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Platelet-activating factor downregulates the expression of liver X receptor-α and its target genes in human neutrophils

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Liver X receptors (LXRs) are ligand-activated members of the nuclear receptor superfamily that regulate the expression of genes involved in lipid metabolism and inflammation, although their role in inflammation and immunity is less well known. It has been reported that oxysterols/LXRs may act as anti-inflammatory molecules, although opposite actions have also been reported. In this study, we investigated the effect of platelet-activating factor (PAF), a proinflammatory molecule, on LXRα signalling in human neutrophils. We found that PAF exerted an inhibitory effect on mRNA expression of TO901317-induced LXRα, ATP-binding cassette transporter A1, ATP-binding cassette transporter G1, and sterol response element binding protein 1c. This negative action was mediated by the PAF receptor, and was dependent on the release of reactive oxygen species elicited by PAF, as it was enhanced by pro-oxidant treatment and reversed by antioxidants. Current data also support the idea that PAF induces phosphorylation of the LXRα molecule in an extracellular signal-regulated kinase 1/2-mediated fashion. These results suggest that a possible mechanism by which PAF exerts its proinflammatory effect is through the downregulation of LXRα and its related genes, which supports the notion that LXRα ligands exert a modulatory role in the neutrophil-mediated inflammatory response.

Introduction

Liver X receptors (LXRs) are ligand-activated transcription factors of the nuclear receptor superfamily. They were first identified in 1994 by screening a rat liver cDNA library, and were initially classified as orphan nuclear receptors, given that their natural ligands were unknown [1]. Later, their natural endoge-

Abbreviations
ABCA1, ATP-binding cassette transporter A1; ABC, ATP-binding cassette; ABCG1, ATP-binding cassette transporter G1; aPAF, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phospho-(*N*,*N*,*N*-trimethyl)hexanolamine; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione; HRP, horseradish peroxidase; IL, interleukin; JNK, c-Jun N-terminal kinase; LXR, liver X receptor; MAPK, mitogen-activated protein kinase; NAC, *N*-acetylcysteine; NF-κB, nuclear factor-κB; oxLDL, oxidized low-density lipoprotein; PAF, platelet-activating factor; PAFR, platelet-activating factor receptor; ROS, reactive oxygen species; SEM, standard error of the mean; SREBP1c, sterol response element-binding protein 1c; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxyl.

nous agonists were identified, and are now known to consist mainly of a variety of oxidized cholesterol derivatives, referred to as oxysterols, and intermediate products of the cholesterol biosynthetic pathway [2]. LXRs act as key sensors of intracellular sterol levels that trigger a series of adaptive mechanisms in response to cholesterol overload and positively regulate the expression of an array of genes involved in almost all aspects of cholesterol transport and metabolism, such as those encoding the ATP-binding cassette (ABC) transporters ABC transporter A1 (ABCA1) and ABC transporter G1 (ABCG1), and sterol response element-binding protein 1c (SREBP1c) [3]. Moreover, they are involved in the modulation of other cellular functions, such as fatty acid synthesis and metabolism, glucose homeostasis, steroidogenesis, and neuronal homeostasis [4].

In addition, LXRs have been more recently associated with the modulation of the inflammatory response and innate immunity [2]. LXRs negatively regulate the transcription of proinflammatory genes by antagonizing the actions of transcription factors such as nuclear factor-κB (NF-κB) [5,6]. However, the relationship between LXRs and the anti-inflammatory response is not well established, and the activation of LXRs has also been related to positive regulation of inflammation in human macrophages [7] and mouse neutrophils [8].

A novel role of LXRs in the oxidative stress response has been recently described [9], and positive regulation of the expression of antioxidant enzymes by these receptors has been found in the lung. Also, oxidative stress limits cholesterol efflux in human macrophages through a molecular cascade involving inhibition of LXR gene expression, which, in turn, leads to decreased ABCA1 expression [10].

Platelet-activating factor (PAF) is a potent proinflammatory mediator that links the haemostatic and innate immune systems. Although PAF is minimally expressed under normal physiological conditions, several cell types, such as neutrophils and monocytes, release significant amounts of PAF in particular conditions, such as oxidative stress [11]. PAF contributes to increasing the permeability of the endothelium, induces the release of active oxygen species, and contributes to low-density lipoprotein oxidation [12]. PAF has been proposed to be a key factor and initial trigger in atherosclerosis, and modulation of PAF metabolism by bioactive food constituents has recently been associated with reduced atherosclerosis [13]. PAF exerts its physiological activities through a unique G-protein-coupled receptor, the PAF receptor (PAFR). Oxidized low-density lipoprotein (oxLDL) particles are known to activate platelets through PAFR [14,15].

PAF is an inducer of NF-κB activation *in vivo* and *in vitro* [16]. This transcription factor is present in the cytoplasm of a variety of unstimulated cell types, forming a complex with its inhibitory protein, IκB. When cells are stimulated with PAF, IκB is phosphorylated by the action of several kinases. Whereas, in its reduced state, IκB is protected from phosphorylation, upon oxidative modification IκB is inactivated by phosphorylation and subsequent ubiquitination, allowing the translocation of NF-κB (p50 and p65 subunits) to the nucleus [17].

LXRs have been linked mainly to macrophages, because of their direct relationship with atherosclerosis. However, increasing knowledge is accumulating on the role that neutrophils, which constitute the most abundant cell type in the immune system [18], play in the pathogenesis of vascular diseases [19]. In this context, we have very recently reported that transcription of the LXRα gene is downregulated by 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 through oxidative stress in human neutrophils [20]. In the present study, we extended our knowledge on the regulation of LXRα in neutrophils by using the proinflammatory molecule PAF. The present data show that, when LXRα is activated by synthetic ligands, mRNA expression of LXRα and its target genes, encoding ABCA1, ABCG1, and SREBP1c, becomes increased. Also, we have found that treatment of this cell type with PAF downregulates transcriptional activation of these genes, and this modulatory process is exerted through extracellular signal-regulated kinase (ERK)1/2-mediated phosphorylation.

Results

Effect of PAF on mRNA expression of LXRα and target genes in human neutrophils

LXRs comprise two isoforms: the inducible LXRα, which is highly expressed in the liver, adrenal glands, intestine, adipose tissue, macrophages, lung, and kidney; and LXRβ, which is ubiquitously expressed [21]. We previously found that LXRα mRNA was induced in human neutrophils by a synthetic ligand, TO901317, whereas LXRβ mRNA was constitutively expressed by these cells, and its levels remained unchanged [20]. In preliminary experiments, we attempted to transfect human circulating neutrophils with plasmids containing cloned LXRα genes or a short interfering RNA specific for human LXRα. However, these experiments were unsuccessful, because

of the difficulty in applying standard transfection and microinjection techniques to primary cultures of neutrophils, given that these are small, short-lived and terminally differentiated cells [22].

In the present study, we first tested whether the addition of the pro-oxidant molecule PAF to neutrophils influenced LXRα mRNA expression induced by TO901317. We found that TO901317-induced LXRα transcription was inhibited by 10 nM PAF, and that this response was time-dependent (Fig. 1A). In order to assess whether this effect was mediated through PAFR, the antagonist 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol-3-phospho-(*N*,*N*,*N*-trimethyl)hexanolamine (aPAF) was used [23]. This compound effectively cancelled the inhibitory effect of PAF on TO901317-induced LXRα expression in human neutrophils, as shown in Fig. 1B.

We next performed assays of dose-dependent PAF effects on the mRNA expression of LXRα and of a series of genes activated by this transcription factor, such as those encoding the cholesterol efflux transporters ABCA1 and ABCG1, and SREBP1c. We observed that 10 nM PAF produced a clear inhibition of TO901317-induced LXRα mRNA expression and a decrease in the mRNA levels of its target genes, encoding ABCA1, ABCG1, and SREBP1c. At doses of PAF < 10 nM, we did not observe any effect of this ligand (data not shown), whereas at higher doses, such as 100 nM, the PAF inhibitory effect on all genes was lost, except for LXRα TO901317 induction (Fig. 1D). Assays performed with the LXRα natural agonist, 22*R*-OH-cholesterol, which is chemically different from TO901317, elicited a similar response as the latter (Fig. 1C). Similar inhibitory effects of PAF were observed in other human immune cells, namely lymphocytes and macrophages (Fig. 1E).

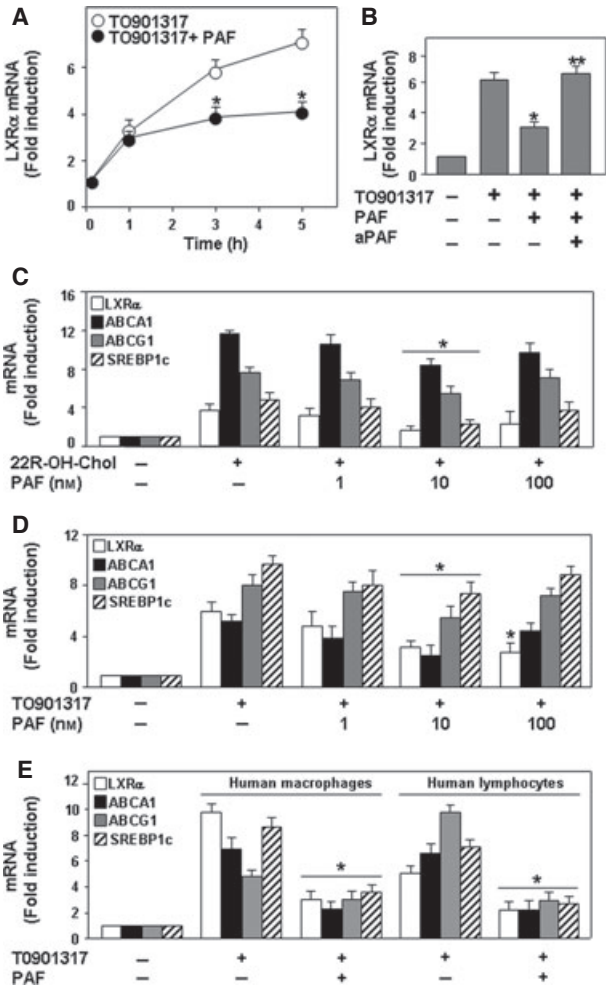

Fig. 1. Effect of PAF on mRNA expression of LXRα and its target genes in human neutrophils, macrophages, and lymphocytes. Neutrophils (A–D), macrophages and lymphocytes (E) were incubated at 37 °C with or without 10 nM PAF for the indicated times (A) or for 1 h (B–E). In (B), the cells were additionally preincubated at 37 °C with or without 10 nM aPAF for 30 min prior to PAF addition. Then, 1 μM TO901317 (A, B, D, E) or 22*R*-OH-cholesterol (22R-OH-Chol) (C) was added, and cells were incubated as above for a further 5 h. Control cells were cultured for the same times as treated cells, but without any additions. Each panel is representative of a set of three experiments yielding similar results, and values are plotted as the mean ± SEM. Statistical data from experiments investigating mRNA levels measured by real-time PCR were corrected for differences in β-actin mRNA levels, and are expressed as fold induction: $*P < 0.05$ for PAF-treated and TO901317-treated or 22R-OH-Chol-treated versus PAF-untreated; $**P < 0.01$ for aPAF-treated, PAF-treated and TO901317-treated versus aPAF-untreated.

Effect of PAF and TO901317 on interleukin (IL)-8 release, cellular migration, and reactive oxygen species (ROS) production

With the aim of evaluating the effects of LXRα on the proinflammatory properties of PAF, we studied the release of IL-8, chemotaxis and ROS production by human neutrophils treated with this ligand. IL-8 is the main cytokine produced by this cell type under pro-oxidant conditions. Figure 2A shows that PAF strongly induced significant IL-8 release to the medium by human neutrophils, and that this effect was hindered by previous TO901317 treatment. Another important role of PAF is enhancing neutrophil extravasation at nanomolar levels. Thus, chemotaxis was also assayed, and it was found that TO901317 treatment also inhibited PAF-induced neutrophil migration activity (Fig. 2B). Treatment with the natural agonist 22*R*-OH-cholesterol also reduced this migration, but to a lesser extent than TO901317. Therefore, for both

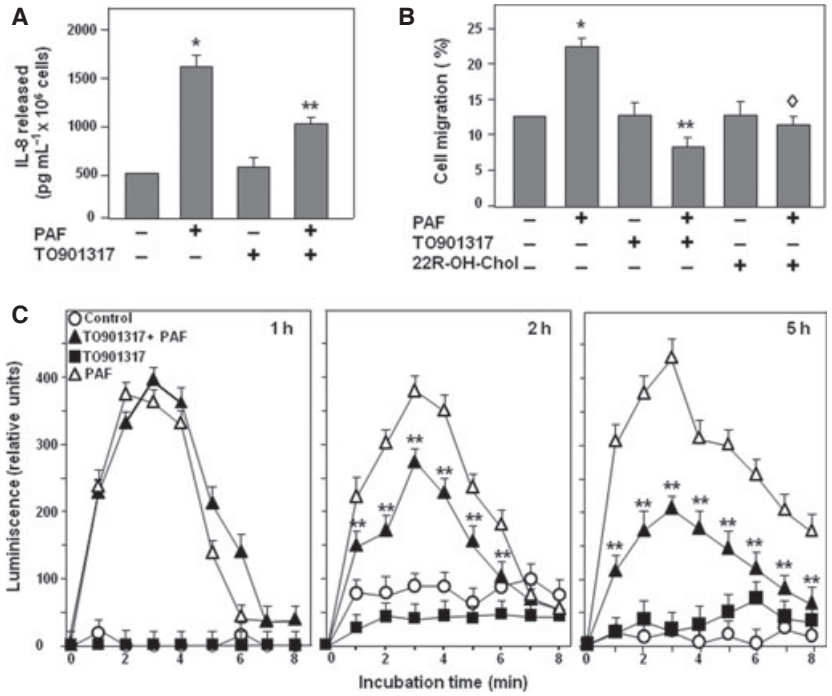

Fig. 2. Effects of PAF and TO901317 on IL-8 release, cellular migration, and ROS production. (A) Neutrophils were preincubated at 37 °C with or without 1 μM TO901317 for 5 h. Then, the cells were treated or not treated with 10 nM PAF for 1 h, and the levels of IL-8 released were analysed by ELISA. (B) Cells were stimulated or not stimulated with 1 μM TO901317 or 22*R*-OH-cholesterol (22R-OH-Chol) for 5 h, and PAF was then added for 1 h. Neutrophil migration activity was assayed in Transwell chambers, and the results were expressed as the percentage of cells that migrated from the upper to the lower compartment. (C) Neutrophils were treated with 1 μM TO901317 for 1 h, 2 h, or 5 h. Then, 10 μg/mL HRP and 40 μM luminol were added, and cells were stimulated with 10 nM PAF. The production of ROS by neutrophils was detected in a luminescence analyser. Control cells were incubated for the same times as treated cells, without any additions. Each panel is representative of a set of three experiments yielding similar results, and values are plotted as the mean ± SEM. Statistical data from experiments: $*P < 0.01$ for PAF-treated versus PAF-untreated; $**P < 0.01$ for PAF-treated and TO901317-treated versus TO901317-untreated; $\lozenge P < 0.01$ for PAF-treated and 22R-OH-Chol-treated versus 22R-OH-Chol-untreated.

parameters measured, i.e. IL-8 levels and chemotaxis, PAF clearly showed proinflammatory properties in human neutrophils, whose mobility was diminished when LXRα was activated by its agonists.

PAF activates NADPH oxidase in neutrophils. Therefore, subsequent experiments were performed to investigate whether the downregulatory effect of PAF on LXRα mRNA expression affected the release of ROS elicited by PAF. With this aim, we measured ROS production in neutrophils pretreated with TO901317 for 1 h, 2 h and 5 h before PAF addition. We observed that the ability of PAF to induce ROS production was decreased in a time-dependent fashion (Fig. 2C). When this pretreatment period was < 1 h, we did not observe any change (data not shown), and TO901317 did not induce any ROS production by itself (Fig. 2C). These facts suggested that TO901317 did alter the intracellular redox status in human neutrophils.

Effect of intracellular redox status on mRNA expression of LXRα and its target genes in human neutrophils

Next, we investigated whether the downregulation of LXRα mRNA expression by PAF was affected by the intracellular redox status. Neutrophils were separately treated with both antioxidant and pro-oxidant molecules. Antioxidants with thiol-reducing groups, such as glutathione (GSH) or *N*-acetylcysteine (NAC), reversed the inhibitory effect of PAF on LXRα expression, as well as on its target genes, encoding ABCA1, ABCG1, and SREBP1c (Fig. 3A). Other chemically different antioxidants, such as vitamin E and 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO), also counteracted the inhibitory effect of PAF on the mRNA expression of LXRα and its target genes (Fig. 3B). In contrast, under pro-oxidant conditions, i.e. treatment with Fe^{2+}/Cu^{2+} (Fenton reaction), the inhibitory

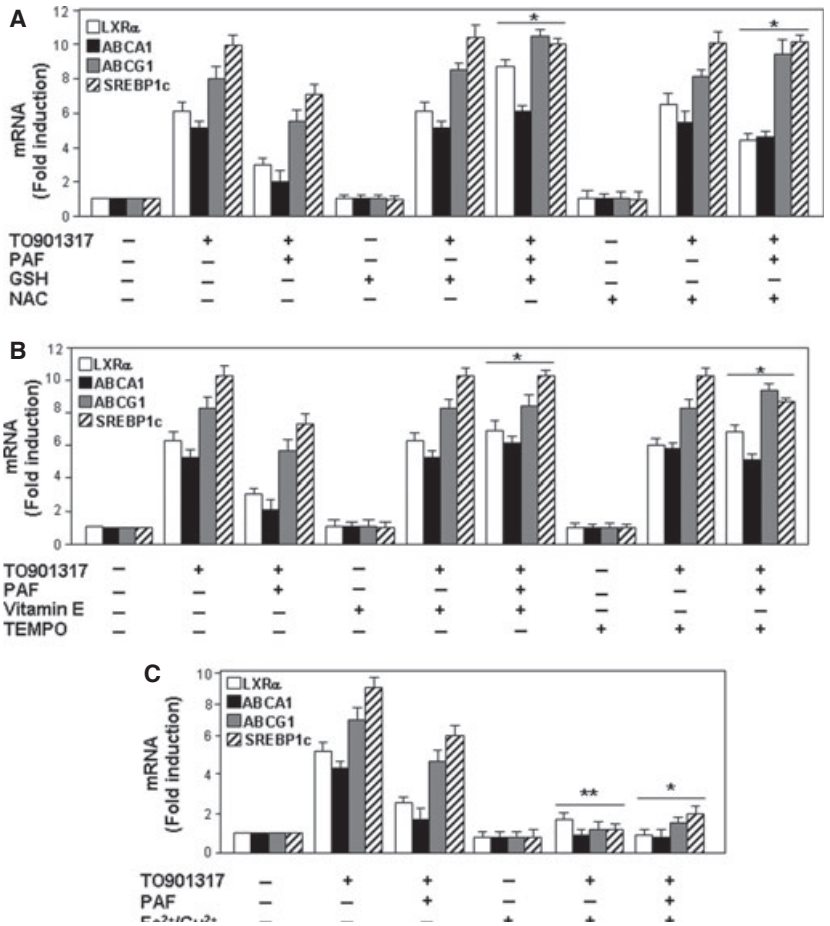

Fig. 3. Effect of the intracellular redox status on mRNA expression of LXRα and its target genes in human neutrophils. Cells were preincubated with 5 mM GSH, 1 mM NAC, 0.1 mM vitamin E and 0.1 mM TEMPO (A, B) or 0.1 mM $FeSO_4$ plus $CuSO_4$ (C) at 37 °C for 1 h. Then, 10 nM PAF was added, the cells were incubated for a further 1 h, and then stimulated or not stimulated with 1 μM TO901317 for 5 h. Control cells were cultured for the same times without any additions. Finally, the levels of mRNA were analysed by real-time PCR, corrected for differences in β-actin mRNA levels, and indicated as fold induction. Each panel is representative of a set of three experiments yielding similar results, and values are plotted as the mean ± SEM. Statistical data: $*P < 0.01$ for TO901317-treated, PAF-treated and GSH-treated, NAC-treated, vitamin E-treated, TEMPO-treated or $FeSO_4$-treated plus $CuSO_4$-treated versus antioxidant-treated or pro-oxidant-untreated; $**P < 0.01$ for $FeSO_4$-treated plus $CuSO_4$-treated and TO901317-treated versus $FeSO_4$-untreated plus $CuSO_4$-untreated.

effect of PAF on LXRα and LXRα-responsive gene expression was found to be potentiated (Fig. 3C).

Possible involvement of mitogen-activated protein kinases (MAPKs) in mRNA expression of LXRα and its target genes in human neutrophils

LXRα is phosphorylated at Ser198 by MAPKs [24]. Thus, we analysed the possible participation of MAPK pathways in the modulation of LXRα mRNA expression and its downregulation promoted by PAF in human neutrophils. Neutrophils were incubated, prior to addition of PAF to the medium, with different kinase inhibitors, such as PD098059 (an inhibitor of MEK1/2, the upstream activator of ERK1/2), SB203580 (a p38 MAPK inhibitor), and SP600125 [an inhibitor of c-Jun N-terminal kinase (JNK)1/2]. Neither SB203580 nor SP600125 had any significant effect on TO901317-promoted LXRα transcription (Fig. 4A), and nor did they alter the negative effect of PAF on this process (Fig. 4A). Therefore, neither p38 nor JNK1/2 MAPKs seemed to be involved in the regulation of LXRα gene transcription in human neutrophils. Nevertheless, when these cells were preincubated with the MEK1/2 inhibitor PD098059, LXRα mRNA synthesis induced by TO901317 was considerably enhanced (Fig. 4A). In fact, this inhibitor was able by itself to induce LXRα mRNA expression (Fig. 4A). The effect observed on LXRα with the inhibitor PD098059 was also seen with its target genes (Fig. 4B). These data suggested that ERK1/2 activation downregulated the mRNA transcription induced by TO901317 of LXRα and its target genes, and that the inhibitory effect of PAF on this process could be mediated by ERK1/2 activation.

Next, we investigated the effect of PAF on the phosphorylation status of ERK1/2 (p42/44) in neutrophils at different times of incubation, up to 1 h. We observed that PAF induced maximum levels of ERK1/2 phosphorylation at 3 min, and that this effect progressively decreased at longer times (Fig. 5A). Moreover, when a dose–response study was performed at PAF concentrations ranging from 1 nM to 100 nM, this ligand induced ERK1/2 phosphorylation in a dose-dependent manner,

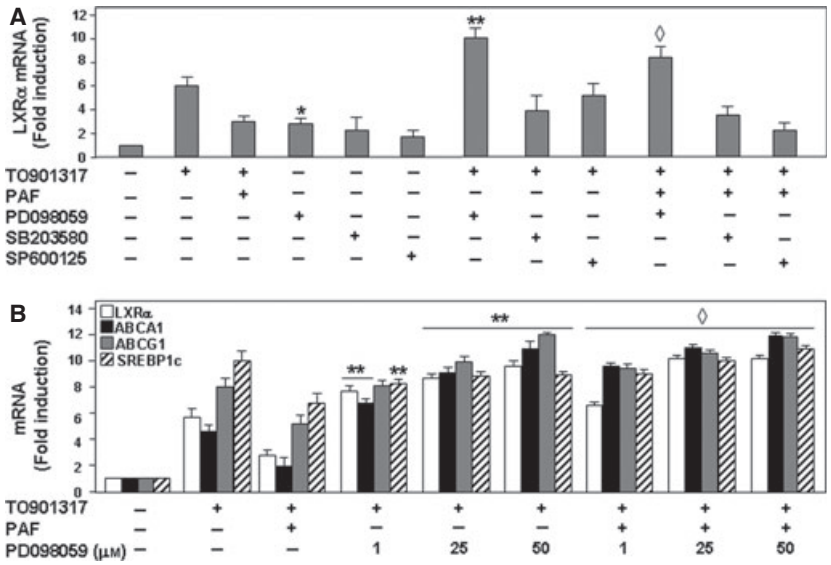

Fig. 4. Possible involvement of MAPKs in mRNA expression of LXRα and its target genes in human neutrophils. (A) Cells were preincubated at 37 °C with or without 25 μM PD98059, 10 μM SB203580 or 1 μM SP600125 for 30 min. Then, they were treated with 10 nM PAF for 1 h, and, after addition of 1 μM TO901317, incubated at 37 °C for a further 5 h. (B) PD98059 was added to cells at the indicated concentrations. After 30 min, 10 nM PAF was added for 1 h, and the cells were then incubated with 1 μM TO901317 for 5 h. The levels of mRNA were analysed by real-time PCR, corrected for differences in β-actin mRNA levels, and indicated as fold induction. Each panel is representative of a set of three experiments yielding similar results, and values are plotted as the mean ± SEM. Statistical data: $*P < 0.01$ for PD098059-treated versus PD098059-untreated; $**P < 0.01$ for PD098059-treated and TO901317-treated versus PD098059-untreated; $\lozenge P < 0.01$ for PD098059-treated, PAF-treated and TO901317-treated versus PD098059-untreated.

as illustrated in Fig. 5B, with the highest values being observed at 10 nM PAF. In contrast, TO901317 did not induce any ERK1/2 phosphorylation by itself. Interestingly, pretreatment with TO901317 inhibited ERK1/2 phosphorylation induced by PAF.

Involvement of NF-κB in mRNA expression of LXRα and its target genes in human neutrophils

PAF is known to act as an activator of NF-κB *in vivo* and *in vitro* [16] by promoting the phosphorylation of IκB inhibitory proteins. These then become ubiquitinated, releasing NF-κB, which thereafter translocates to the nucleus to activate its target genes [17]. Many kinases have been suggested to potentially phosphorylate IκB, which led us to study whether the inhibition of ERK phosphorylation by PD098059 affected NF-κB activation induced by PAF. Neutrophils were therefore preincubated at 37 °C in the presence or absence of PD98059 or MG132 (a specific proteasome inhibitor that blocks NF-κB activation). The cells were then treated with PAF, and IκB levels were measured in total cell extracts. PAF induced IκB degradation, with the levels found being 30% lower than in untreated neutrophils (Fig. 6A). However, when the cells were treated with both PAF and MG132, the inhibitory effect of PAF on IκB disappeared, as expected (Fig. 6A). Treatment of neutrophils with PAF and PD098059 also reversed the degradation of IκB observed upon PAF treatment. We thus concluded that ERK inhibition blocked the NF-κB activation induced by PAF. Furthermore, in order to analyse whether this IκB decrease correlated with NF-κB p65 subunit nuclear translocation, immunocytochemical experiments were performed. Figure 6B shows that, in resting neutrophils, the p65 subunit was exclusively located in the cytosol, whereas in PAF-treated cells this polypeptide also appeared in the nucleus (albeit remaining partly cytosolic). Noticeably, IκB disappearance from the cytosol was observed to occur prior to p65 nuclear translocation (Fig. 6A).

Subsequent experiments were performed to determine whether NF-κB was involved in the PAF inhibition of the expression of LXRα and its target genes. The cells were therefore preincubated at 37 °C with or without the NF-κB inhibitor MG132 before they were treated with PAF, and LXRα expression was finally induced by TO901317 addition. As shown in Fig. 6C, we observed that the NF-κB inhibitor was able to reverse the negative PAF effect on the expression of all four genes, i.e. the LXRα gene and its targets, encoding ABCA1, ABCG1, and SREBP1c.

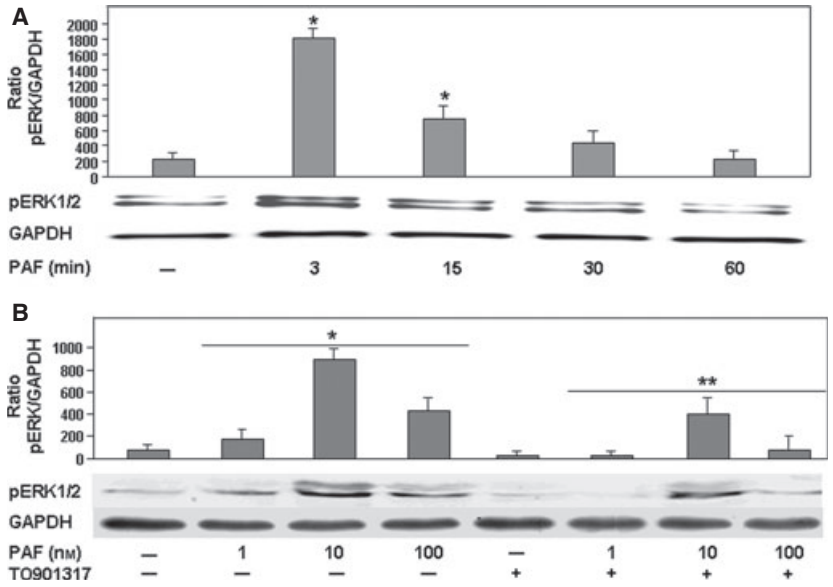

Fig. 5. Effect of PAF on ERK1/2 phosphorylation status in human neutrophils. Cells were left untreated (A) or incubated with 1 μM TO901317 for 5 h (B). Then, PAF was added at 10 nM for the indicated times (A) or at the indicated concentrations for 15 min (B). Phosphorylated ERK1/2 (pERK1/2) levels were analysed in cell lysates by western blotting, and are given in arbitrary units. Control cells were cultured for the same times without any additions. Each panel is representative of a set of three experiments yielding similar results, and values are plotted as the mean ± SEM. Statistical data: $*P < 0.05$ for PAF-treated versus untreated; $**P < 0.01$ for PAF-treated and TO901317-treated versus TO901317-untreated.

Discussion

LXRs regulate lipid metabolism and exert anti-inflammatory effects, although the latter are not well established. Most studies on LXRs have focused on macrophages, with little attention being paid to neutrophils. We have recently reported that mRNA expression of LXRα (but not LXRβ) is induced in human neutrophils by a synthetic LXR ligand, TO901317 [20]. In the present study, we investigated the effect on LXRα mRNA expression in neutrophils exerted by PAF, a strong proinflammatory and prooxidant mediator that links the haemostatic and innate immune systems. We observed that PAF at 10 nM produced a clear inhibition of TO901317-induced LXRα mRNA expression, and this was also seen for the LXR target genes encoding ABCA1, ABCG1, and SREBP1c. Similar behaviour was observed when the natural LXR ligand 22*R*-OH-cholesterol was used instead of TO901317.

Neutrophils are activated by PAF via PAFR, which is a member of the seven-transmembrane domain receptor family (also termed G-protein-coupled receptors), which participate in various second messenger systems, including those involving phospholipase A_2, C and D activation and the MAPK cascade [25]. By using a PAFR antagonist, we found evidence that the effects on LXRα activation induced by PAF were mediated by this receptor. In this context, PAFR has been associated with foam cell formation, because it plays a pivotal role in mediating oxLDL uptake, and could thus constitute a promising target for atherosclerosis treatment [26].

Next, possible changes in the proinflammatory effects of PAF on neutrophils after oxysterol activation of LXRα were evaluated. PAF mediates the effects of different proinflammatory cytokines [27], and IL-8 is an important cytokine produced by neutrophils under oxidative conditions. In this context, we found in the present study that IL-8 release is significantly decreased in PAF-stimulated neutrophils when LXRα is activated by TO901319. Interaction of oxLDL with the PAFR also induces IL-8 release in macrophages [26], and other authors have reported that LXRα may regulate cytokine release in human monocytes [28] and pancreatic islets [29]. A recent study by Hong *et al.* [30] demonstrated that LXRs are involved in the control of neutrophil homeostasis and LXR activation in neutrophils by directly repressing the IL-23–IL-17–granulocyte colony-stimulating factor granulopoietic cytokine cascade. PAF also induces chemotaxis in neutrophils, and this important proinflammatory action was also found to be reduced in LXRα-activated neutrophils in this study. Similar results have been obtained in TO901317-treated neutrophils from the lung [31]. PAF can directly stimulate the production of ROS by inflammatory cells, such as neutrophils, eosinophils, and macrophages, both *in vitro* and *in vivo* [32]. We have also assessed this proinflammatory capacity of PAF, and found that the pretreatment of neutrophils with TO901319 diminished the ROS release induced by PAF in a time-dependent fashion. However, when PAF pretreatment was performed for < 1 h, we did not observe any change. The long pretreatment time needed to induce changes, as described above, suggested that PAF might act by altering gene expression.

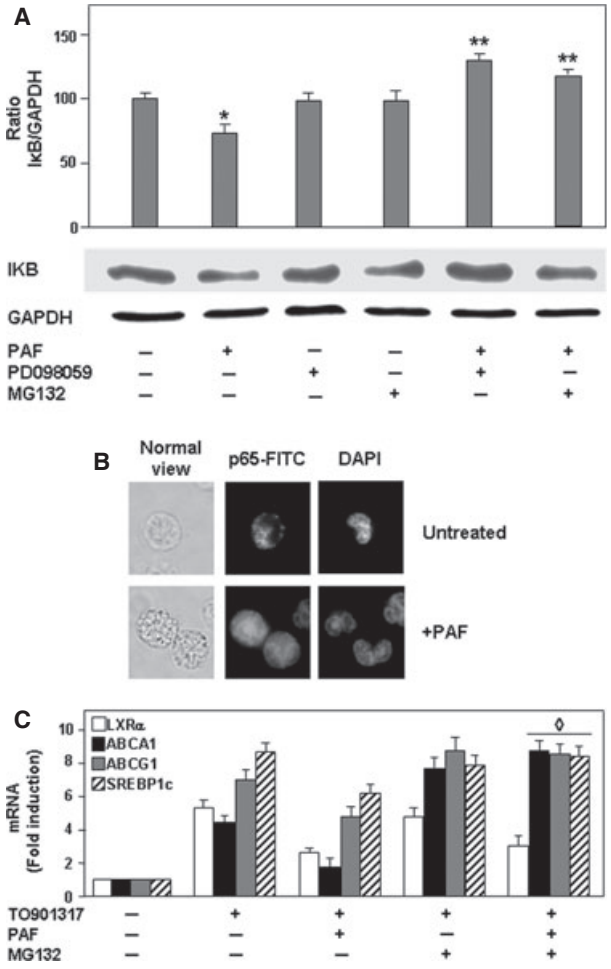

Fig. 6. Effect of PAF on NF-κB activation in human neutrophils. Cells were preincubated at 37 °C with or without 25 μM PD98059 (A) or 25 μM MG132 (A, C) for 30 min. They were then incubated with 10 nM PAF for 3 min (A), 15 min (B), or 1 h (C), and then with or without 1 μM TO901317 for a further 5 h (C). Levels of IκB and GAPDH were assessed by western blotting and expressed as the IκB/GAPDH ratio (A). p65 nuclear translocation was assessed by using antibodies against p65 and FITC-conjugated anti-rabbit IgG. Immunostained and 4′,6-diamidino-2-phenylindole (DAPI)-stained cells were photographed under a Nikon EFD-3 fluorescence microscope (B). The levels of mRNA were analysed by real-time PCR, corrected for differences in β-actin mRNA levels, and expressed as fold induction (C). Each panel is representative of a set of three experiments yielding similar results, and values are plotted as the mean ± SEM. Statistical data: $*P < 0.01$ for PAF-treated versus untreated; $**P < 0.01$ for PAF-treated and PD098059-treated or MG132-treated versus PD098059-untreated or MG132-untreated; $^{\circ}P < 0.05$ for TO901317-treated and PAF-treated and MG132-treated versus MG132-untreated.

LXR activity appears to be regulated by phosphorylation. In studies carried out on macrophages, Chen *et al.* [24] provided the first evidence that LXRs are phosphorylated proteins, and that LXRα is phosphorylated by MAPKs on Ser198, a residue located in its hinge region [24]. It was later found that PAF preferentially activates p38 MAPK, as well as ERK to a lower extent, but that it does not activate JNKs in neutrophils [11]. We thus examined the potential participation of MAPKs in the modulation of LXRα mRNA expression in these cells. We found that treatment of neutrophils with PD098059, an inhibitor of the ERK upstream activator MEK1/2, resulted in a great increase in the mRNA synthesis of LXRα and other target genes induced by TO901317. Even PD098059 alone was able to augment the transcription of these genes, and we also observed that PD098059 reversed the effect of PAF on TO901317-induced LXRα, ABCA1, ABCG1 and SREBP1c mRNA expression in a dose-dependent manner. These data strongly suggest that ERK1/2 activation is involved in the downregulation of TO901317-induced transcription of these genes exerted by PAF in human neutrophils. Therefore, LXRα activity appears to be regulated in these cells through post-translational modification (i.e. phosphorylation) induced by TO901317 and PAF.

Post-translational modifications of nuclear receptors provide a rapid cellular response to environmental changes, the main changes being phosphorylation, acetylation, and SUMOylation [33]. Post-translational modification by phosphorylation has been described for nuclear receptors other than LXRα [34,35], although the functional significance of phosphorylation in the modulation of LXR activity has not been clarified so far. Chen *et al.* [24] found that a phosphorylation-deficient LXRα mutant, S198A, responds to ligands as effectively as does the wild-type on activation of the expression of the genes encoding ABCA1 or ABCG1, suggesting that phosphorylation at Ser198 is not required for the expression of these genes under basal conditions. In contrast, Torra *et al.* [36] revealed a previously unrecognized role of phosphorylation in restricting the repertoire of LXRα-responsive genes. Expression of some established LXRα target genes, such as those encoding apoptosis inhibitor of macrophages and lipoprotein lipase, but not others, such as those encoding ABCA1 or SREBP1c, is increased in RAW 264.7 macrophages expressing the S198A phosphorylation-deficient mutant LXRα as compared with those expressing the wild-type receptor. Other authors have reported LXRα phosphorylation by kinases other than MAPKs. In this context, Yamamoto *et al.* [35] found, in liver, that protein kinase A directly phosphorylates LXRα and inhibits SREBP1c transcription, suggesting a relationship between phosphorylation of LXRα by protein kinase A in fasting conditions and the reduction in expression of the lipogenic gene

encoding SREBP1c. Also, Delvecchio *et al.* [37] reported that transcriptional activation of the gene encoding LXRα and its target genes is decreased by activation of the protein kinase C signalling pathway in monkey kidney COS-1 cells.

Several mechanistic effects have been associated with the phosphorylation of LXRα, such as a decreased ability to dimerize with its obligate partner, the retinoid X receptor, decreased binding of the phosphorylated heterodimer to LXR-responsive DNA elements, or altered cofactor recruitment to its regulated promoters [35]. A possible candidate for preferential binding to the Ser198-phosphorylated LXRα could be nuclear receptor corepressor (NCoR), for which reduced recruitment by the nonphosphorylatable S198A LXRα has been observed [36].

PAF is extracellularly released almost immediately in response to inflammatory stimuli and induced NF-κB activation. This transcription factor is a key regulator of the levels of many proteins involved in the inflammatory process, such as receptors, enzymes, and cytokines [27]. Many of the genes inhibited by LXRs are established targets of NF-κB signalling [38], and activation by PAF of this transcription factor is exerted through the generation of ROS [32]. In fact, our results indicate that PAF acts as an NF-κB activator in an ERK1/2-dependent manner. Also, NF-κB is involved in the negative effects of PAF on the expression of LXRα and its target genes. Taking these results together, we suggest that PAF, through ROS production, induces ERK1/2 phosphorylation, and that this, in turn, inhibits the expression of LXRα and its target genes in an NF-κB-dependent manner.

Intracellular thiols (or oxidants) could also regulate NF-κB activation at other points in this signal transduction pathway [39]. PAF modulates the redox status in U-937 monocyte cells, and antioxidants mostly inhibit PAF-induced NF-κB activation. In this regard, the NF-κB inhibitor pyrrolidine dithiocarbamate, a thiol-containing agent known to be a stable antioxidant, totally abolished PAF-induced secretion of MCP-1 by U-937 monocytes [40].

In summary, the results herein presented show that a possible mechanism for the proinflammatory effect of PAF may be the downregulation of LXRα and its target genes. Despite increasing knowledge on the physiological function and mechanisms of action of LXRs, little is known about the mechanisms by which LXRα expression is regulated. In this light, the results presented in this study point to LXRα phosphorylation as an additional modulatory mechanism for its transcriptional activity. They also support the potential usefulness of LXRα ligands in the therapeutic assessment of neutrophil inflammatory responses.

Experimental procedures

Materials

TO901317 and PAF were obtained from Cayman Chemical (Ann Arbor, MI, USA), and aPAF, SP600125, SB203580 and PD098059 were obtained from Calbiochem (San Diego, CA, USA). RPMI-1640 was obtained from Biomedia (Boussens, France); Dextran T-500 and lymphocyte separation medium (Ficoll-Paque) were obtained from GE Healthcare (Barcelona, Spain), and poly(vinylidene difluoride) membranes were obtained from Pall (Madrid, Spain). Rabbit polyclonal antibodies against phosphorylated (Thr202/Tyr204) ERK1/2 and total (unphosphorylated plus phosphorylated) ERK1/2 were obtained from New England Biolabs (Beverly, MA, USA). Antibodies against p65 and IκB were obtained from Santa Cruz Biotechnology; horseradish peroxidase (HRP)-conjugated goat anti-(rabbit IgG) and goat anti-(mouse IgG) were obtained from Promega (Madison, WI, USA). Mouse mAb against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Chemicon International (Madrid, Spain). TEMPO, luminol, GSH, NAC, $FeSO_4$, $CuSO_4$, protease inhibitor cocktail, vitamin E, MG132, IL-8, HRP and diisopropyl fluorophosphate were obtained from Sigma-Aldrich (Madrid, Spain). Fetal bovine serum, L-glutamine, streptomycin, penicillin and amphotericin B were obtained from BioWhittaker (Basel, Switzerland).

Isolation and culture of human leukocytes

Human peripheral blood neutrophils and lymphocytes were isolated from fresh heparinized blood of human donors by Ficoll-Paque gradient centrifugation and hypotonic lysis of residual erythrocytes [41]. Human monocyte-derived macrophages were obtained from buffy coat preparations by Ficoll-Paque density gradient centrifugation, followed by adhesion-mediated purification on tissue culture or gelatin-coated plastic. Monocytes were induced to differentiate into macrophages *in vitro* by culturing them in medium containing autologous human fibrin-depleted plasma [42]. For experiments, 2×10^7 cells were cultured in RPMI complete medium (RPMI-1640; Life Technologies BRL, Rockville, MD, USA) containing 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine,100 μg/mL streptomycin, 100 IU/mL penicillin, and 250 ng/mL amphotericin B. Before all stimulations, neutrophil suspensions were preincubated at room temperature with 1 mM diisopropyl fluorophosphate (to minimize proteolysis) for 5 min [43], and then incubated at 37 °C for the times indicated in each experiment. In all experiments, cell viability ranged between

90% and 97%, as estimated from the lactate dehydrogenase release assay after treatment [44].

Real-time PCR quantitation of mRNA levels

Real-time PCR analysis was performed with SYBR Green and the ABI Prism 7300 sequence detection system from Applied Biosystems (Foster City, CA, USA) under the specific thermocycler conditions recommended by the manufacturer for the primers used. PCR reactions were performed in triplicate. Each sample was also analysed for β-actin transcript levels to normalize for RNA input amounts. For the relative quantification of gene expression, the comparative threshold cycle method was used, as described in the ABI Prism 7700 User Bulletin 2 [45]. Primers were designed with Primer Express (Applied Biosystems) and synthesized by Roche Diagnostics, and their sequences were as follows: LXRα forward, 5′-AAG CCCTGCATGCCTACGT-3′; LXRα reverse, 5′-TGCAGA CGCAGTGCAAACA-3′; SREBP1c forward, 5′-CATG TCTTCGATGTCGGTCAG-3′; SREBP1c reverse 5′-TCC-TGTTGCCCATATGAAATCA-3′; ABCA1 forward, 5′-CC CTGTGGAATGTACCTATGTG-3′; ABCA1 reverse, 5′-G AGGTGTCCCAAAGATGCAA-3′; ABCG1 forward, 5′-C AGTCGCTCCTTAGCACCA-3′; ABCG1 reverse 5′-TCCA TGCTCGGACTCTCTG-3′; β-actin forward, 5′-CCAGCT CACCATGGATGATG-3′; and β-actin reverse, 5′-ATG CCGGAGCCGTTGTC-3′.

ELISA quantitation of IL-8 release

Cell culture supernatants were collected after treatments, and the levels of secreted IL-8 were quantified with the Human IL-8 ELISA Kit (Raybiotech, Norcross, GA, USA). Plates were read on a Wallac 1420 Victor^2 spectrofluorometer (Perkin Elmer, Madrid, Spain).

Chemotaxis assay

Migration of neutrophils was assessed in Transwell migration chambers (diameter, 6.5 mm; pore size, 5 μm; Costar plates type 3421). Cells were preincubated with or without TO901317 for 4 h at 37 °C. Chemoattractants were deposited in the lower compartment in a final volume of 0.6 mL of RPMI-1640, and the plates were prewarmed at 37 °C. Then, 0.1 mL of medium containing 10^6 neutrophils was deposited on each detachable insert, which was placed over the chemoattractant solution. Loaded chambers were incubated for 2 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. In separate wells, neutrophils were added to the lower compartment and used as controls representing 100% migration. At the end of the incubation period, the cells that had migrated into the bottom chambers were collected and centrifuged at 400 ***g*** for 5 min. After being stained with fluorescein isothiocyanate (FITC)-conjugated mAbs against CD16 (Immunotech, Marseille, France), the cells were fixed with 1% paraformaldehyde and finally counted on a flow cytometer. The results are presented as the mean ± standard error of the mean (SEM) from three separate experiments, and are expressed as the percentage of total neutrophils initially added to each chamber.

Measurement of ROS production

Cells were incubated with or without TO901317 for 4 h at 37 °C. They were then treated with 10 μg/mL HRP and 40 μM luminol, and ROS assays were carried out as indicated previously [46]. The luminol-plus-HRP-derived chemiluminescence was initiated by adding 3 mM NADH as a substrate. The production of ROS in neutrophils was detected in the Wallac 1420 Victor^2 luminescence analyser.

Western blotting analysis of phosphorylated ERK1/2 and IκB

Cells were rinsed once with ice-cold NaCl/P_i, resuspended in a lysis solution containing 50 mM Tris/HCl (pH 7.4), 10 mM EDTA, 50 mM NaF, 10% glycerol, 1% Triton X-100, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM phenylmethanesulfonyl fluoride, and kept on ice for 30 min. The cells were then disrupted by sonication on ice, and, after centrifugation at 12 000 ***g*** for 5 min at 4 °C, the protein concentration in the supernatant was determined with the Bradford method [47], with BSA as a standard. Proteins were boiled in Laemmli loading buffer, resolved by SDS/PAGE (10% polyacrylamide), and transferred to PVDF membranes as previously described [46]. The blots were probed, without need for prior blocking, with rabbit polyclonal antibody against phospho-ERK1/2, at a 1 : 1000 dilution, or rabbit polyclonal antibody against IκB, at a 1 : 2000 dilution, in NaCl/P_i plus 0.5% BSA and 0.02% Tween-20 [48]. Thereafter, HRP-conjugated anti-rabbit IgG or anti-mouse IgG was used at a 1 : 5000 or a 1 : 20 000 dilution, respectively, in NaCl/P_i plus 0.5% casein, and detection was carried out with enhanced chemiluminescence [41]. To verify even protein loading, the blots were subsequently stripped and reprobed with mouse mAbs against GAPDH at a 1: 5000 dilution. Band intensities were measured by scanning densitometry analysis with Scion IMAGE software (Frederick, MD, USA).

Immunofluorescence microscopy analysis of NF-κB p65 nuclear translocation

Nuclear translocation of the NF-κB p65 subunit in human neutrophils was assessed by immunofluorescence cell staining, as described previously [49], with minor modifications. After stimulation with PAF for 15 min, neutrophils

(10^7 cells) were harvested, washed with $NaCl/P_i$, smeared onto poly(L-lysine)-coated glass slides, and fixed at room temperature with 2% paraformaldehyde for 30 min. After washing with $NaCl/P_i$, unspecific binding was blocked with $NaCl/P_i$ containing 10% (v/v) heat-inactivated fetal bovine serum, 0.1% Triton X-100, and 1 mg/mL BSA. Then, the cells were permeabilized with 0.1% Triton X-100 for 10 min, and incubated with rabbit anti-p65 IgG at a 1 : 100 dilution overnight at 4 °C, washed extensively, and stained with FITC-conjugated anti-rabbit IgG at a 1 : 500 dilution for 30 min. After final washing, coverslips were mounted on the slides with 50% glycerol in $NaCl/P_i$. Immunostained cells were observed and photographed with a Nikon EFD-3 fluorescence microscope.

Statistical analysis

mRNA levels measured by real-time PCR are expressed as fold induction relative to untreated cells (mean ± SEM from a minimum of three independent experiments performed with similar results). Protein levels measured from western blots are expressed in arbitrary units. The results were statistically analysed with STATGRAPHICS PLUS 5.0 (Manugistic, Rockville, MD, USA) by means of ANOVA and Student's paired *t*-test.

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References

1 Song C, Kokontis JM, Hiipakka RA & Liao S (1994) Ubiquitous receptor: a receptor that modulates gene activation by retinoic acid and thyroid hormone receptors. *Proc Natl Acad Sci USA* **91**, 10809–10813.
2 Pascual-Garcia M & Valledor AF (2012) Biological roles of liver X receptors in immune cells. *Arch Immunol Ther Exp (Warsz)* **60**, 235–249.
3 Calkin AC & Tontonoz P (2012) Transcriptional integration of metabolism by the nuclear sterol-activated receptors LXR and FXR. *Nat Rev Mol Cell Biol* **13**, 213–224.
4 Viennois E, Mouzat K, Dufour J, Morel L, Lobaccaro JM & Baron S (2012) Selective liver X receptor modulators (SLiMs): what use in human health? *Mol Cell Endocrinol* **351**, 129–141.
5 Glass CK & Ogawa S (2006) Combinatorial roles of nuclear receptors in inflammation and immunity. *Nat Rev Immunol* **6**, 44–55.
6 Ghisletti S, Huang W, Jepsen K, Benner C, Hardiman G, Rosenfeld MG & Glass CK (2009) Cooperative NCoR/SMRT interactions establish a corepressor-based strategy for integration of inflammatory and anti-inflammatory signaling pathways. *Genes Dev* **23**, 681–693.
7 Fontaine C, Rigamonti E, Nohara A, Gervois P, Teissier E, Fruchart JC, Staels B & Chinetti-Gbaguidi G (2007) Liver X receptor activation potentiates the lipopolysaccharide response in human macrophages. *Circ Res* **101**, 40–49.
8 Korf H, Vander Beken S, Romano M, Steffensen KR, Stijlemans B, Gustafsson JA, Grooten J & Huygen K (2009) Liver X receptors contribute to the protective immune response against Mycobacterium tuberculosis in mice. *J Clin Invest* **119**, 1626–1637.
9 Gong H, He J, Lee JH, Mallick E, Gao X, Li S, Homanics GE & Xie W (2009) Activation of the liver X receptor prevents lipopolysaccharide-induced lung injury. *J Biol Chem* **284**, 30113–30121.
10 Marcil V, Delvin E, Sane AT, Tremblay A & Levy E (2006) Oxidative stress influences cholesterol efflux in THP-1 macrophages: role of ATP-binding cassette A1 and nuclear factors. *Cardiovasc Res* **72**, 473–482.
11 Penna C, Bassino E & Alloatti G (2011) Platelet activating factor: the good and the bad in the ischemic/reperfused heart. *Exp Biol Med (Maywood)* **236**, 390–401.
12 Tselepis AD & John Chapman M (2002) Inflammation, bioactive lipids and atherosclerosis: potential roles of a lipoprotein-associated phospholipase A2, platelet activating factor-acetylhydrolase. *Atheroscler Suppl* **3**, 57–68.
13 Nasopoulou C, Tsoupras AB, Karantonis HC, Demopoulos CA & Zabetakis I (2011) Fish polar lipids retard atherosclerosis in rabbits by down-regulating PAF biosynthesis and up-regulating PAF catabolism. *Lipids Health Dis* **10**, 213.
14 Akkerman JW (2008) From low-density lipoprotein to platelet activation. *Int J Biochem Cell Biol* **40**, 2374–2378.
15 Chen R, Chen X, Salomon RG & McIntyre TM (2009) Platelet activation by low concentrations of intact oxidized LDL particles involves the PAF receptor. *Arterioscler Thromb Vasc Biol* **29**, 363–371.
16 Borthakur A, Bhattacharyya S, Alrefai WA, Tobacman JK, Ramaswamy K & Dudeja PK (2010) Platelet-activating factor-induced NF-kappaB activation and

IL-8 production in intestinal epithelial cells are Bcl10-dependent. *Inflamm Bowel Dis* **16**, 593–603.
17 Brigelius-Flohe R & Flohe L (2011) Basic principles and emerging concepts in the redox control of transcription factors. *Antioxid Redox Signal* **15**, 2335–2381.
18 Arbel Y, Finkelstein A, Halkin A, Birati EY, Revivo M, Zuzut M, Shevach A, Berliner S, Herz I, Keren G *et al.* (2012) Neutrophil/lymphocyte ratio is related to the severity of coronary artery disease and clinical outcome in patients undergoing angiography. *Atherosclerosis* **225**, 456–460.
19 Mazor R, Shurtz-Swirski R, Farah R, Kristal B, Shapiro G, Dorlechter F, Cohen-Mazor M, Meilin E, Tamara S & Sela S (2008) Primed polymorphonuclear leukocytes constitute a possible link between inflammation and oxidative stress in hyperlipidemic patients. *Atherosclerosis* **197**, 937–943.
20 Alba G, Reyes ME, Santa-Maria C, Ramirez R, Geniz I, Jimenez J, Martin-Nieto J, Pintado E & Sobrino F (2012) Transcription of liver X receptor is down-regulated by 15-deoxy-delta(12,14)-prostaglandin J(2) through oxidative stress in human neutrophils. *PLoS ONE* **7**, e42195.
21 Bensinger SJ & Tontonoz P (2008) Integration of metabolism and inflammation by lipid-activated nuclear receptors. *Nature* **454**, 470–477.
22 Gaines P, Chi J & Berliner N (2005) Heterogeneity of functional responses in differentiated myeloid cell lines reveals EPRO cells as a valid model of murine neutrophil functional activation. *J Leukoc Biol* **77**, 669–679.
23 Rouis M, Nigon F & Chapman MJ (1988) Platelet activating factor is a potent stimulant of the production of active oxygen species by human monocyte-derived macrophages. *Biochem Biophys Res Commun* **156**, 1293–1301.
24 Chen M, Bradley MN, Beaven SW & Tontonoz P (2006) Phosphorylation of the liver X receptors. *FEBS Lett* **580**, 4835–4841.
25 Tsuda M, Tozaki-Saitoh H & Inoue K (2011) Platelet-activating factor and pain. *Biol Pharm Bull* **34**, 1159–1162.
26 Rios FJ, Koga MM, Ferracini M & Jancar S (2012) Co-stimulation of PAFR and CD36 is required for oxLDL-induced human macrophages activation. *PLoS ONE* **7**, e36632.
27 Fernandes ES, Passos GF, Campos MM, de Souza GE, Fittipaldi JF, Pesquero JL, Teixeira MM & Calixto JB (2005) Cytokines and neutrophils as important mediators of platelet-activating factor-induced kinin B1 receptor expression. *Br J Pharmacol* **146**, 209–216.
28 Myhre AE, Agren J, Dahle MK, Tamburstuen MV, Lyngstadaas SP, Collins AJ, Foster SJ, Thiemermann C, Aasen AO & Wang JE (2008) Liver X receptor is a key regulator of cytokine release in human monocytes. *Shock* **29**, 468–474.
29 Scholz H, Lund T, Dahle MK, Collins JL, Korsgren O, Wang JE & Foss A (2009) The synthetic liver X receptor agonist GW3965 reduces tissue factor production and inflammatory responses in human islets in vitro. *Diabetologia* **52**, 1352–1362.
30 Hong C, Kidani Y, A-Gonzalez N, Phung T, Ito A, Rong X, Ericson K, Mikkola H, Beaven SW, Miller LS *et al.* (2012) Coordinate regulation of neutrophil homeostasis by liver X receptors in mice. *J Clin Invest* **122**, 337–347.
31 Crisafulli C, Mazzon E, Paterniti I, Galuppo M, Bramanti P & Cuzzocrea S (2010) Effects of liver X receptor agonist treatment on signal transduction pathways in acute lung inflammation. *Respir Res* **11**, 19.
32 Choi JH, Chung WJ, Han SJ, Lee HB, Choi IW, Lee HK, Jang KY, Lee DG, Han SS, Park KH *et al.* (2000) Selective involvement of reactive oxygen intermediates in platelet-activating factor-mediated activation of NF-kappaB. *Inflammation* **24**, 385–398.
33 Anbalagan M, Huderson B, Murphy L & Rowan BG (2012) Post-translational modifications of nuclear receptors and human disease. *Nucl Recept Signal* **10**, e001.
34 Rochette-Egly C (2003) Nuclear receptors: integration of multiple signalling pathways through phosphorylation. *Cell Signal* **15**, 355–366.
35 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. *J Biol Chem* **282**, 11687–11695.
36 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.
37 Delvecchio CJ & Capone JP (2008) Protein kinase C alpha modulates liver X receptor alpha transactivation. *J Endocrinol* **197**, 121–130.
38 Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ & Tontonoz P (2003) Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nat Med* **9**, 213–219.
39 Staal FJ, Roederer M, Herzenberg LA & Herzenberg LA (1990) Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. *Proc Natl Acad Sci USA* **87**, 9943–9947.
40 Verouti SN, Fragopoulou E, Karantonis HC, Dimitriou AA, Tselepis AD, Antonopoulou S, Nomikos T & Demopoulos CA (2011) PAF effects on MCP-1 and IL-6 secretion in U-937 monocytes in

comparison with oxLDL and IL-1beta effects. *Atherosclerosis* **219**, 519–525.
41 Carballo M, Marquez G, Conde M, Martin-Nieto J, Monteseirin J, Conde J, Pintado E & Sobrino F (1999) Characterization of calcineurin in human neutrophils. Inhibitory effect of hydrogen peroxide on its enzyme activity and on NF-kappaB DNA binding. *J Biol Chem* **274**, 93–100.
42 Davies JQ & Gordon S (2005) Isolation and culture of human macrophages. *Methods Mol Biol* **290**, 105–116.
43 Gilbert C, Rollet-Labelle E & Naccache PH (2002) Preservation of the pattern of tyrosine phosphorylation in human neutrophil lysates. II. A sequential lysis protocol for the analysis of tyrosine phosphorylation-dependent signalling. *J Immunol Methods* **261**, 85–101.
44 Gualberto A, Marquez G, Carballo M, Youngblood GL, Hunt SW, 3rd, Baldwin AS & Sobrino F (1998) p53 transactivation of the HIV-1 long terminal repeat is blocked by PD 144795, a calcineurin-inhibitor with anti-HIV properties. *J Biol Chem* **273**, 7088–7093.
45 Alba G, El Bekay R, Chacon P, Reyes ME, Ramos E, Olivan J, Jimenez J, Lopez JM, Martin-Nieto J, Pintado E *et al.* (2008) Heme oxygenase-1 expression is down-regulated by angiotensin II and under hypertension in human neutrophils. *J Leukoc Biol* **84**, 397–405.
46 El Bekay R, Alvarez M, Carballo M, Martin-Nieto J, Monteseirin J, Pintado E, Bedoya FJ & Sobrino F (2002) Activation of phagocytic cell NADPH oxidase by norfloxacin: a potential mechanism to explain its bactericidal action. *J Leukoc Biol* **71**, 255–261.
47 Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
48 Mansfield MA (1995) Rapid immunodetection on polyvinylidene fluoride membrane blots without blocking. *Anal Biochem* **229**, 140–143.
49 El Bekay R, Alba G, Reyes ME, Chacon P, Vega A, Martin-Nieto J, Jimenez J, Ramos E, Olivan J, Pintado E *et al.* (2007) Rac2 GTPase activation by angiotensin II is modulated by Ca^{2+}/calcineurin and mitogen-activated protein kinases in human neutrophils. *J Mol Endocrinol* **39**, 351–363.