Involvement of PGE₂ and the cAMP signalling pathway in the up-regulation of COX-2 and mPGES-1 expression in LPS-activated macrophages

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INTRODUCTION

PGs (prostaglandins) and TXs (thromboxanes) are important lipid mediators involved in physiological and pathological processes. These agents are generated from the conversion of AA (arachidonic acid) into the intermediate mediator PGH₂ by two different COXs (cyclo-oxygenases), COX-1 and COX-2 (reviewed in [1,2]). These enzymes are the target of NSAIDs (non-steroidal anti-inflammatory drugs) [3]. COX-1 has been defined as a constitutive enzyme that generates PGs within physiological homeostasis. In contrast, COX-2 is an inducible enzyme responsible for PG production in different pathological processes involving inflammation, such as infectious diseases, cancer, arthritis and atherosclerosis [4–7]. PGH₂ is the substrate of different PG and TX synthases that, in turn, generate a range of prostanoids with potent and diverse biological effects, such as PGD₂, PGE₂, PGF₂α, PG₁ and TXA₂. Three types of PGESs (PGE synthases) participating in the synthesis of PGE₂ have been described: one cPGES (cytosolic PGES) and two membrane-associated PGESs, mPGES (microsomal PGES)-1 and -2 [3,8]. mPGES-1, which belongs to the MAPEG (membrane-associated PGES) family, is inducible by similar stimuli that induce COX-2, its induction also being suppressed by glucocorticoids. Moreover, mPGES-1 is inducible by similar stimuli that induce COX-2 and its induction is usually co-ordinated with COX-2 [9]. In macrophages, large amounts of PGE₂ are generated during the inflammatory process, due to up-regulation of both COX-2 and mPGES-1 enzymes. Co-ordinated induction of the expression of COX-2 and mPGES-1 by pro-inflammatory stimuli, such as LPS (lipopolysaccharide), IL ( interleukin)-1β or TNFα (tumour necrosis factor α), has been reported in several cell types [10–13].

Prostanoids released into the extracellular medium exert their biological effects in an autocrine or paracrine fashion upon interaction with prostanoid receptors present in target cells. PGE₂ signals through four G-protein-coupled receptors named EP (E-prostanoid) 1, EP2, EP3 and EP4 (reviewed in [14–17]). EP receptors are linked to different transduction pathways that may even give rise to opposite effects, i.e. activation or inhibition, on cellular responses. Thus EP3 induces the inhibition of adenylate cyclase, leading to a decrease in cAMP, whereas EP2 and EP4 receptors activate this enzyme. On the other hand, EP1 is coupled to Gₐα and its activation results in an increase in intracellular calcium.

cAMP is thought to be the main intracellular second messenger of PGE₂ signalling in macrophages, playing a crucial role in the modulation of the functional activity of macrophages and monocytes. In fact, PGE₂-dependent elevation of intracellular cAMP in LPS-stimulated macrophages results in a decreased synthesis of pro-inflammatory cytokines, including TNFα [18,19] and IL-1β [20], and in an increased production of the inflammatory cytokine IL-10 [21]. On the other hand, stimuli known to elevate intracellular cAMP levels, such as PGE₂, may positively modulate COX-2 expression [22,23].
We have previously reported an essential role of NF-κB (nuclear factor κ B) and EGR-1 (early growth-response gene product 1) as key factors involved in co-ordinated up-regulation of COX-2 and mPGES-1 expression in macrophages in response to LPS, leading to increased PGE_2 production [13]. In the present study, we explore the importance of cAMP signalling in the regulation of COX-2 and mPGES-1 in LPS-stimulated macrophages, analysing the role of PGE_2-dependent signalling. Our results show that PGE_2 induces transcriptional activation of both COX-2 and mPGES-1 via mechanisms involving EP2 receptor activation and the cAMP/PKA (protein kinase A)/CREB [CRE (cAMP-response element)-binding protein] signalling pathway. This positive-feedback regulation of the PGE_2-synthesizing enzymes COX-2 and mPGES-1 in macrophages constitutes an amplification signalling that may play an important role in the modulation of the inflammatory process and the immune response.

EXPERIMENTAL

Cell culture and reagents

The mouse macrophage cell line RAW 264.7 (A.T.C.C., Rockville, MD, U.S.A.) was cultured in RPMI 1640 medium (Invitrogen) supplemented with 5% fetal calf serum (Lonza), 100 units/ml penicillin, 100 μg/ml streptomycin, 1000 units/ml gentamycin, 2 mM l-glutamine and 0.1 mM non-essential amino acids. Cells were treated with LPS from Escherichia coli serotype 026:B6 (Sigma–Aldrich) at 1 μg/ml. The selective COX-2 inhibitors Celecoxib and NS398 (Alexis Biochemicals) were used at final concentrations between 0.01 and 1 μM. The PGE_2, EP2 and EP4 agonists CAY10399 and PGEOH, and the EP2 antagonist AH689, were purchased from Cayman Chemical. dbcAMP (dibutyryl-cAMP; 100 μM) and forskolin (10 μM) were from Sigma. NSAIDs and PKA inhibitors H89 (10 μM) and KT5720 (1 μM) (Enzo Life Sciences) were added 1 h before cell stimulation with PGE_2 or LPS.

Plasmid constructs

The COX2 promoter–luciferase construct PGHS-2 Medium (−1844) (PGHS is PGH endoperoxide synthase) was provided by Dr Stephanie Vogel (University of Maryland Medical Center, Baltimore, MD, U.S.A.) [24]. PGHS-2-400 and PGHS-2-250 constructs were kindly provided by Dr Harvey Herschman (Molecular and Medical Pharmacology, University of California at Los Angeles, Los Angeles, CA, U.S.A.) [25]; PGHS-2-150, PGHS-2-88 and CRE-mutated PGHS-2-400 and PGHS-2-88 constructs were generated by Dr Virginia Vila-del Sol (Centro de Biología Molecular Severo Ochoa, Universidad Autónoma de Madrid, Madrid, Spain) [26]. Luciferase constructs containing different deletions of the murine promoter of mPGES-1 (mPGES-1-895, mPGES-1-694, mPGES-1-483 and mPGES-1-154) were as described previously [13]. The CRE-LUC (luciferase) plasmid contained four copies of the CRE site of the human choriongonadotropin gene promoter (−147 to −129) [27]. An EP2 expression vector (pcDNA3.1-EP2) was obtained from Missouri S&T UMR cDNA Resource Center. The expression vector for the catalytic subunit of PKA was as described previously [28].

Transient transfection

COX2 and mPGES-1 promoter activity was analysed by luciferase reporter gene assays. RAW 264.7 cells were transiently transfected with 0.5–2 μg of the different luciferase constructs along with 250 ng of pcDNA3 or pcDNA 3.1-EP2 plasmids using Lipofectamine™ 2000 reagent (Invitrogen). After 5 h of transfection, cells were treated with different stimuli for an additional 18 h. Then, cells were harvested and lysed, and luciferase activity was determined by using a luciferase assay kit (Promega) in a Monolight 2010 luminometer (Analytical Luminescence Laboratory). Transfection experiments were performed in triplicate. Results are expressed as mean fold induction ± S.D. [observed experimental RLUs (relative luminescence units)/basal RLUs in the absence of any stimulus].

mRNA analysis

Total RNA was obtained from cells using the TRIzol® reagent RNA isolation protocol (Invitrogen). For standard RT (reverse transcription)–PCR, RNA (1 μg) was reverse-transcribed by the RNA PCR core kit (PerkinElmer). cDNA was used for PCR amplification to analyse EP expression by standard RT–PCR using specific primers: EP1, sense 5′-TTAACCTGAGCCTAGCAGATG-3′ and antisense 5′-CCGTCGAGCTTATggCACACTA-3′; EP2, sense 5′-CCACGTGCTTCTGCTGTTAT-3′ and antisense 5′-CAGCCCCCTTACACCTTCAAA-3′; EP3, sense 5′-TGACCTTTGCGCCACCT-3′ and antisense 5′-GGCGCCAGAAAGATCT-3′; EP4, 5′-CTTCTACTTGCCCACTCTCTGGT-3′ and antisense 5′-TGGTGGCCTCCTTTACTCCAC-3′; and β-actin, sense 5′-CTCTTTGATGTCAGCAGATTTC-3′ and antisense 5′-GGGGCGCTCTTAGGCCACAA-3′. Template was amplified by 25–30 cycles of denaturation at 94°C for 30 s, annealing at 55 or 60°C for 45 s, and extension at 72°C for 45 s. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Results shown correspond to a number of cycles at which the amount of amplified product is proportional to the abundance of starting material.

For quantitative real-time RT–PCR analysis, total RNA was reverse-transcribed using the components of the High-Capacity cDNA Archive Kit (Applied Biosystems). Amplification of the COX2 and mPGES-1 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 MGB (minor groove binder) probes for COX1, COX2, mPGES-1, EP1, EP2, EP3, EP4 and 18S rRNA (Applied Biosystems). All samples were run in triplicate. Quantification of gene expression by real-time RT–PCR was calculated by the ΔΔC_T (comparative threshold cycle) method, following the manufacturer’s instructions. RQ (relative quantification) of mRNA levels was determined using endogenous expression of 18S rRNA and is shown in all of the experiments as RQ ± S.D.

Western blotting

Protein extracts were obtained as described previously [13]. Protein concentration was determined by the BCA method (Thermo Scientific). Cell lysates were subjected to Western blot analysis using conventional SDS/PAGE and protein transfer to nitrocellulose filters. Membranes were incubated with the indicated antibodies and developed by the ECL (enhanced chemiluminescence) system (Thermo Scientific). COX2 and mPGES-1 protein expression was detected using a monoclonal anti-COX-2 antibody (BD Transduction Laboratories) and a polyclonal rabbit anti-mPGES-1 antibody (Cayman Chemical). Antibodies against CREB and pCREB were purchased from...
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Figure 1 Inhibition of PGE2 synthesis by COX-2-specific inhibitors reduces COX2 and mPGES-1 gene expression in macrophages

(A) RAW 264.7 cells were treated with LPS (1 μg/ml) for 24 h and COX1, COX2 and mPGES-1 mRNA levels were analysed by real-time quantitative RT-PCR, normalized to the expression of 18S rRNA. Results are shown as the fold induction ± S.D. over the levels in the absence of LPS treatment. (B) RAW 264.7 cells were treated with LPS (1 μg/ml) for 24 h in the presence or absence of increasing doses (1–100 nM) of the COX-2 inhibitors NS398 or Celecoxib (Celec). PGE2 production in cell supernatants of RAW 264.7 cells was determined by a standard EIA assay as described in the Experimental section. Analysis of COX2 (C and D) and mPGES-1 (E and F) mRNA levels by real-time quantitative RT–PCR in RAW 264.7 cells treated with LPS for 24 h along with increasing doses (1–100 nM) of Celecoxib (C and E) or NS398 (D and F) in the presence or absence of PGE2 (5 μM) as indicated. COX2 and mPGES-1 mRNA levels were normalized to the expression of 18S rRNA and are shown as percentage of induction ± S.D., considering the induction obtained upon LPS treatment to be 100 %. *P < 0.05; **P < 0.01. ns, not significant.

Upstate Signalling. The β-actin level was used as a loading control in each lane.

ChIP (chromatin immunoprecipitation) assay

Specific binding of CREB and pCREB to COX2 and mPGES-1 promoters was determined by ChIP assays as described previously [13]. Briefly, RAW 264.7 cells treated with PGE2 (5 μM) at the indicated times were fixed with 1% formaldehyde and lysed in ice-cold lysis buffer [10 mM Hepes, pH 7.6, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT (dithiothreitol) and 0.1% NP-40 (Nonidet P40) with protease inhibitors]. The pellet of nuclei was suspended in nuclear lysis buffer (50 mM Tris/HCl, pH 8, 10 mM EDTA, 1% SDS and protease inhibitors) and then chromatin DNA was sheared by sonication. Lysates were pre-cleared with salmon sperm/Protein A–agarose. A sample of input DNA was collected. Protein–DNA complexes were immunoprecipitated overnight at 4°C with anti-CREB or anti-pCREB polyclonal antibodies or non-immune rabbit antiserum as a control. Antibody–protein–DNA complexes were incubated with salmon sperm DNA/Protein A–agarose for 30 min followed by washes with wash buffer (20 mM Tris/HCl, pH 8, 2 mM EDTA, 0.1% SDS, 1% NP-40 and 500 mM NaCl) and TE buffer (20 mM Tris/HCl, pH 8.0, and 2 mM EDTA). Protein–DNA complexes were eluted and disrupted by incubating at 65°C followed by proteinase K treatment. DNA was extracted with a QIAquick PCR Purification kit (Qiagen). PCR was conducted using promoter-specific primers for COX2 and mPGES-1, and amplified bands were analysed by 2% agarose gel electrophoresis.

PGE2 determination

After various treatments, PGE2 levels were measured in culture supernatants of RAW 264.7 cells using a competitive immunoassay PGE2 EIA (enzyme immunoassay) kit, following the manufacturer’s instructions (Cayman Chemical).

Statistics

Results are expressed as means ± S.D. from at least three independent experiments performed in duplicate or triplicate.
RESULTS

Effect of the inhibition of PGE\textsubscript{2} production on LPS-mediated induction of COX-2 and mPGES-1 expression

Expression of the enzymes COX-1, COX-2 and mPGES-1 involved in PGE\textsubscript{2} biosynthesis was assessed by quantitative real-time RT–PCR in the murine macrophage cell line RAW 264.7 after LPS treatment (Figure 1A). Low levels of expression of COX-1 (average $C_T = 33.11$), COX-2 (average $C_T = 29.28$) and mPGES-1 (average $C_T = 32.15$) were observed in these cells in basal conditions. Upon LPS treatment for 24 h, COX-2 and mPGES-1 mRNA levels were up-regulated 20- and 5-fold respectively, whereas COX1 expression was reduced by 2-fold, thus pointing to COX-2 and mPGES-1 as the main enzymes involved in enhanced PGE\textsubscript{2} production in LPS-treated RAW 264.7 cells. Accordingly, treatment with increasing doses (1–100 nM) of the COX-2 inhibitors Celecoxib or NS398 led to a decrease in the production of PGE\textsubscript{2} induced by LPS (Figure 1B).

In order to study the effect of COX-2 inhibitors on the expression of COX-2 and mPGES-1, mRNA levels were determined by quantitative real-time RT–PCR in RAW 264.7 cells treated with LPS for 24 h in the presence of increasing doses of NS398 or Celecoxib. As shown in Figures 1(C)–1(F), LPS-mediated increase in COX-2 and mPGES-1 expression was
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Figure 3 Involvement of EP2 receptor on the transcriptional activation of COX-2 and mPGES-1

(A) Left-hand panel: expression of EP receptors EP1, EP2, EP3 and EP4 in unstimulated RAW 264.7 macrophages. An aliquot of the amplified DNA was separated on an agarose gel and stained with ethidium bromide for qualitative comparison. The average Ct value for each gene obtained by real-time RT–PCR in basal conditions is indicated. β-Actin mRNA levels are shown as a loading control. Middle and right-hand panels: mRNA levels of EP receptors were analysed by quantitative real-time RT–PCR in RAW 264.7 cells stimulated with PGE2 (5 μM) or LPS (1 μg/ml) for the time indicated (h). (B) Analysis by real-time RT–PCR of COX-2 and mPGES-1 expression in response to the EP2 agonist CAY10399 (1 μM) or the EP4 agonist PGE1OH (1 μM). (C) Cells were treated with PGE2 (5 μM) in the presence or absence of EP2 antagonist AH6809 (1 or 5 μM), and COX2 and mPGES-1 mRNA levels were analysed by real-time RT–PCR. Relative mRNA levels were determined using endogenous expression of 18S rRNA and is shown as fold induction +− S.D. (**P < 0.01). Cont, control.

Furthermore, PGE2 co-operated with LPS in the induction of COX2 and mPGES-1 mRNA levels (Figure 2D).

EP2 participates in PGE2-dependent transcriptional induction of COX-2 and mPGES-1

Four different EP receptors mediate PGE2-dependent intracellular signalling [14–16]. In basal conditions, murine macrophage cell lines express mainly EP2 and EP4 receptors [29–31]. Analysis of mRNA levels for the EP receptors in control RAW 264.7 cells confirmed the presence of EP2 and EP4 transcripts (Figure 3A, left-hand panel). Quantitative RT–PCR analysis showed C_T values above 38 for EP1 (38.15 ± 0.43) and EP3 (38.73 ± 0.01) receptors, thus indicating low to negligible levels of expression. C_T values for basal expression of EP2 (35.56 ± 0.12) and EP4 (30.53 ± 0.03) receptors were in the low to moderate range. Upon stimulation with either PGE2 or LPS, EP2 mRNA levels

attenuated by increasing doses of NS398 and Celecoxib (1–100 nM), suggesting that suppression of endogenous PGE2 may cause a reduction in LPS-induced expression of mPGES-1 and COX2 mRNA. Moreover, attenuation of mPGES-1 and COX-2 expression by NSAIDs was effectively restored by exogenous PGE2 (Figures 1C–1F).
dbcAMP increases COX-2 and mPGES-1 expression and PGE2 production in RAW 264.7 cells

(A) COX2 and mPGES-1 mRNA levels from RAW 264.7 cells stimulated with dbcAMP (100 μM) at the indicated times were determined by real-time RT–PCR. Results are shown as the mean fold induction ± S.D. for two independent experiments performed in triplicate. (B) Western blot analysis of COX-2 and mPGES-1 protein expression in response to dbcAMP (100 μM) at different times of treatment (h). (C) PGE2 production by RAW 264.7 cells after stimulation with dbcAMP for different times. The results shown are the means ± S.D. of replicate determinations for three independent assays (*P < 0.01; **P < 0.05).

increased in a time-dependent manner. On the other hand, both PGE2 and LPS treatment promoted a decrease in EP4 mRNA levels (Figure 3A).

dbcAMP co-operates with LPS in the induction of COX-2 and mPGES-1

Combined treatment of RAW 264.7 cells with LPS and dbcAMP strongly enhanced transcription of COX2 and mPGES-1 genes. Induction of COX2 and mPGES mRNA levels in

**Figure 4**

**Figure 5**

dbcAMP increases COX-2 and mPGES-1 expression

PGE2 signalling through the EP2 receptor promoted an increase in intracellular levels of cAMP due to activation of adenylate cyclase [15]. Incubation of RAW 264.7 cells with the cell-permeant cAMP analogue dbcAMP induced an early accumulation of COX2 mRNA at 3 and 8 h, whereas a significant increase in mPGES-1 mRNA levels was detected after 24 h of treatment (Figure 4A). Analysis of protein levels by Western blotting showed an increase in COX-2 and mPGES-1 protein levels after dbcAMP treatment, with maximal induction at 24 h (Figure 4B).

A co-ordinated increase in COX-2 and mPGES-1 expression after dbcAMP stimulation led to enhanced production of PGE2 by these cells (Figure 4C).

dbcAMP co-operates with LPS in the induction of COX-2 and mPGES-1

Figure 5 shows the results of luciferase assays using COX-2 and mPGES-1 mouse promoter constructs (PGHS-2 Medium and mPGES-1-895) that showed transient expression of EP2 receptor mediated transcriptional activation of COX-2 and mPGES-1 after PGE2 stimulation of RAW 264.7 cells (Figure 3D).

A co-ordinated increase in COX-2 and mPGES-1 expression after dbcAMP stimulation led to enhanced production of PGE2 by these cells (Figure 4C).
Figure 6  Involvement of PKA on COX-2 and mPGES-1 expression

(A) COX2 and mPGES-1 mRNA levels were analysed by quantitative real-time RT–PCR in cells treated with forskolin (Forsk) (10 μM) or PGE2 (5 μM) for 24 h in the presence of the PKA inhibitor H89 (10 μM). Results for two independent experiments performed in triplicate were normalized to the levels of the endogenous control 18S rRNA and are shown as fold induction ± S.D. (B) Luciferase activity of COX-2 Luc and mPGES-1 Luc constructs were analysed in RAW 264.7 cells transiently transfected with the EP2 receptor. Cells were pre-treated with H89 1 h prior to PGE2 stimulation for 18 h. Luciferase activity is shown as fold induction ± S.D. A representative for three independent experiments performed in triplicate is shown. (C) Activity of COX-2 Luc and mPGES-1 Luc constructs in RAW 264.7 cells transiently transfected with an expression vector for the catalytic subunit of PKA and treated or not with LPS for 18 h. Luciferase activity is shown as fold induction ± S.D. Statistical difference over the control group is shown (*** P < 0.005; **P < 0.01; *P < 0.05). (D) Analysis of PGE2 production in supernatants of RAW 264.7 cells pretreated with the PKA inhibitors H89 or KT5720 before stimulation with LPS + dbcAMP for 24 h. Results are means ± S.D. for three experiments. Paired Student’s t tests indicate a significant difference between stimulated and H89- or KT5720-treated groups (***P < 0.005; **P < 0.01; *P < 0.05).

these cells by LPS was enhanced in the presence of dbcAMP (Figure 5A), resulting in augmented PGE2 production by these cells (Figure 5B). The effect of dbcAMP on LPS-mediated transcriptional activation was also analysed on COX2 and mPGES-1 promoter activity. As shown in Figure 5(C), LPS and dbcAMP co-operated in the induction of the transcriptional activity of both promoters compared with the induction after stimulation with either dbcAMP or LPS.

PKA regulates COX-2 and mPGES-1 expression by PGE2

Signalling through the cAMP pathway leads to the activation of PKA, which in turn may activate cAMP-dependent gene transcription [32,33]. We next analysed the involvement of this signalling pathway in the activation of COX-2 and mPGES-1 expression in RAW 264.7 macrophages. As shown in Figure 6(A), inhibition of PKA by H89 produced a decrease in the induction of COX-2 and mPGES-1 promoted by PGE2 treatment or by activation of adenylate cyclase by forskolin. PKA inhibition was also able to abolish EP2-mediated induction of COX2 and mPGES-1 promoter activity after PGE2 treatment (Figure 6B). Interestingly, overexpression of an expression vector for the catalytic subunit of PKA significantly increased activity of the COX2 and mPGES-1 promoters and co-operated with LPS to further induce transcriptional activation of these promoters (Figure 6C). Moreover, inhibition of PKA by either H89 or KT5720 promoted a substantial reduction in LPS + dbcAMP induction of PGE2 production by RAW 264.7 cells (Figure 6D).
were stimulated with PGE2, and CRE-dependent transcriptional activation was assayed. The
levels of phosphorylated CREB were detected by immunoblotting with specific antibodies. Relative levels of CREB phosphorylation are shown in the lower panel.

RAW 264.7 cells were transiently transfected with a CRE-dependent luciferase reporter plasmid and treated with PGE2 (5 μM), forskolin (Forsk; 10 μM) or dbcAMP (100 μM) for 18 h. Results are means ± S.D. of replicate determinations expressed as fold induction over the RLUs of unstimulated controls (Cont). Results are representative for at least two independent experiments. (C) RAW 264.7 cells were transfected with CRE-Luc reporter plasmid along with empty vector (pcDNA3) or an expression vector for EP2 receptor. Transfected cells were stimulated with PGE2, and CRE-dependent transcriptional activation was assayed. The means of replicate determinations expressed as fold induction ± S.D. are shown. Results are representative for at least three independent assays. *P < 0.05; **P < 0.005.

**Figure 7** PGE2 induces CRE-mediated transcriptional activation

(A) RAW 264.7 macrophages were incubated in the absence or presence of PGE2 (5 μM) for the indicated period of time (min). Protein extracts were separated by SDS/PAGE and levels of phosphorylated (P) and total CREB were detected by immunoblotting with specific antibodies. Relative levels of CREB phosphorylation are shown in the lower panel. (B) RAW 264.7 cells were transiently transfected with a CRE-dependent luciferase reporter plasmid (CRE-Luc) and treated with PGE2 (5 μM), forskolin (Forsk; 10 μM) or dbcAMP (100 μM) for 18 h. Results are means ± S.D. of replicate determinations expressed as fold induction over the RLUs of unstimulated controls (Cont). Results are representative for at least two independent experiments. (C) RAW 264.7 cells were transfected with CRE-Luc reporter plasmid along with empty vector (pcDNA3) or an expression vector for EP2 receptor. Transfected cells were stimulated with PGE2, and CRE-dependent transcriptional activation was assayed. The means of replicate determinations expressed as fold induction ± S.D. are shown. Results are representative for at least three independent assays. *P < 0.05; **P < 0.005.

**PGE2 induces CRE-mediated COX-2 and mPGES-1 expression**

Since CREB is the main transcriptional mediator of the cAMP/PKA signal [32,33], we evaluated the effect of PGE2 on CREB phosphorylation and CRE-mediated transcriptional activation. Incubation of RAW 264.7 cells with PGE2 led to efficient CREB phosphorylation (Figure 7A). Furthermore, PGE2 as well as forskolin and dbcAMP activated CRE-dependent gene transcription of a luciferase reporter construct (Figure 7B). CRE-dependent transcription upon PGE2 stimulation was enhanced in cells co-transfected with an EP2 expression vector (Figure 7C).

Previous studies have described a functional CRE-binding site in the murine COX2 promoter [34]. We have analysed the functional significance of this CRE-binding site in PGE2-dependent COX-2 transcriptional activity by using different deletions of the COX2 promoter in cells co-transfected with the EP2 receptor. Mutation of the sequence containing the CRE site within the COX2 gene abolished the induction of COX2 promoter activity by PGE2 in RAW 264.7 cells (Figure 8A). Analysis of the activity of different deletions of the murine mPGES-1 promoter (mPGES-1-895, mPGES-1-694, mPGES-1-483 and mPGES-1-154) showed that deletion of the region located at position −483/−154 of the mPGES-1 transcription start site resulted in a clear reduction of the inducibility by PGE2 (Figure 8C). Sequence analysis of this region in the mPGES-1 murine gene revealed potential CRE elements with the TRANSFAC Database and P-Match software [35].

LPS treatment of RAW 264.7 cells induces the synthesis of COX-2 and mPGES-1, which is accompanied by a significant increase in the release of PGE2. The results of the present study show that COX-2-selective inhibitors down-regulate the expression of these enzymes at a concentration dose that abrogated LPS-mediated PGE2 production in RAW 264.7 cells, suggesting a positive regulation of this PG on the expression of COX-2 and mPGES-1. In fact, exogenous PGE2 treatment reversed the effect of COX-2 inhibitors in LPS-stimulated RAW.264.7 cells. Moreover, PGE2 treatment in unstimulated RAW 264.7 cells was able to induce COX2 and mPGES-1 transcriptional induction. The ability of PGE2 to promote COX-2 and mPGES-1 expression in unstimulated cells was weak compared with LPS, but PGE2 was able to enhance LPS-mediated up-regulation of both enzymes. Induction of COX-2 and mPGES-1 showed different kinetics,
with a delay in mPGES-1 induction in comparison with COX-2. Although COX-2 and mPGES expression vary similarly in response to a variety of stimuli (IL-1β, LPS, TNFα etc.), there are multiple reports showing differences in the specific timing for induction, in such a way that induction of mPGES-1 is generally delayed with respect to COX-2 in several cell systems [10–13]. These observations suggest a differential regulation of these enzymes in terms of the up-regulation and maintenance of steady-state expression levels. Some differences between these two genes that could be relevant to explain divergences in the timing of responses to the same stimuli have been reported. The promoter of human mPGES-1 lacks a TATA box, unlike the COX2 promoter. Furthermore, mPGES-1 mRNA does not contain AUUUA instability motifs that are present in COX2 mRNA [44]. The delayed induction of mPGES-1 mRNA compared with COX2 upon a stimulatory treatment can thus be explained on the basis of a smaller increase in the rate of gene transcription in the setting of a relatively stable mRNA.

PGE2 exerts its effects through a family of G-protein-coupled receptors named EP-1, -2, -3 and -4 that differ in their signal transduction pathways. EP2 and EP4 receptors are coupled to the Gs protein and activate adenylate cyclase, increasing cAMP levels [14–16]. In agreement with previous reports analysing the expression of EP receptors in murine macrophages, we have detected the presence of overexpressed EP2 receptor. CREB at Ser133, resulting in the regulation of cAMP-dependent transcriptional up-regulation of COX-2 and mPGES-1 expression, resulting in augmented PGE2 production. These agents co-operated with LPS to further up-regulate COX-2 and mPGES-1 expression and PGE2 production by RAW 264.7 cells. Involvement of PKA was revealed by the use of the PKA inhibitors H89 and KT5720, which down-regulated cAMP-dependent transcriptional up-regulation of COX-2 and mPGES-1. Furthermore, overexpression of a catalytic active PKA was able to induce the activity of COX2 and mPGES-1 promoters, both in basal and LPS-stimulated RAW 264.7 cells. Activation of PKA by cAMP can result in the phosphorylation of CREB, which interacts with CREs, resulting in the regulation of cAMP-responsive gene expression [32,33]. The present study shows that PGE2 treatment of RAW 264.7 cells induced phosphorylation of CREB at Ser133, resulting in the transcriptional activation of a CRE–luciferase reporter. Induction of CREB-mediated transcriptional activation of this reporter construct could be observed in the presence of overexpressed EP2 receptor. CREB-dependent regulation of COX2 promoter activity in response to a variety of stimuli has been observed in different cell types [34,50–52].
ChIP assays showed that PGE2 treatment led to CREB binding to the murine COX2 promoter, but also to the mPGES-1 promoter, involving for the first time this transcription factor in the regulation of this gene in response to cAMP-elevating agents. Moreover, analysis of the regulation of COX2 as well as mPGES-1 promoter activity by PGE2 in RAW 264.7 cells not only confirmed the role of the CRE sequence in the COX2 promoter, but also localized a PGE2-responsive region (−483 to −154) within the murine mPGES-1 promoter containing two putative CRE elements.

In summary, the results of the present study point to an essential role of the EP2 receptor in PGE2-mediated regulation of COX-2 and mPGES-1 expression though the cAMP/PKA/CREB signalling pathway in RAW 264.7 cells. An autocrine PGE2 positive feedback involving both EP2 as well as EP4 receptors has been suggested previously as a mechanism modulating COX-2 induction and PGE2 production [51–54]. The results of the present study cannot entirely discard the involvement of EP4 receptor-mediated signalling in these effects. EP2 and EP4 receptors apparently act redundantly in some processes, although they show important functional differences that may explain their distinct roles in others [14,15]. Even though these receptors share common signal transduction pathways through the activation of adenylate cyclase, they differ in their desensitization and internalization [55,56] as well as in the signalling properties [15,57]. Gβγ-mediated increases in cAMP seem to play a less important role for EP4 receptor signalling compared with the EP2 receptor in such a way that inhibition of PKA by H89 is able to attenuate PGE2-increases in cAMP seems to play a less important role for EP4 receptor-mediated signalling in these effects. EP2 and EP4 receptors play an important role in the actions of this PG in the modulation of the inflammatory process and the immune response.

AUTHOR CONTRIBUTION
Manuel Díaz-Muñoz designed and performed experiments and analysed data. Inés Osma-García performed experiments. Manuel Fresno contributed with financial support, supervised the interpretation of data and edited the paper prior to submission. Miguel Iñiguez contributed with financial support, and participated in the conception and design of the experiments, and data analysis and interpretation, and wrote the paper. All authors discussed the results and implications and commented on the paper at all stages prior to submission.

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