or RXR ligands.

4. Discussion

In addition to the well-known actions of LXRs in the regulation of lipid homeostasis, increasing evidence supports a role of LXRs as anti-inflammatory transcription factors through their antagonism with the induction of pro-inflammatory genes [14–16,19]. In this sense, various studies have clearly pointed out that LXRs play a pivotal role in innate immunity modulating macrophage activation. Ligand activation of these receptors modulates macrophage apoptosis [17,20,22] and negatively regulates expression of pro-inflammatory genes in activated macrophages [14,23–26,47]. LXR receptor activation plays a key role in modulating the function of macrophages on the regulation of cholesterol metabolism by inducing the expression of various genes involved in cholesterol homeostasis as apolipoprotein E and members of the ABC transporter family [5–7,48]. Our studies confirm that LXR activation with specific ligands as TO901317, GW3965 or 25-hydroxycholesterol was able to induce transcriptional activation mediated by LXXRE elements and the induction of LXR target genes as ABCA1 and ABCG1 in the human and murine

Fig. 9. Effects of LXR/RXR activation on COX-2 and mPGES-1 promoter activity. THP-1 cells were transfected with reporter plasmids containing the promoter region of COX-2 (COX2-luc) or mPGES-1 (mPGES1-luc) genes. After transfection, cells were incubated with either the LXR ligand GW3965 (GW) or the RXR agonist 9cisRA (2 μM) before LPS (2 μg/ml) for 18 h and assayed for luciferase activity. Data are shown as means of fold induction (sample RLUs per mg of protein/control RLUs per mg of protein) ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 compared to LPS-treated cells.

Fig. 10. Interference of LXR/RXR activation with NFκB and Egr-1-mediated transcriptional activation. (A) THP-1 cells were co-transfected with the plasmid NFκB-luc along with expression vectors for LXRα or RXRα. After treatment with 1 μM of either LXR ligands TO901317 (TO) and GW3965 (GW) or RXR ligands 9cisRA and SR11237 (SR), cells were stimulated with LPS (2 μg/ml) for 18 h and then luciferase activity was measured. (B) THP-1 cells were transiently transfected with the construct Egr1-luc and treated with the indicated ligands TO and SR11237 (SR) at 1 μM before stimulation with LPS for 18 h. Results from a representative experiments out of three are shown as fold induction over untreated control cells (sample RLUs per mg of protein/control RLUs per mg of protein) ± SD. *p<0.05, **p<0.01, ***p<0.001 compared to LPS-treated cells.
monocyte-macrophage cell lines RAW264.7 and THP-1, as well as in mouse peritoneal macrophages. LXRα form permissive heterodimers with RXR and thus can exert their regulatory effects on gene expression in macrophages, upon activation by either LXR or RXR ligands [1–4]. Accordingly, we found that both LXR ligands and RXR agonists were able to induce gene expression through LXREs. Moreover, combination of both types of ligands produced a substantial increase in such induction. In turn, over-expression of both RXRα and LXRα receptors significantly increased ligand-dependent transcriptional activation in RAW264.7 cells.
We have also shown that, in addition to their ability to up-regulate LXR-dependent expression of target genes in murine and human macrophages, LXR ligands led to an inhibition of the LPS-induced expression of pro-inflammatory genes such as IL-6, IL1-β and MCP-1. Several reports have shown LXR-dependent negative regulation of the induction of pro-inflammatory genes in response to different stimuli in macrophages, including IL-6, MCP-1, iNOS as well as enzymes involved in prostanoit production [14,23–25, 49,50]. Here, we have analyzed the effects of different LXR and RXR ligands on the expression of COX-2 and mPGES-1 enzymes. Coordinated induction of these genes is responsible for the increase in the production of PGE2 that takes place in response to inflammatory stimuli in different cell types, including activated macrophages [33,35,51,52]. Induction of COX-2 and mPGES-1 transcriptional activation in response to stimulation with LPS was reduced significantly by activation of LXR/RXR heterodimers. Consequently, LXR-mediated inhibition of these two enzymes led to a decrease in the production of PGE2 in these cells. Furthermore, although to a lesser extent, a reduction in other prostanoids such as PGF2α was detected in the supernatant of LPS-activated macrophages treated with LXR agonists. Since there is no previous evidence about LXR-mediated regulation of the expression of other terminal prostanoit synthases, reduction in PGE2 synthesis could be a consequence of the inhibition of COX-2 expression, which regulates the limiting step in the conversion of arachidonic acid to PGH2, essential for prostanoit biosynthesis.

In addition to the previously reported anti-inflammatory effect of LXR ligands, our results also support an anti-inflammatory role of RXR ligands. To determine RXR-mediated effects, we have used 9cisRA as the RA stereoisomer with higher affinity for RXRs [43]. As 9cisRA can also act through binding to RA receptors, we have confirmed the results obtained with the use of the specific RXR agonist SR11237. This ligand allows the specific activation of RXR-selective response pathways without inducing RAR-dependent response pathways [53]. Treatment of LPS-activated macrophages with these RXR ligands resulted in a reduction of COX-2 and mPGES-1 transcriptional induction similar to that observed with LXR ligands. Recent reports have demonstrated the existence of an RXR-selective signaling pathway, independent of heterodimerization with other nuclear receptors in macrophages and able to control innate inflammatory responses by up-regulating the transcription of chemokine expression [54].

Most of the evidence about the anti-inflammatory effects of nuclear receptor activation has been explained by the existence of a mechanism of transrepression that results in the interference with the activation of transcription factors implicated in the expression of pro-inflammatory genes [47,55]. Thus, LXR-dependent transcriptional repression is thought to occur, at least in part, by interference with the pro-inflammatory transcription factor NFκB [14,23,27,56]. In agreement with that, we have shown that the LPS-induced activity of NFκB, is reduced in the presence of LXR and RXR ligands. In addition to NFκB, other transcription factors including Egr-1 are known to participate in transcriptional induction of COX-2 and mPGES-1 in response to LPS [32–35]. Our results show that, besides interfering with NFκB-activation, LXR and RXR ligands modulate Egr-1-mediated signaling. LXR and RXR agonists down regulated induced expression of Egr-1 upon LPS-treatment in macrophages. Moreover, Egr-1-dependent transcriptional activation was also affected by these ligands, thus suggesting a role of LXR and RXR signaling in the transrepression of Egr-1-dependent genes involved in inflammation such as COX-2 and mPGES-1, but also other as MCP-1, TNFα or IL-1β. Egr-1 expression is induced under pro-inflammatory conditions and this transcription factor has been implicated in the regulation of a wide range of genes that are involved in macrophage responses [36,37,57,58]. Regulation of Egr-1 expression depends on signaling by mitogen-activated kinases (MAPK), and on activation of other transcription factors such as NFκB [59,60]. In addition, a positive feedback mechanism for Egr-1 has been recently identified [61]. Further work will be necessary to determine if the inhibition of Egr-1 activation by LXR/RXR heterodimers takes place by direct interference with the transcription factor, or indirectly, through inhibition of other factors involved in Egr-1 expression, as NFκB. Nevertheless, considering the fact that this transcription factor is involved in the regulation of a variety of genes involved in inflammatory processes, LXR/RXR-mediated interference with Egr-1 could contribute to explain anti-inflammatory actions of drugs targeting these nuclear receptors.

The discoveries of LXR-regulated inflammatory pathways have open new promises for these receptors and their target genes for therapeutic intervention in human diseases. LXR activation in cellular and animal models has proven to have anti-inflammatory effects, reducing inflammatory responses in several diseases with an important inflammatory component, including rheumatoid arthritis, atherosclerosis, Alzheimer’s disease and skin disorders [262]. The dual role of LXRs as cholesterol sensors and regulators of inflammation has been demonstrated to be essential for the attenuation of atherosclerotic plaque development by LXR agonists in animal models of atherosclerosis [17–19,63]. However, these treatments also promoted an LXR-mediated increase in lipogenesis in the liver, resulting in increased levels of serum and hepatic triglycerides. In order to overcome these undesirable effects of LXR activation in the liver after the systemic administration of agonists, new strategies for pharmacological intervention are currently aimed to increase the selectivity regarding LXR subtype and pathway, as well as the cell type or tissue where they exert their actions. New generation of compounds as the intestine-specific agonist GW6340 retain the capacity to modulate cholesterol levels, avoiding liver-related side effects [64]. The first LXR ligand of this new class of agonists used in a phase I clinical trial was the compound LXR-623. This LXR agonist produced an increase in the expression of target genes involved in reverse cholesterol transport in healthy volunteers without increased hepatic triglyceridaemia when administered at low doses [65]. Unfortunately, these clinical trials were discontinued because of adverse neurological side effects. Although similar adverse effects have not been reported for other LXR agonists, some other LXR ligands that have entered in clinical trials have also had limited success [66]. Thus, both increase in the knowledge on new LXR/RXR target genes and pathways, on their potential contribution to pathophysiology, as well as the development of safer and therapeutically efficacious drugs targeting these receptors, will contribute to define their potential as drugs for therapeutic intervention.

In this context, our results provide new evidence on the anti-inflammatory actions of LXR, but also RXR activation, through the regulation of the LPS-mediated transcriptional induction of proinflammatory genes in macrophages. Thus, it will be therefore relevant to achieve a deeper understanding of the possible differences or the common traits of LXR and RXR-mediated anti-inflammatory effects in different cell types or tissues in both pathological and physiological conditions. These results contribute to the overall knowledge on the LXR-mediated transrepression effects by furnishing new data on the interference of LXR and RXR agonists with the Egr-1-dependent signaling.

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