Involvement of PGE_2 and cyclic AMP signaling pathway in the up-regulation of COX-2 and mPGES-1 expression in LPS -activated macrophages

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Running title: Regulation of COX-2 and mPGES-1 by PGE_2 in macrophages

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Abbreviations

AA, Arachidonic acid; ChIP, Chromatin Immunoprecipitation; COX, Cyclooxygenase; dbcAMP, dibutyryl-cyclic AMP; CRE, cyclic AMP response element; CREB, Cyclic AMP response element binding protein; EP, PGE_2 receptor; LPS, lipopolysaccharide, LUC, luciferase; PG, prostaglandin; mPGES-1, microsomal Prostaglandin E synthase 1; cPGES, cytosolic Prostaglandin E synthase; PGHS, Prostaglandin H endoperoxide synthase; PKA, Protein kinase A; TX, Thromboxane.

Abstract
Prostaglandin (PG) E_2 plays an important role in the modulation of the immune response and the inflammatory process. In this study, we describe a PGE_2 positive feedback for Cyclooxygenase (COX) -2 and microsomal PGE Synthase (mPGES) -1 expression in the macrophage cell line RAW 264.7. Our results show that PGE_2 induces COX-2 and mPGES-1 expression, an effect mimicked by dibutyryl-cAMP (dbcAMP) or Forskolin. Furthermore, cAMP signaling pathway cooperates with LPS in the induction of COX-2 and mPGES-1 transcriptional activation. Analysis of the involvement of EP receptors showed that incubation with EP2 agonists up-regulated both COX-2 and mPGES-1 mRNA levels. Moreover, EP2 receptor over expression enhanced the transcriptional activation of COX-2 and mPGES-1 promoters, being this induction abolished by the PKA inhibitor, H89. Activation of PGE_2/EP2/PKA signaling pathway induced the phosphorylation of the cAMP response element-binding protein (CREB) in macrophages and stimulated the specific binding of this transcription factor to COX-2 and mPGES-1 promoters. Deletion or mutation of potential CRE sites in both promoters diminished their transcriptional activity. In summary, our data demonstrate that activation of PKA/CREB signaling through the EP2 receptor by PGE_2 plays a key role in the expression of COX-2 and mPGES-1 in activated macrophages.

Key words: COX-2, mPGES-1, EP receptors, Lipid mediators, Inflammation, Gene Regulation.

INTRODUCTION

Prostaglandins (PGs) and Thromboxanes (TXs) are important lipid mediators involved in physiological and pathological processes. These agents are generated from the conversion of arachidonic acid (AA) into the intermediate mediator PGH_2 by two different cyclooxygenases, COX-1 and COX-2 (reviewed in [1, 2]). These enzymes are the target of non anti-inflammatory drugs (NSAIDs) [3]. COX-1 has been defined as a constitutive enzyme that generates PGs within physiological homeostasis. On the contrary, COX-2 is an inducible enzyme responsible for PG production in different pathologic processes involving inflammation such as infectious diseases, cancer, arthritis and atherosclerosis [4-7]. PGH_2 is the substrate of different PG and TX synthases that, in turn, generate a range of prostanoids with potent and diverse biological effects, as PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2. Three types of PGE synthases (PGES) participating in the synthesis of PGE_2 have been described: one cytosolic (cPGES) and two membrane-associated PGE synthases (mPGES)-1 and -2 [3, 8]. mPGES-1, which belongs to the Membrane-Associated Proteins involved in Eicosanoid and Glutathione metabolism (MAPEG) superfamily, is inducible by similar stimuli that induce COX-2, being its induction also suppressed by glucocorticoids. Moreover, mPGES-1 appears functionally coupled with COX-2 and its induction is usually coordinated with COX-2 [9]. In macrophages, large amounts of PGE_2 are generated during the inflammatory process, due to up-regulation of both COX-2 and mPGES-1 enzymes. Coordinated induction of the expression of COX-2 and mPGES-1 has been reported upon pro-inflammatory stimuli as LPS, IL-1β or TNFα, 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. PGE_2 signals through four G protein coupled receptors named EP1, EP2, EP3 and EP4 (reviewed in [14-17]). EP receptors are linked to different transduction pathway that may even give rise to opposite effects, activation or inhibition, on cellular responses. Thus, EP1 induces inhibition of adenylate cyclase, leading to a decrease in cAMP, whereas EP2 and EP4 receptors activate this enzyme. On the other hand, EP3 is coupled to Gαq and its activation results in intracellular calcium increase.

cAMP is thought to be the main intracellular second messenger of PGE_2 signaling in macrophages playing a crucial role in the modulation of the functional activity of macrophages and monocytes. In fact, PGE_2 –dependent elevation of intracellular cAMP in LPS -stimulated macrophages results in a decreased synthesis of pro-inflammatory cytokines, including tumor necrosis factor (TNF) –α [18, 19], Interleukin (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 PGE_2 may positively modulate COX-2 expression [22, 23].

We have previously reported an essential role of NF-κB and Egr-1 as key factors involved in coordinated up-regulation of COX-2 and mPGES-1 expression in macrophages in response to LPS, leading to increased PGE_2 production [13]. In this study we explore the importance of cAMP signaling in the regulation of COX-2 and mPGES-1 in LPS –stimulated macrophages, analyzing the role of PGE_2 -dependent signaling. Our data show that PGE_2 induces transcriptional activation of both COX-2 and mPGES-1 via mechanisms involving EP2 receptor activation and cAMP/PKA/CREB signaling pathway. This positive feedback regulation of PGE_2-synthesizing enzymes COX-2 and mPGES-1 in macrophages constitutes an amplification signaling that may play an important role in the modulation of the inflammatory process and the immune response.

EXPERIMENTAL PROCEDURES

Cell culture and reagents

The mouse macrophage cell line RAW 264.7 (American Type Culture Collection, Rockville, MD) was cultured in RPMI 1640 medium (Invitrogen) supplemented with 5% fetal calf serum (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. Cells were treated with LPS from E. coli, serotype 026:B6 (Sigma-Aldrich), at 1 μg/ml. Selective COX-2 inhibitors Celecoxib and NS398 (Alexis Biochemicals) were used at final concentrations between 0.01 and 1 μM. PGE_2, EP2 and EP4 agonists CAY10399 and PGE_1OH, and EP2 antagonist AH6809, were purchased to Cayman BioChemical. dbcAMP (100 μM) (Sigma) and Forskolin (10 μM) (Biomol International) PKA inhibitors H89 (10 μM) and KT5720 (1 μM). Inhibitors were added 1 hour before cell stimulation with PGE_2 or LPS.

Plasmid constructs

COX-2 promoter luciferase constructs PGHS-2 medium (-1844) was provided by Dr. S. Vogel (University of Maryland, Baltimore, MD, USA) [24]. PGHS-2-400 PGHS-2-250 constructs were kindly provided by Dr. H. Herschman (University of California at Los Angeles, Los Angeles, CA, USA) [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 [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 have been previously described [13]. The cAMP responsive element (CRE)-LUC plasmid contains four copies of the CRE site of the human choriogonadotropin gene promoter (−147 to −129) [27]. EP2 expression vector (pcDNA 3.1-EP2) were obtained from Missouri S&T UMR cDNA Resource Center. The expression vector for the catalytic subunit of PKA has been previously described [28].

Transient transfection

COX-2 and mPGES-1 promoter activity was analyzed by luciferase reporter gene assays. RAW 264.7 cells were transiently transfected with 0.5 to 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 hours of transfection, cells were treated with different stimuli for additional 18 h. 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. Results are expressed as fold induction ± SD (observed experimental RLU/basal RLU in absence of any stimulus).

mRNA Analysis

Total RNA was obtained from cells by the TRIzol reagent RNA protocol (Invitrogen). For standard RT-PCR, RNA (1 μg) was reverse transcribed by the RNA PCR core kit (Perkin-Elmer). cDNA was used for PCR amplification to analyze EPs expression by standard RT-PCR using specific primers for EP1: sense 5'-TTAACCTGAGCCTAGCGGATG-3' and anti-sense 5'-CGCTGAGCGTATTGCACACTA-3'; EP2: sense 5'-CCACGATGCTCTCCTGCTGCTTAT-3' and anti-sense 5'-CAGCCCCTTACACTTCTCCAATGA-3'; EP3: sense 5'-TGAC CTTTGCCTGCAACCTG-3' and anti-sense 5'-GACCCAGGGAAACAGGTACT-3'; EP4, sense 5'-CTTACTCATCGCCACCTCTCTGGT-3' and anti-sense 5'-TGTGGCTCCCACTAACCTCATCCAC-3'; and β-actin, sense 5´-CTCTTTGATGTCACGCACGATTTC-3´and antisense 5´-GTGGGCCGCTCTAGGCACCAA-3´. PCR reaction was 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. PCR products were separated on agarose gel electrophoresis and 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 COX-2 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 probes for COX-1, COX-2, 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 comparative threshold cycle (ΔΔCT) method following the manufacturer's instructions. Relative quantification (RQ) of mRNA levels was determined using endogenous expression of rRNA 18S and is shown in all the experiments as RQ ± SD.

Western blotting

Protein extracts were obtained as previously described [13]. 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 a monoclonal anti-COX-2 antibody (BD Transduction Laboratories) and a polyclonal rabbit anti-mPGES-1 antibody (Cayman Chemical). Antibodies against CREB and P-CREB were purchased from Upstate Signaling. β-Actin level was used as a control of loading in each lane.

Chromatin Immunoprecipitation (ChIP) assay

Specific binding of CREB and P-CREB to COX-2 and mPGES-1 promoters was determined by Chromatin immunoprecipitation assay (ChIP) assays as previously described [13]. Briefly, RAW 264.7 cells treated with PGE_2 (5 μM) at indicated times were fixed with 1% formaldehyde and lysed in ice-cold lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% NP-40 with protease inhibitors). Nuclei pellet 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 precleared with salmon-sperm/protein A agarose. A sample of *input DNA* was collected. Protein-DNA complexes were immunoprecipitated overnight at 4°C with CREB or phospho-CREB polyclonal antibodies or non-immune rabbit serum 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, 500 mM NaCl) and TE buffer (20 mM Tris-HCl, 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 COX-2 and mPGES-1 and amplified bands were analyzed by 2% agarose gel electrophoresis.

PGE_2 determination

PGE_2 levels were measured in culture supernatants of RAW 264.7 cells after different treatment by a competitive immunoassay PGE_2 EIA kit following manufacturer's instructions (Cayman Chemical)

Statistics

Results are expressed as mean ± standard deviation (SD) from at least three independent experiments performed by duplicate or triplicate. Data were analyzed by ANOVA followed by Tukey´s test and by Student´s paired *t*-test to compare different assay groups using OriginPro 7.5 software. $p < 0.05$ was considered statistically significant.

RESULTS

Effect of the inhibition of PGE_2 production on LPS-mediated induction of COX-2 and mPGES-1 expression.

Expression of enzymes involved in PGE_2 biosynthesis as COX-1, COX-2 and mPGES-1 were assessed by quantitative real time RT-PCR in the murine macrophage cell line RAW 264.7 after LPS treatment (Fig. 1*A*). Low levels of expression of COX-1 (average Ct = 33,11), COX-2 (average Ct =29,28) and mPGES-1 (average Ct= 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 by 20 and 5 fold respectively whereas COX-1 expression was reduced by 2 fold, thus pointing to COX-2 and mPGES-1 as the main enzymes involved in enhanced PGE_2 production in LPS treated RAW264.7 cells. Accordingly, treatment with increasing doses (1 to 100 nM) of COX-2 inhibitors Celecoxib or NS398, led to a decrease in the production of PGE_2 induced by LPS (Fig. 1*B*).

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 hours in the presence of increasing doses of NS398 or Celecoxib. As shown in Figures 1*C,D,E* and *F*, LPS -mediated increase of COX-2 and mPGES-1 expression was attenuated by increasing doses of NS398 and Celecoxib (1 to 100 nM) , suggesting that suppression of endogenous PGE_2 may cause a reduction in LPS-induced expression of mPGES-1 and COX-2 mRNA. Moreover, attenuation of mPGES-1 and COX-2 expression by NSAIDs was effectively restored by exogenous PGE_2 (Fig 1*C,D,E* and *F*).

Induction of COX-2 and mPGES-1 expression by PGE_2

In order to analyze the effect of PGE_2 on COX-2 and mPGES-1 expression, we treated murine macrophages RAW 264.7 with increasing doses of PGE_2 (0.1 to 10 μM) for 24 h. Quantitative RT-PCR and Western blots showed that COX-2 and mPGES-1 expression was significantly enhanced in a dose-dependent manner by PGE_2 (Figs. 2*A* and *2B*). PGE_2 treatment led to an early induction of COX-2 mRNA whereas mPGES-1 mRNA reached peak levels at 24 hr after stimulation (Fig. *2C*). Furthermore, PGE_2 cooperated with LPS in the induction of COX-2 and mPGES-1 mRNA levels (Fig. *2D*).

EP2 participates in PGE_2-dependent transcriptional induction of COX-2 and mPGES-1.

Four different EP receptors mediate PGE_2-dependent intracellular signaling [14-16]. In basal conditions, murine macrophage cell lines cells express mainly EP2 and EP4 receptors [29-31]. Analysis of mRNA levels for the EP receptors in control RAW264.7 cells confirmed the presence of EP2 and EP4 transcripts with very low levels of EP1 and EP3 ones (Fig 3*A, left panel*). Quantitative RT-PCR analysis showed Ct values above 38 for EP1 (38.15 ± 0.43) and EP3 (38.73 ± 0.01) receptors thus indicating low to negligible levels of expression. Ct 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 PGE_2 or LPS, EP2 mRNA levels increased in a time-dependent manner. On the other hand, both PGE_2 and LPS treatment promoted a decrease in EP4 mRNA levels (Fig. 3*A*).

Treatment of RAW 264.7 cells with the EP2 agonist, CAY10399 or with the EP4 agonist PGE_1OH revealed that EP2 triggering participate in the induction of both COX-2 and mPGES-1 mRNA levels (Fig. 3*B*). Involvement of EP2 signaling in the induction of the expression of these enzymes was confirmed with the use of the EP2 antagonist AH6809, which was able to reverse PGE_2-mediated effects (Fig. 3*C*).

Moreover, luciferase assays using COX-2 and mPGES-1 mouse promoter constructs (PGHS-2 Medium and pmPGES-1:-895) showed that transient expression of EP2 receptor mediated transcriptional activation of COX-2 and mPGES-1 after PGE_2 stimulation of RAW 264.7 cells (Fig. 3*D*).

dbcAMP increases COX-2 and mPGES-1 expression

PGE_2 signaling 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 permeable-cAMP analogue dbcAMP induced an early accumulation of COX-2 mRNA at 3 and 8 h., whereas a significant increase in mPGES-1 mRNA levels was detected after 24 h of treatment (Fig. 4*A*). Analysis of protein levels by Western Blot showed an increase of COX-2 and mPGES-1 protein levels after dbcAMP treatment with maximal induction at 24 h (Fig. 4*B*). Coordinated increase in COX-2 and mPGES-1 expression after dbcAMP stimulation led to enhanced production of PGE_2 by these cells (Fig. 4*C*).

dbcAMP cooperates 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 COX-2 and mPGES-1 genes. Induction of COX-2 and mPGES mRNA levels in these cells by LPS was enhanced in the presence of dbcAMP (Fig *5A*), resulting in augmented PGE_2 production by these cells (Fig 5*B*). The effect of dbcAMP on LPS-mediated transcriptional activation was also analyzed on COX-2 and mPGES-1 promoter activity. As shown in figure 5C, LPS and dbcAMP cooperated in the induction of the transcriptional activity of both promoters compared to the induction after stimulation with either dbcAMP or LPS.

PKA regulates COX-2 and mPGES-1 expression by PGE_2.

Signaling through the cAMP pathway leads to the activation of PKA, that in turns may activate cAMP –dependent gene transcription [32, 33]. We next analyze the involvement of this signaling pathway in the activation of COX-2 and mPGES-1 expression in RAW 264.7 macrophages. As shown in figure 6A, inhibition of PKA by H89, produced a decrease in the induction of COX-2 and mPGES-1 promoted by PGE_2 treatment or by activation of the adenylate cyclase by Forskolin. PKA inhibition was also able to abolish EP2-mediated induction of COX-2 and mPGES-1 promoter activity after PGE_2 treatment (Fig. 6*B*). Interestingly, overexpression of an expression vector for the catalytic subunit of PKA significantly increased activity of COX-2 and mPGES-1 promoters and cooperated with LPS to further induce transcriptional activation of these promoters (Fig. *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 (Fig. *6D*).

PGE_2 induces CRE –mediated COX-2 and mPGES-1 expression

Since CREB is the main transcriptional mediator of cAMP/PKA signal [32, 33], we evaluated the effect PGE_2 on CREB phosphorylation and CRE –mediated transcriptional activation. Incubation of RAW 264.7 cells with PGE_2 led to efficient CREB phosphorylation (Fig. 7*A*). Furthermore, PGE_2 as well as Forskolin and dbcAMP activated CRE-dependent gene transcription of a luciferase reporter construct (Fig. 7*B*). CRE-dependent transcription upon PGE_2 stimulation was enhanced in cells co-transfected with an EP2 expression vector (Fig. 7*C*).

Previous studies have described a functional CRE binding site in the murine COX-2 promoter [34]. We have analyzed the functional significance of this CRE binding site in PGE_2-depended COX-2 transcriptional activity by using different deletions of the COX-2 promoter in cells co-transfected with the EP2 receptor. Mutation of the sequence containing the CRE site within the COX-2 gene abolished the induction of COX-2 promoter activity by PGE_2 in RAW 264.7 cells (Fig. 8*A*). 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 between the position –483/-154 of the mPGES-1 transcription start site resulted in a clear reduction of the inducibility by PGE_2 (Fig. 8*C*). Sequence analysis of this region in the mPGES-1 murine gene to identify potential CRE elements with the TRANSFAC Database and P-Match software [35] revealed the presence of two putative CRE elements (5´-tcagTGATAtgc-3´ and 5´-gtccTGAGCcaa-3´) located at the positions –301/-290 and -217/-206 of the mPGES-1 transcription start site, with a high score of core similarity (0.976 and 0.988) and matrix similarity (0.916 and 0.894). The capital letters indicate the positions in the sequence which match with the core sequence of the matrix, while the lower cases refer to the remaining position of a matrix.

To confirm the involvement of CREB in the induction of COX-2 and mPGES-1 by PGE_2, we examined CREB binding to mouse COX-2 and mPGES-1 promoters by ChIP assays in PGE_2-treated RAW 264.7 cells. As shown in figure 8*B* and 8*D*, PGE_2 stimulation resulted in increased binding of CREB to both COX-2 and mPGES-1 promoters.

DISCUSSION

PGE_2 plays an important role in the modulation of the inflammatory and immune response through autocrine and paracrine signaling participating in the regulation of cytokine production, leukocyte migration, proliferation and differentiation [36-38]. Activation of specific EP receptors by this prostaglandin has been shown to regulate the function of many cell types including macrophages, dendritic cells, T and B lymphocytes, leading to both pro- and anti-inflammatory effects. Emerging data reveal that regulatory effects of PGE_2 in inflammation depend on receptor subtype, cell population, and context of activation. Although mostly implicated as a pro-inflammatory agent, PGE_2 is also able to down-regulate the expression of inflammatory cytokines and chemokines from activated macrophages and dendritic cells [18-21, 39, 40]. Macrophages are the main source of PGE_2 generated in settings of inflammation upon activation by stimulus such as LPS, TNFα or IL-1β. Enhanced release of PGE_2 in response to these stimuli is due to coordinated up-regulation of COX-2 and mPGES-1 enzymes [41, 42]. The molecular mechanisms that regulate PGE_2 synthesis by macrophages involved transcriptional and post-transcriptional processes with NF-κB, CREB AP-1 and c/EBP transcription factors as critical modulators of COX-2 gene transcription [23, 26, 34, 43]. On the other hand, induced expression of mPGES-1 depends essentially on Egr-1 transcription factor, although other factors as NF-κB and SP1 have been also shown to participate in its regulation [13, 41, 44-46]. In this study we provide evidence that PGE_2 is able to enhance transcriptional activation of COX-2 and mPGES-1 in LPS -activated RAW 264.7 murine macrophage cell line. This PGE_2 -mediated positive loop involves the activation of EP2/cAMP/PKA signaling pathway resulting in CREB –dependent transcriptional activation of COX-2 and mPGES genes.

LPS treatment of RAW 264.7 cells induces the synthesis of COX-2 and mPGES-1 that is accompanied by a significant increase in the release of PGE_2. Our results show that COX-2 selective inhibitors down-regulate the expression of these enzymes at a concentration doses that abrogated LPS-mediated PGE_2 production in RAW 264.7 cells, suggesting a positive regulation of this prostaglandin on the expression of COX-2 and mPGES-1. In fact, exogenous PGE_2 treatment reversed the effect of COX-2 inhibitors in LPS stimulated RAW.264.7 cells. Moreover, PGE_2 treatment in unstimulated RAW 264.7 cells was able to induce COX-2 and mPGES-1 transcriptional induction. The ability of PGE_2 to promote COX-2 and mPGES-1 expression in unstimulated cells was weak compared to LPS, but PGE_2 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 to 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 {Diaz-Munoz, 2010 #365; Stichtenoth, 2001 #161; Thoren, 2000 #162; Inada, 2006 #193; Kojima, 2002 #447; Han, 2002 #448}. These observations suggest a differential regulation of these enzymes in terms of the up-regulation and maintenance of steady-state expression levels. It has been reported some differences between these two genes that could be relevant to explain divergences in the timing of response to the same stimuli. The promoter of the human mPGES-1 gene lacks a TATA box unlike the COX-2 gene promoter. Furthermore, mPGES-1 mRNA does not contain AUUUA instability motifs that are present in the COX-2 mRNA {Forsberg, 2000 #195}. The delayed induction of mPGES mRNA with respect to COX-2 upon a stimulatory treatment thus can be explained on the basis of a smaller increase in the rate of gene transcription in the setting of a relatively stable mRNA.

PGE_2 exert 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 analyzing the expression of EP receptors in murine macrophages, we have

detected the expression of EP2 and EP4 in RAW 264.7 cells with negligible levels of EP1 and EP3 receptors [29, 30, 47-49]. Our results show that expression levels of EP2 and EP4 receptors change inversely in such a manner that basal low levels of EP2 receptor are increased in a time dependent manner upon LPS treatment while EP4 expression diminished in stimulated RAW 264.7 cells. Moreover, a similar profile on the regulation of EP2 and EP4 mRNA levels was observed upon PGE_2 treatment. Differential regulation of EP2 and EP4 receptors upon activation or PGE_2 treatment involving cAMP signaling have been observed in murine macrophages [30, 47-49]. In the present study we provide evidences indicating the involvement of EP2 receptor in the PGE_2 positive loop by the use of EP2 receptor agonists and antagonists as well as overexpression of this receptor in RAW 264.7 cells. EP2 receptor couples to $G_{\alpha s}$ leading to the activation of adenylyl cyclase and increased formation of intracellular cAMP and thus activation of the cAMP -dependent protein kinase PKA. Here we show that treatment of unstimulated RAW 264.7 cells with cAMP analogues or adenylate cyclase activators induced COX-2 and mPGES-1 expression resulting in augmented PGE_2 production. These agents cooperated with LPS to further up-regulate COX-2, mPGES-1 expression and PGE_2 production by RAW 264.7 cells. Involvement of PKA was revealed by the use of 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 COX-2 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 that interacts with CREs, resulting in the regulation of cAMP responsive gene expression [32, 33]. Our study show that PGE_2 treatment of RAW 264.7 cells induced phosphorylation of CREB at Ser-133, 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 overexpresed EP2 receptor. CREB –dependent regulation of COX-2 promoter activity in response to a variety of stimuli have been observed in different cell types [34, 50-52]. Our Chip assays show that PGE_2 treatment lead to CREB binding to the murine COX-2 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 COX-2 as well as mPGES-1 promoters activity by PGE_2 in RAW 264.7 cells not only confirmed the role of the CRE sequence in the COX-2 promoter but localize a PGE_2 responsive region (-483 to -154) within the murine mPGES-1 promoter containing two putative CRE elements.

In summary, our results point to an essential role of the EP2 receptor in PGE_2 -mediated regulation of COX-2 and mPGES-1 expression though the cAMP/PKA/CREB signaling in RAW 264.7 cells. An autocrine PGE_2 positive feedback involving both EP2 as well as EP4 receptors have been suggested previously as a mechanism modulating COX-2 induction and PGE_2 production [51-54]. Our results cannot entirely discard the involvement of EP4 receptor –mediated signaling 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 signaling properties [15, 57]. Gs-mediated increases in cAMP seem to play a less important role for EP4 receptor signaling compared with the EP2 receptor in such a way that inhibition of PKA by H89 is able to attenuate PGE_2 mediated phosphorylation of CREB in EP2 expressing cells but not in EP4 expressing cells [57]. Furthermore, the existence of a functional switch from EP4 to EP2 upon macrophage treatment with LPS or with cAMP elevating agents, in such a way that EP4 receptor is down-regulated while EP2 expression levels are increased, points to EP2 as the predominant isoform in activated macrophages (this report and[47, 49]).

In summary, our study demonstrates the existence of a positive feedback regulation of PGE_2-synthesizing enzymes as COX-2 but also mPGES-1 in macrophages, which may play an

important role in the actions of this prostaglandin in the modulation of the inflammatory process and the immune response.

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REFERENCES

1 Smith, W. L., DeWitt, D. L. and Garavito, R. M. (2000) Cyclooxygenases: structural, cellular, and molecular biology. Annu. Rev. Biochem. **69**, 145-182

2 Morita, I. (2002) Distinct functions of COX-1 and COX-2. Prostaglandins Other Lipid Mediat. **68-69**, 165-175

3 Murakami, M. and Kudo, I. (2006) Prostaglandin E synthase: a novel drug target for inflammation and cancer. Curr. Pharm. Des. **12**, 943-954

4 Mitchell, J. A. and Evans, T. W. (1998) Cyclooxygenase-2 as a therapeutic target. Inflamm. Res. **47 Suppl 2**, S88-92

5 Liao, Z., Mason, K. A. and Milas, L. (2007) Cyclo-oxygenase-2 and its inhibition in cancer: is there a role? Drugs. **67**, 821-845

6 Funk, C. D. and Fitzgerald, G. A. (2007) COX-2 Inhibitors and Cardiovascular Risk. J. Cardiovasc. Pharmacol. **50**, 470-479

7 Iniguez, M. A., Rodriguez, A., Volpert, O. V., Fresno, M. and Redondo, J. M. (2003) Cyclooxygenase-2: a therapeutic target in angiogenesis. Trends Mol. Med. **9**, 73-78

8 Park, J. Y., Pillinger, M. H. and Abramson, S. B. (2006) Prostaglandin E2 synthesis and secretion: the role of PGE2 synthases. Clin Immunol. **119**, 229-240

9 Samuelsson, B., Morgenstern, R. and Jakobsson, P. J. (2007) Membrane prostaglandin E synthase-1: a novel therapeutic target. Pharmacol. Rev. **59**, 207-224

10 Stichtenoth, D. O., Thoren, S., Bian, H., Peters-Golden, M., Jakobsson, P. J. and Crofford, L. J. (2001) Microsomal prostaglandin E synthase is regulated by proinflammatory cytokines and glucocorticoids in primary rheumatoid synovial cells. J. Immunol. **167**, 469-474

11 Thoren, S. and Jakobsson, P. J. (2000) Coordinate up- and down-regulation of glutathione-dependent prostaglandin E synthase and cyclooxygenase-2 in A549 cells. Inhibition by NS-398 and leukotriene C4. Eur. J. Biochem. **267**, 6428-6434

12 Inada, M., Matsumoto, C., Uematsu, S., Akira, S. and Miyaura, C. (2006) Membrane-Bound Prostaglandin E Synthase-1-Mediated Prostaglandin E2 Production by Osteoblast Plays a Critical Role in Lipopolysaccharide-Induced Bone Loss Associated with Inflammation. J. Immunol. **177**, 1879-1885

13 Diaz-Munoz, M. D., Osma-Garcia, I. C., Cacheiro-Llaguno, C., Fresno, M. and Iniguez, M. A. (2010) Coordinated up-regulation of cyclooxygenase-2 and microsomal prostaglandin E synthase 1 transcription by nuclear factor kappa B and early growth response-1 in macrophages. Cell. Signal. **22**, 1427-1436

14 Sugimoto, Y. and Narumiya, S. (2007) Prostaglandin E receptors. J. Biol. Chem. **282**, 11613-11617

15 Regan, J. W. (2003) EP2 and EP4 prostanoid receptor signaling. Life Sci. **74**, 143-153

16 Hata, A. N. and Breyer, R. M. (2004) Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. Pharmacol. Ther. **103**, 147-166

17 Alfranca, A., Iniguez, M. A., Fresno, M. and Redondo, J. M. (2006) Prostanoid signal transduction and gene expression in the endothelium: role in cardiovascular diseases. Cardiovasc. Res. **70**, 446-456

18 Seldon, P. M., Barnes, P. J., Meja, K. and Giembycz, M. A. (1995) Suppression of lipopolysaccharide-induced tumor necrosis factor-alpha generation from human peripheral blood monocytes by inhibitors of phosphodiesterase 4: interaction with stimulants of adenylyl cyclase. Mol. Pharmacol. **48**, 747-757

19 Kunkel, S. L., Spengler, M., May, M. A., Spengler, R., Larrick, J. and Remick, D. (1988) Prostaglandin E2 regulates macrophage-derived tumor necrosis factor gene expression. J. Biol. Chem. **263**, 5380-5384

20 Hurme, M. (1990) Modulation of interleukin-1 beta production by cyclic AMP in human monocytes. FEBS Lett. **263**, 35-37

21 Strassmann, G., Patil-Koota, V., Finkelman, F., Fong, M. and Kambayashi, T. (1994) Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E2. J. Exp. Med. **180**, 2365-2370
22 Vichai, V., Suyarnsesthakorn, C., Pittayakhajonwut, D., Sriklung, K. and Kirtikara, K. (2005) Positive feedback regulation of COX-2 expression by prostaglandin metabolites. Inflamm. Res. **54**, 163-172
23 Kang, Y. J., Wingerd, B. A., Arakawa, T. and Smith, W. L. (2006) Cyclooxygenase-2 gene transcription in a macrophage model of inflammation. J. Immunol. **177**, 8111-8122
24 Blanco, J. C., Contursi, C., Salkowski, C. A., DeWitt, D. L., Ozato, K. and Vogel, S. N. (2000) Interferon regulatory factor (IRF)-1 and IRF-2 regulate interferon gamma-dependent cyclooxygenase 2 expression. J. Exp. Med. **191**, 2131-2144
25 Xie, W., Fletcher, B. S., Andersen, R. D. and Herschman, H. R. (1994) v-src induction of the TIS10/PGS2 prostaglandin synthase gene is mediated by an ATF/CRE transcription response element. Mol. Cell. Biol. **14**, 6531-6539
26 Vila-del Sol, V. and Fresno, M. (2005) Involvement of TNF and NF-kappa B in the transcriptional control of cyclooxygenase-2 expression by IFN-gamma in macrophages. J. Immunol. **174**, 2825-2833
27 Schwaninger, M., Blume, R., Oetjen, E., Lux, G. and Knepel, W. (1993) Inhibition of cAMP-responsive element-mediated gene transcription by cyclosporin A and FK506 after membrane depolarization. J. Biol. Chem. **268**, 23111-23115
28 Uhler, M. D. and McKnight, G. S. (1987) Expression of cDNAs for two isoforms of the catalytic subunit of cAMP-dependent protein kinase. J. Biol. Chem. **262**, 15202-15207
29 Arakawa, T., Laneuville, O., Miller, C. A., Lakkides, K. M., Wingerd, B. A., DeWitt, D. L. and Smith, W. L. (1996) Prostanoid receptors of murine NIH 3T3 and RAW 264.7 cells. Structure and expression of the murine prostaglandin EP4 receptor gene. J. Biol. Chem. **271**, 29569-29575
30 Hubbard, N. E., Lee, S., Lim, D. and Erickson, K. L. (2001) Differential mRNA expression of prostaglandin receptor subtypes in macrophage activation. Prostaglandins Leukot. Essent. Fatty Acids. **65**, 287-294
31 Katsuyama, M., Sugimoto, Y., Okano, K., Segi, E., Ikegami, R., Negishi, M. and Ichikawa, A. (1998) Characterization of the gene for the mouse prostaglandin E receptor subtype EP2: tissue-specific initiation of transcription in the macrophage and the uterus. Biochem. J. **330 (Pt 3)**, 1115-1121
32 Mayr, B. and Montminy, M. (2001) Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat. Rev. Mol. Cell Biol. **2**, 599-609
33 Shaywitz, A. J. and Greenberg, M. E. (1999) CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu. Rev. Biochem. **68**, 821-861
34 Eliopoulos, A. G., Dumitru, C. D., Wang, C. C., Cho, J. and Tsichlis, P. N. (2002) Induction of COX-2 by LPS in macrophages is regulated by Tpl2-dependent CREB activation signals. EMBO J. **21**, 4831-4840
35 Chekmenev, D. S., Haid, C. and Kel, A. E. (2005) P-Match: transcription factor binding site search by combining patterns and weight matrices. Nucleic Acids Res. **33**, W432-437
36 Sakata, D., Yao, C. and Narumiya, S. (2010) Prostaglandin E2, an immunoactivator. J. Pharmacol. Sci. **112**, 1-5
37 Tilley, S. L., Coffman, T. M. and Koller, B. H. (2001) Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. J Clin Invest. **108**, 15-23
38 Sakata, D., Yao, C. and Narumiya, S. (2010) Emerging roles of prostanoids in T cell-mediated immunity. IUBMB Life. **62**, 591-596
39 Harizi, H. and Gualde, N. (2005) The impact of eicosanoids on the crosstalk between innate and adaptive immunity: the key roles of dendritic cells. Tissue Antigens. **65**, 507-514

40 Takayama, K., Garcia-Cardena, G., Sukhova, G. K., Comander, J., Gimbrone, M. A., Jr. and Libby, P. (2002) Prostaglandin E2 suppresses chemokine production in human macrophages through the EP4 receptor. J. Biol. Chem. **277**, 44147-44154
41 Catley, M. C., Chivers, J. E., Cambridge, L. M., Holden, N., Slater, D. M., Staples, K. J., Bergmann, M. W., Loser, P., Barnes, P. J. and Newton, R. (2003) IL-1beta-dependent activation of NF-kappaB mediates PGE2 release via the expression of cyclooxygenase-2 and microsomal prostaglandin E synthase. FEBS Lett. **547**, 75-79
42 Ueno, N., Takegoshi, Y., Kamei, D., Kudo, I. and Murakami, M. (2005) Coupling between cyclooxygenases and terminal prostanoid synthases. Biochem. Biophys. Res. Commun. **338**, 70-76
43 Wadleigh, D. J., Reddy, S. T., Kopp, E., Ghosh, S. and Herschman, H. R. (2000) Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages. J. Biol. Chem. **275**, 6259-6266
44 Naraba, H., Yokoyama, C., Tago, N., Murakami, M., Kudo, I., Fueki, M., Oh-Ishi, S. and Tanabe, T. (2002) Transcriptional regulation of the membrane-associated prostaglandin E2 synthase gene. Essential role of the transcription factor Egr-1. J. Biol. Chem. **277**, 28601-28608
45 Ackerman, W. E. t., Summerfield, T. L., Vandre, D. D., Robinson, J. M. and Kniss, D. A. (2008) Nuclear factor-kappa B regulates inducible prostaglandin E synthase expression in human amnion mesenchymal cells. Biol. Reprod. **78**, 68-76
46 Subbaramaiah, K., Yoshimatsu, K., Scherl, E., Das, K. M., Glazier, K. D., Golijanin, D., Soslow, R. A., Tanabe, T., Naraba, H. and Dannenberg, A. J. (2004) Microsomal prostaglandin E synthase-1 is overexpressed in inflammatory bowel disease. Evidence for involvement of the transcription factor Egr-1. J. Biol. Chem. **279**, 12647-12658
47 Ikegami, R., Sugimoto, Y., Segi, E., Katsuyama, M., Karahashi, H., Amano, F., Maruyama, T., Yamane, H., Tsuchiya, S. and Ichikawa, A. (2001) The expression of prostaglandin E receptors EP2 and EP4 and their different regulation by lipopolysaccharide in C3H/HeN peritoneal macrophages. J. Immunol. **166**, 4689-4696
48 Akaogi, J., Yamada, H., Kuroda, Y., Nacionales, D. C., Reeves, W. H. and Satoh, M. (2004) Prostaglandin E2 receptors EP2 and EP4 are up-regulated in peritoneal macrophages and joints of pristane-treated mice and modulate TNF-alpha and IL-6 production. J. Leukoc. Biol. **76**, 227-236
49 Katsuyama, M., Ikegami, R., Karahashi, H., Amano, F., Sugimoto, Y. and Ichikawa, A. (1998) Characterization of the LPS-stimulated expression of EP2 and EP4 prostaglandin E receptors in mouse macrophage-like cell line, J774.1. Biochem. Biophys. Res. Commun. **251**, 727-731
50 Lo, C. J., Fu, M., Lo, F. R. and Cryer, H. G. (2000) Cyclooxygenase 2 (COX-2) gene activation is regulated by cyclic adenosine monophosphate. Shock. **13**, 41-45
51 Hinz, B., Brune, K. and Pahl, A. (2000) Cyclooxygenase-2 expression in lipopolysaccharide-stimulated human monocytes is modulated by cyclic AMP, prostaglandin E(2), and nonsteroidal anti-inflammatory drugs. Biochem. Biophys. Res. Commun. **278**, 790-796
52 Bradbury, D. A., Newton, R., Zhu, Y. M., El-Haroun, H., Corbett, L. and Knox, A. J. (2003) Cyclooxygenase-2 induction by bradykinin in human pulmonary artery smooth muscle cells is mediated by the cyclic AMP response element through a novel autocrine loop involving endogenous prostaglandin E2, E-prostanoid 2 (EP2), and EP4 receptors. J. Biol. Chem. **278**, 49954-49964
53 Hinz, B., Brune, K. and Pahl, A. (2000) Prostaglandin E(2) upregulates cyclooxygenase-2 expression in lipopolysaccharide-stimulated RAW 264.7 macrophages. Biochem. Biophys. Res. Commun. **272**, 744-748
54 Kojima, F., Naraba, H., Sasaki, Y., Beppu, M., Aoki, H. and Kawai, S. (2003) Prostaglandin E2 is an enhancer of interleukin-1beta-induced expression of membrane-

associated prostaglandin E synthase in rheumatoid synovial fibroblasts. Arthritis. Rheum. **48**, 2819-2828
55 Nishigaki, N., Negishi, M. and Ichikawa, A. (1996) Two Gs-coupled prostaglandin E receptor subtypes, EP2 and EP4, differ in desensitization and sensitivity to the metabolic inactivation of the agonist. Mol. Pharmacol. **50**, 1031-1037
56 Desai, S., April, H., Nwaneshiudu, C. and Ashby, B. (2000) Comparison of agonist-induced internalization of the human EP2 and EP4 prostaglandin receptors: role of the carboxyl terminus in EP4 receptor sequestration. Mol. Pharmacol. **58**, 1279-1286
57 Fujino, H., Salvi, S. and Regan, J. W. (2005) Differential regulation of phosphorylation of the cAMP response element-binding protein after activation of EP2 and EP4 prostanoid receptors by prostaglandin E2. Mol. Pharmacol. **68**, 251-259

FIGURE LEGENDS

Figure 1. Inhibition of PGE_2 synthesis by COX-2 specific inhibitors reduces COX-2 and mPGES-1 gene expression in macrophages. *A)* RAW 264.7 cells were treated with LPS (1 μg/ml) for 24 hours and COX-1, COX-2 and mPGES-1 mRNA levels were analyzed by real time quantitative RT-PCR, normalized to the expression of 18S rRNA. Results are shown as the mean of fold induction ± SD over the levels in the absence of LPS treatment. *B)* RAW 264.7 cells were treated with LPS (1 μg/ml) for 24 hours in the presence or absence of increasing doses (1 - 100 nM) of COX-2 inhibitors NS398 or Celecoxib. PGE_2 production in cell supernatants of RAW 264.7 cells was determined by a standard EIA assay as described under "Materials and Methods". Analysis of COX-2 (*C-D)* and mPGES-1 (*E-F*) mRNA levels by real time quantitative RT-PCR in RAW 264.7 cells treated with LPS for 24 hours along with increasing doses (1 to 100 nM) of Celecoxib (*C-E*) or NS398 (*D-F*) in the presence or absence of PGE_2 (5 μM) as indicated. COX-2 and mPGES-1 mRNA levels were normalized to the expression of 18S rRNA and are shown as % of induction ± SD considering 100 % the induction obtained upon LPS treatment. (*p<0.05; **p<0.01).

Figure 2. PGE_2 induces COX-2 and mPGES-1 synthesis in RAW 264.7 cells. *A)* Dose-response effect of PGE_2 on COX-2 and mPGES-1 mRNA expression. RAW 264.7 cells were stimulated with different doses of PGE_2 (0.1 to 10 μM) for 24 h. *B)* Analysis of COX-2 and mPGES-1 protein levels by Western blot in extracts from RAW 264.7 cells treated with increasing doses of PGE_2. β-Actin protein levels were determined as a control of loading. *C)* Time course mRNA induction of COX-2 and mPGES-1 after PGE_2 treatment. RAW 264.7 cells were treated with PGE_2 (5 μM) for the times indicated (hours). *D)* PGE_2 cooperates with LPS to induce COX-2 and mPGES-1 expression in macrophages. RAW 264.7 cells were treated with LPS (1 μg/ml) or LPS + PGE_2 (5 μM) for different times. COX-2 and mPGES-1 mRNA levels were analyzed by real time RT-PCR and normalized to the expression of 18S rRNA. Results are shown as the mean of fold induction over the control group ± SD of at least two independent experiments performed in triplicate (*p<0.05).

Figure 3. Involvement of EP2 receptor on the transcriptional activation of COX-2 and mPGES-1. *A) (left 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 for each gene obtained by real time RT-PCR in basal conditions is indicated. β-actin mRNA levels are shown as a control of loading. *(right)* mRNA levels of EP receptors was analyzed by quantitative real time RT-PCR in RAW 264.7 cells stimulated with PGE_2 (5 μM) or LPS (1 μg/ml) for the time indicated (hours). *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 PGE_1OH (1 μM). *C)* Cells were treated with PGE_2 (5 μM) in the presence or absence of EP2 antagonist AH6809 (1-5 μM) and COX-2 and mPGES-1 mRNA levels were analyzed by real time RT-PCR. Relative quantification of mRNA levels was determined using endogenous expression of rRNA 18S and is shown as fold induction ± SD. *D)* EP2 mediates PGE_2-dependent transcriptional activation of COX-2 and mPGES-1 promoters. RAW 264.7 cells were transfected with PGHS-2 medium (COX-2-LUC) or mPGES-1-895 (mPGES-1-Luc) reporter plasmids along with empty vector (pcDNA3), or an expression vector for EP2. Transfected cells were stimulated with PGE_2 (5 μM), and transcriptional activation of COX-2 and mPGES-1 promoters was assayed. The means of replicate determinations of at least three independent assays expressed as fold induction ± SD are shown. (*p<0.05; **p<0.01).

Figure 4. dbcAMP increases COX-2 and mPGES-1 expression and PGE_2 production in RAW 264.7 cells. *A)* COX-2 and mPGES-1 mRNA levels from RAW 264.7 cells stimulated with dbcAMP (100 μM) at indicated times were determined by real time RT-PCR. Results are shown as the mean of fold induction ± SD of two independent experiments perfomed 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 (hours). *C)* PGE_2 production by RAW 264.7 cells after stimulation with dbcAMP during different times. The results shown are the mean ± SD of replicate determinations of three independent assays (*p<0.01; **p<0.05).

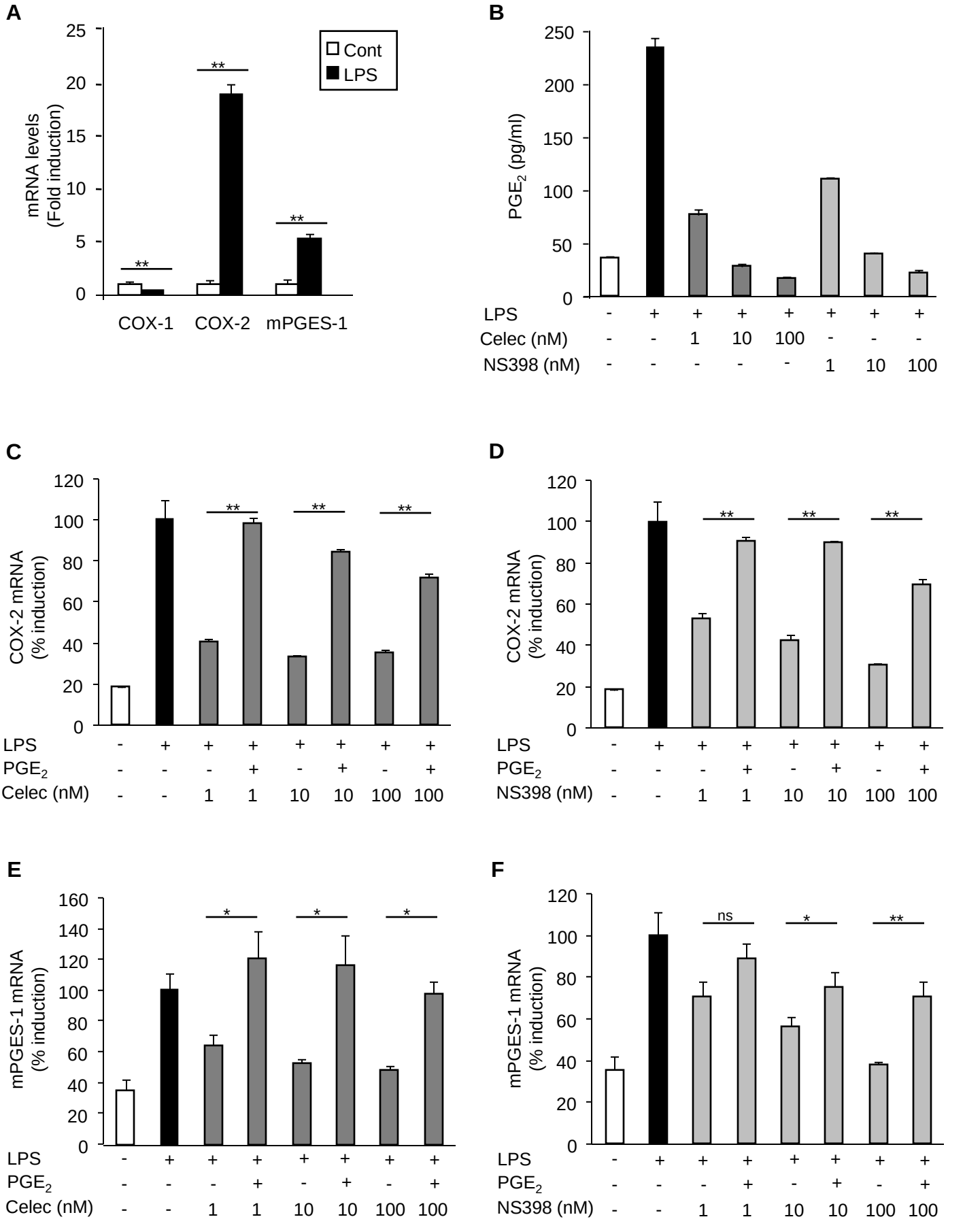
Figure 5. dbcAMP cooperates with LPS in the induction of COX-2 and mPGES-1 expression. *A)* Analysis of COX-2 and mPGES-1 expression in RAW 264.7 cells after stimulation with LPS (1 μg/ml) or LPS + dbcAMP (100 μM) for different times (hours). mRNA levels were analyzed by real time RT-PCR. A representative of three independent experiments is shown. *B)* PGE_2 production in supernatants of RAW 264.7 cells treated with LPS or LPS + dbcAMP for different times. *C)* LPS and dbcAMP induce transcriptional activation of COX-2 and mPGES-1 promoter. Luciferase activity of COX-2 and mPGES-1 luciferase construct containing the proximal promoter regions of these genes in transfected cells treated with dbcAMP, LPS or LPS + dbcAMP for 24 h. Results from three independent experiment are shown as Fold induction (observed experimental RLUs/basal RLUs in absence of any stimuli) ± SD. Paired t student tests indicate a significant difference between LPS and LPS + dbcAMP groups (**p<0.01; *p<0.05).

<u>Figure 6.</u> Involvement of PKA on COX-2 and mPGES-1 expression. *A)* COX-2 and mPGES-1 mRNA levels were analyzed by quantitative real time RT-PCR in cells treated with Forskolin (Forsk) (10 μM) or PGE_2 (5 μM) for 24 h in the presence of PKA inhibitor H89 (10 μM). Data from two independent experiments performed by triplicate were normalized to the levels of the endogenous control 18 S rRNA and are shown as Fold induction ± SD. *B)* Luciferase activity of COX-2 Luc and mPGES-1 Luc constructs were analyzed in RAW 264.7 cells transiently transfected with the EP2 receptor. Cells were pre-treated with H89 1 h prior PGE_2 stimulation for 18 h. Luciferase activity is shown as Fold induction ± SD. A representative of three independent experiments performed by 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 ± SD. Statistical difference over the control group is shown (***p<0.005; **p<0.01; *p<0.05). *D)* Analysis of PGE_2 production in supernatants of RAW 264.7 cells pretreated with PKA inhibitors H89 or KT5720 before stimulation with LPS + dbcAMP for 24 h. Results are means ± SD from three experiments. Paired t student tests indicate a significant difference between stimulated and H89 or KT5720 treated groups (***p<0.005; **p<0.01; *p<0.05).

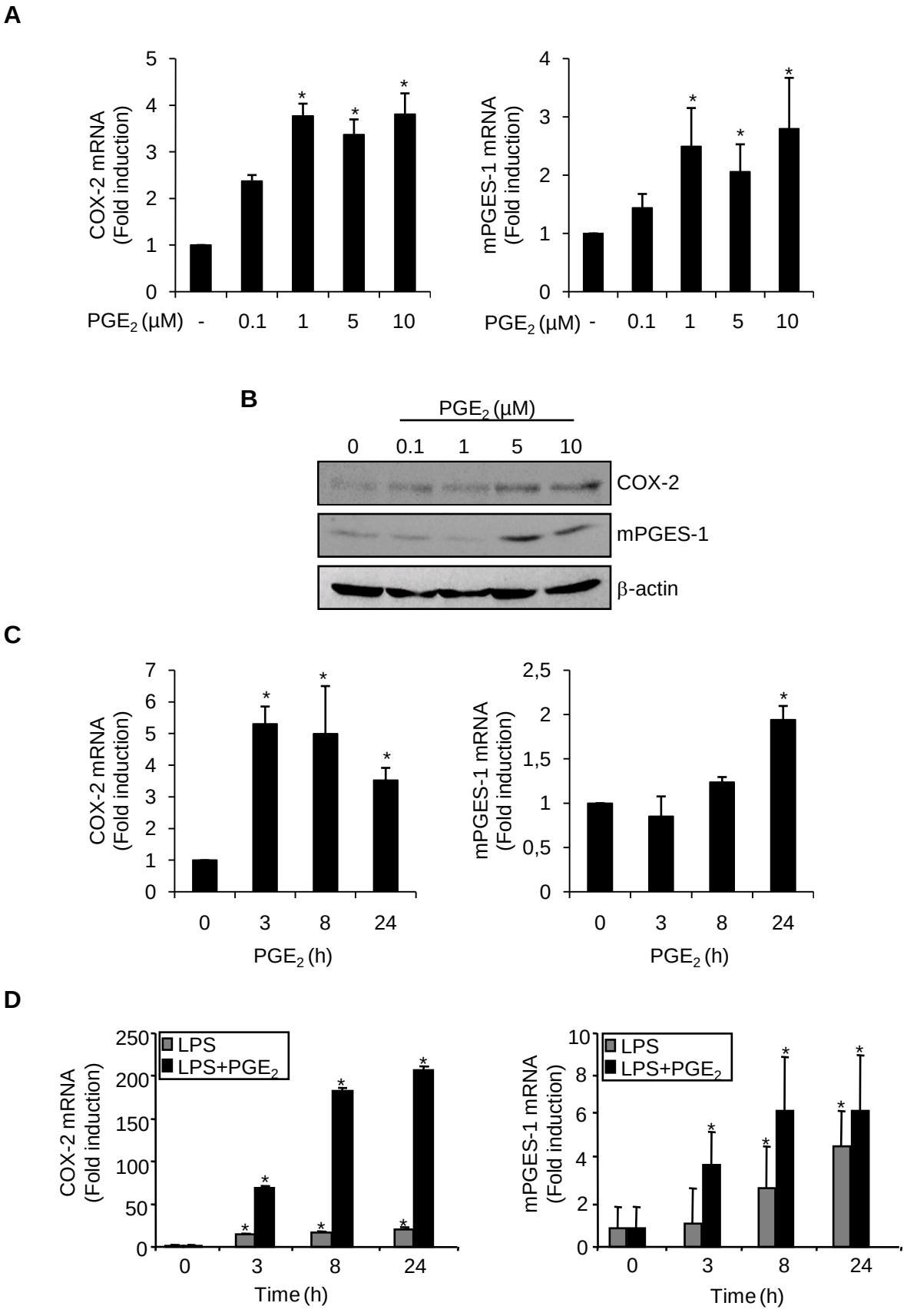
Figure 7. PGE_2 induces CRE-mediated transcriptional activation. *A)* RAW 264.7 macrophages were incubated in the absence or presence of PGE_2 (5 μM) for the indicated period of time (minutes). Protein extracts were separated by SDS-PAGE and levels of phosphorylated (P) and total CREB were detected by immunoblotting with specific antibodies. Relative quantification of CREB phosphorylation is 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 PGE_2 (5 μM), Forskolin (10 μM) or dbcAMP (100 μM) for 18 h. Data are the mean ± SD of replicate determinations expressed as fold induction over the RLUs of unstimulated controls (Cont) Results are representatives of 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 PGE_2 and CRE-dependent transcriptional activation was assayed. The means of replicate determinations expressed as fold induction ± SD are shown. Results are representative of at least three independent assays. (*$p<0.05$; ** $p<0.005$).

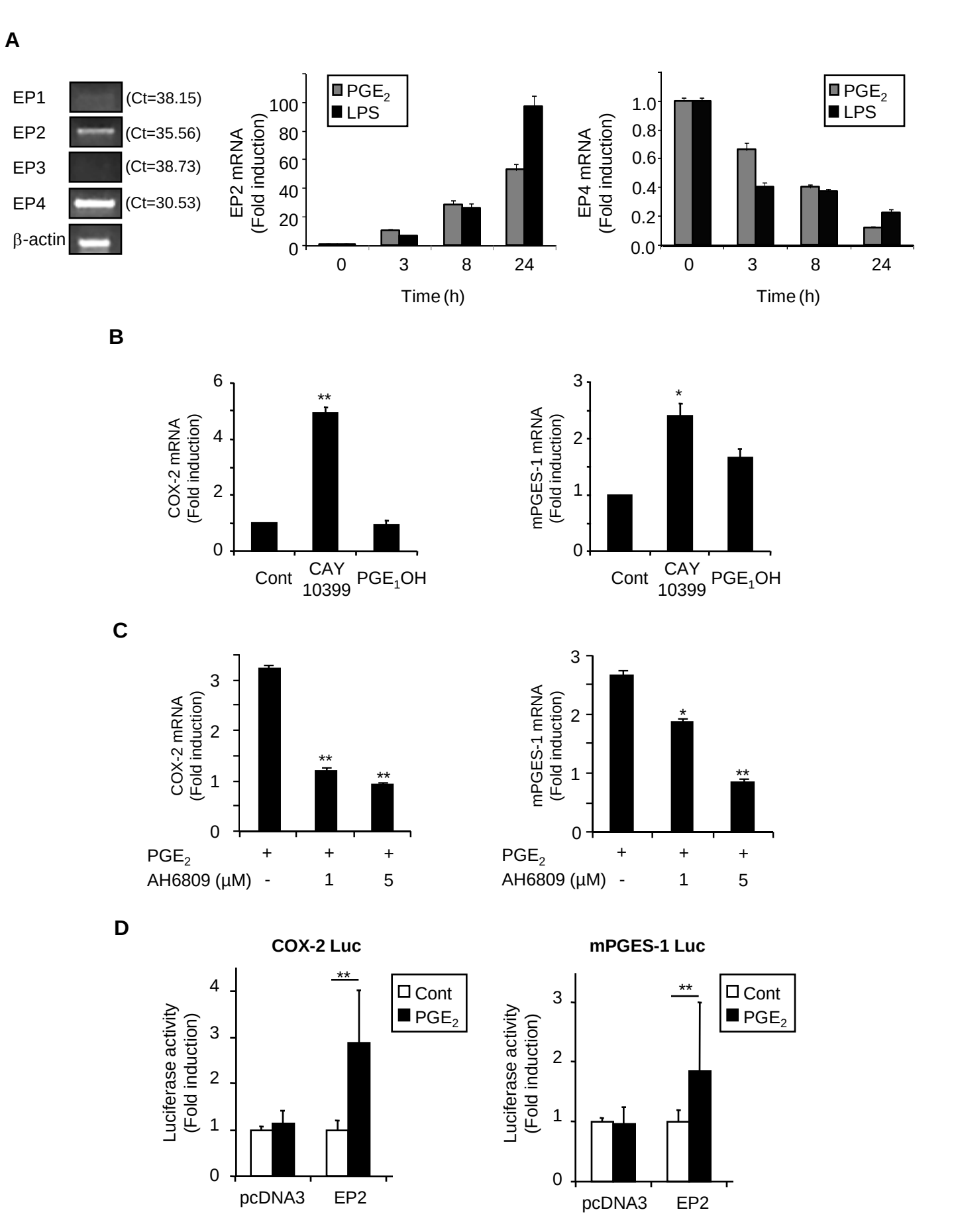
Figure 8. PGE_2 –mediated activation and CREB binding in COX-2 and mPGES-1 promoter regions. RAW 264.7 cells were transfected with different deletions of COX-2 (*A*) or mPGES-1 (*C*) promoter constructs (described in Material and Methods) along with EP2 receptor expression vector. Cis-acting consensus sequences are denoted by boxes. The extent of the 5´ truncations are shown with numbers indicating their length relative to the transcription start site. After transfection, cells were treated with PGE_2 (5 μM) for 18 h and assayed for luciferase activity. Data are shown as means of fold induction (observed experimental RLUs in response to LPS/basal RLUs in absence of any stimuli) ± SD of three different experiments. Analysis of the specific binding of CREB to COX-2 *(B)* and mPGES-1 *(D)* promoter regions in RAW 264.7 macrophages by ChIP assays. CREB transcription factor was immunoprecipitated from cells stimulated with PGE_2 (5μM) for the times indicated (minutes). Immunoprecipitated DNA was amplified with specifics primers for the COX-2 or the mPGES-1 proximal promoter regions. As a control, PCR was performed on chromatin fragments isolated before immunoprecipitation (input). Immunoprecipitation with a normal rabbit serum (NRS) was carried out in parallel as negative control. The results shown are a representative experiment of the three experiment performed.



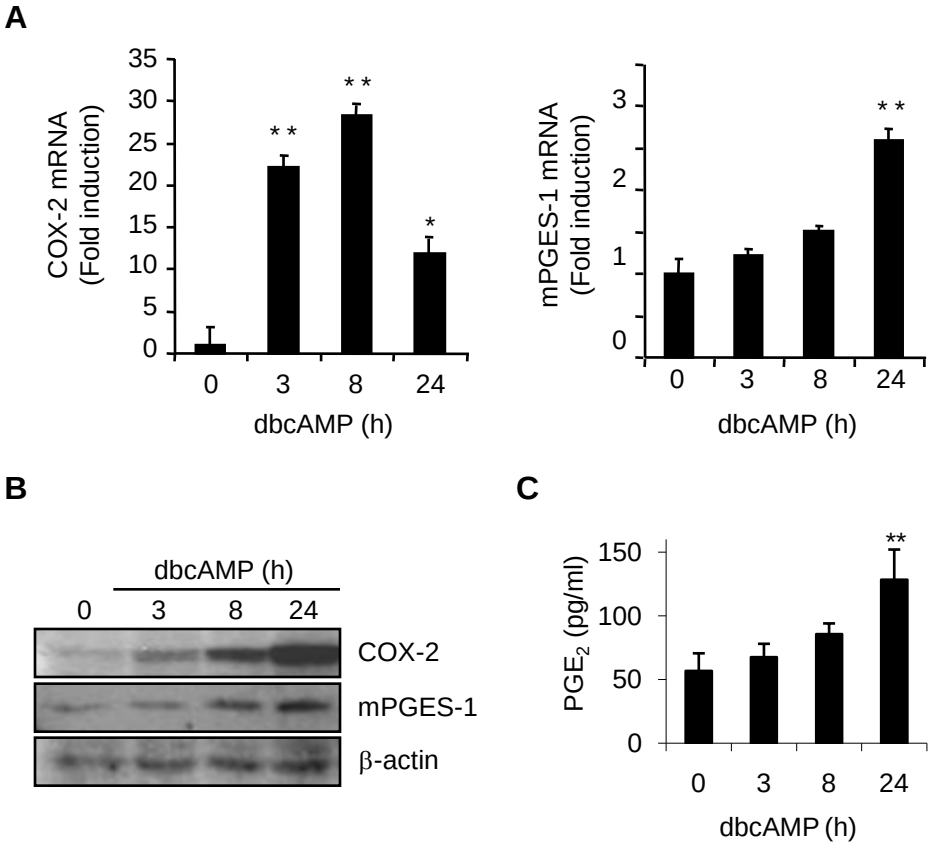
Díaz-Muñoz et al. **Figure 1**



Díaz-Muñoz et al. **Figure 2**



Díaz-Muñoz et al. **Figure 3**



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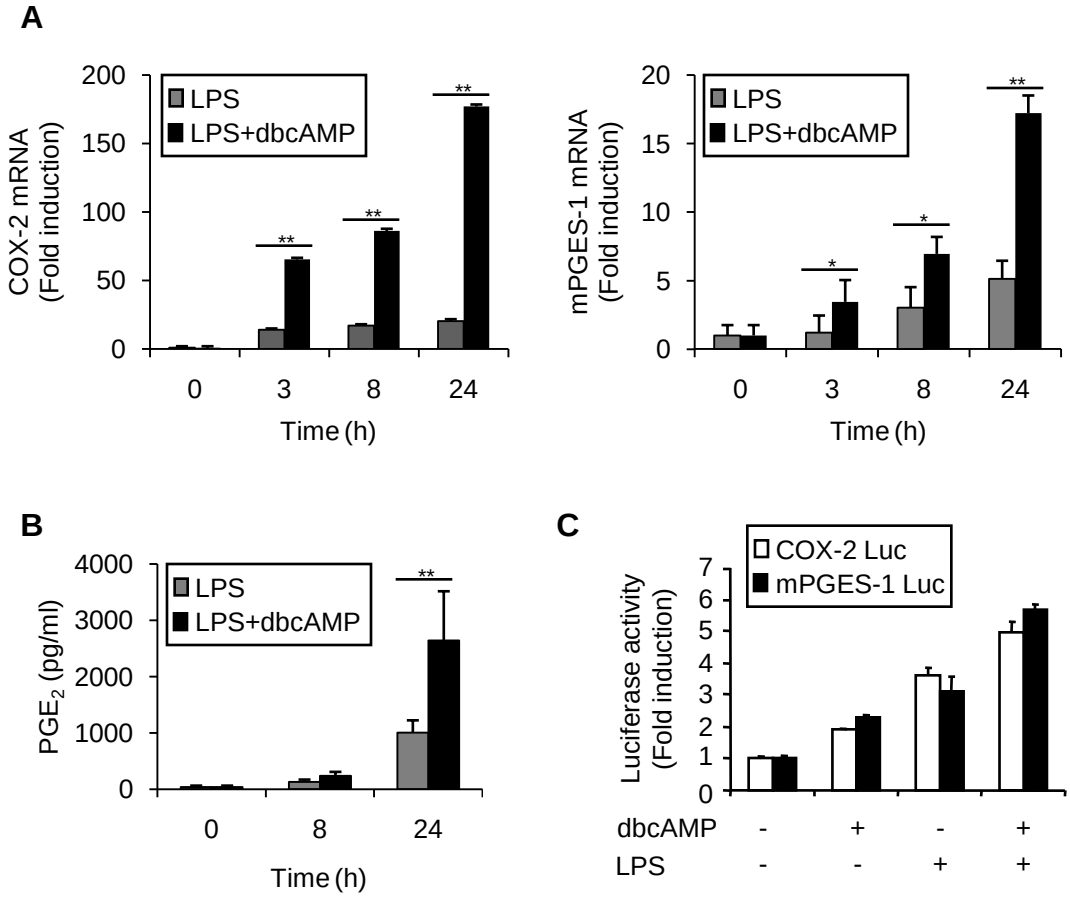

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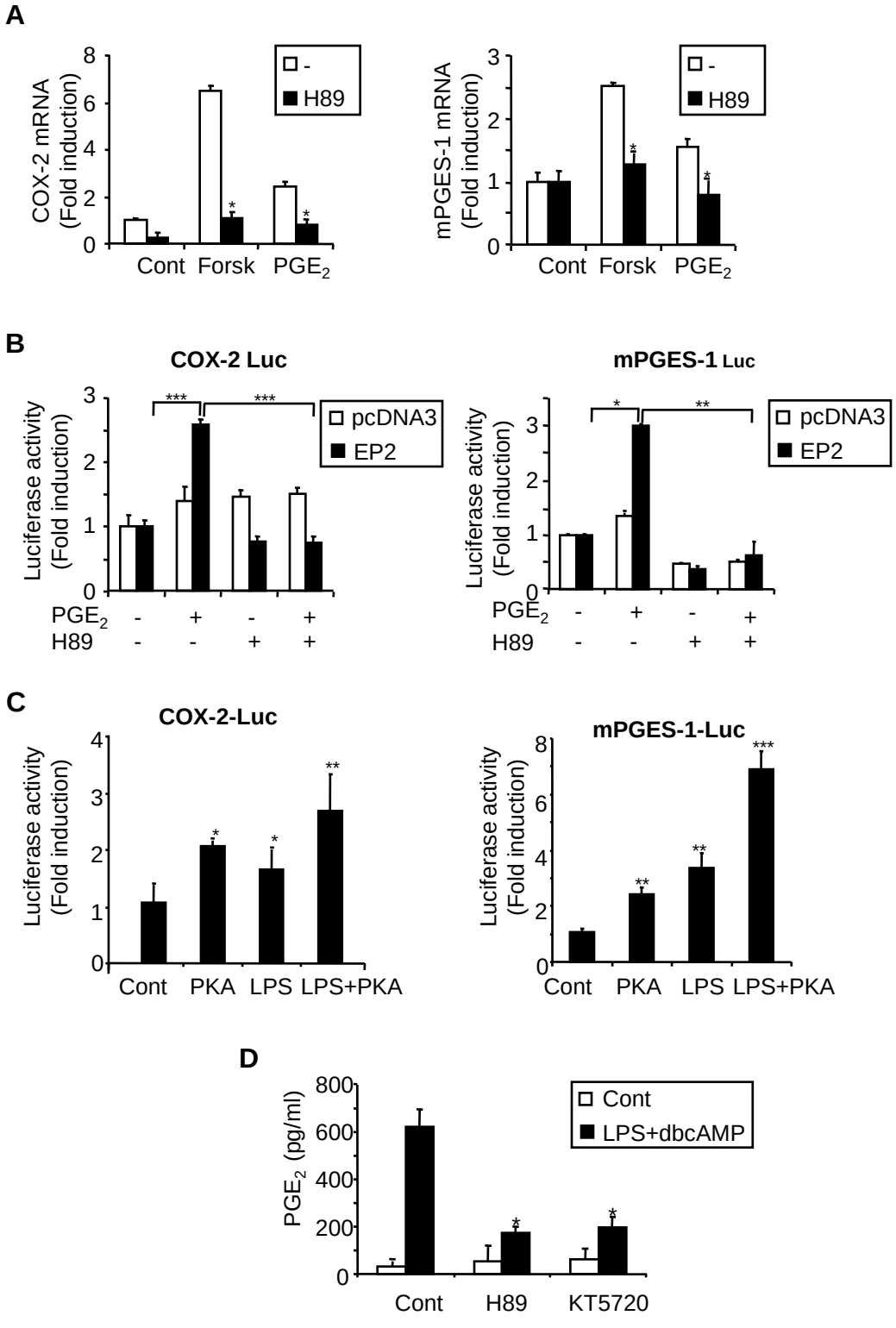
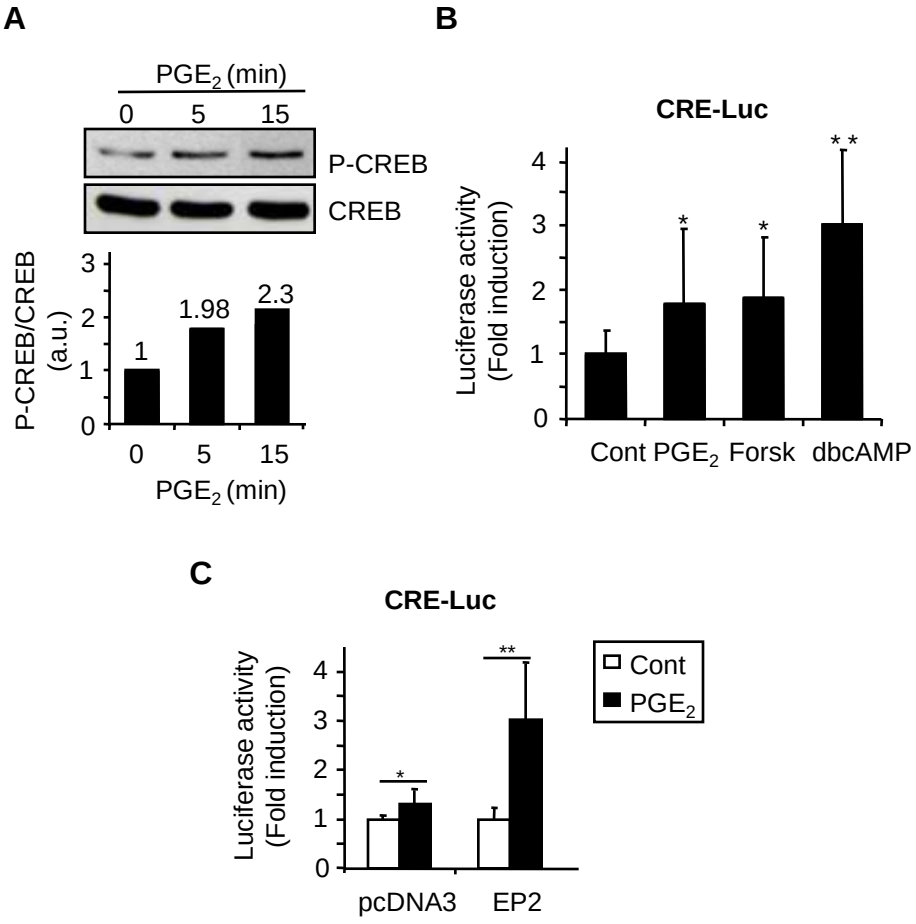
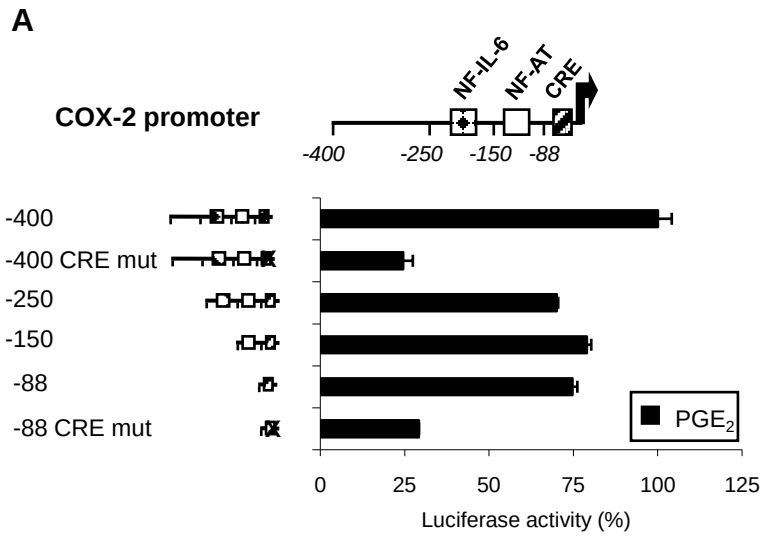
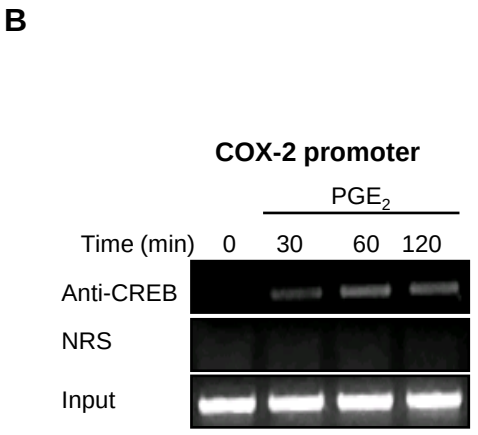


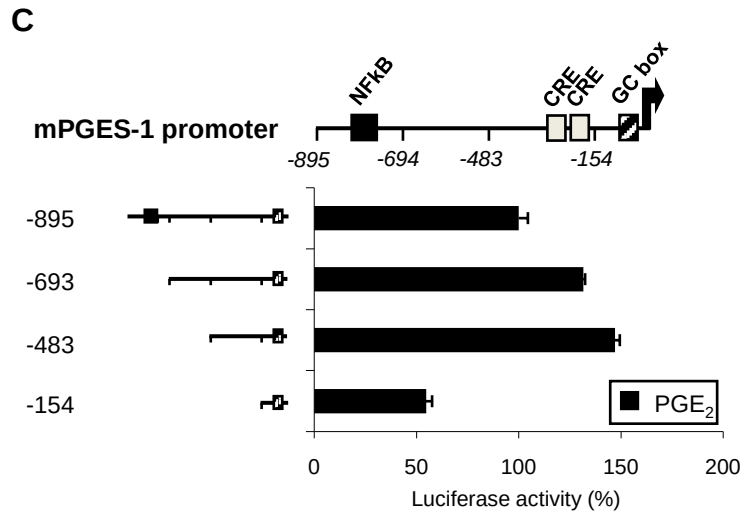
Díaz-Muñoz et al. **Figure 6**

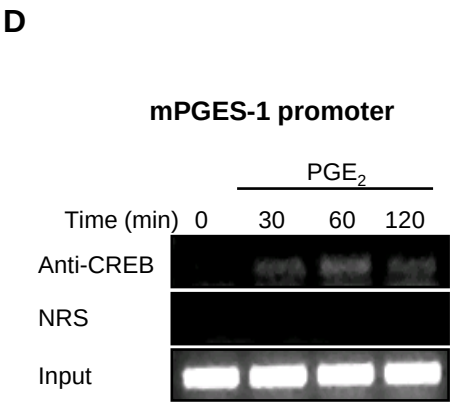


Díaz-Muñoz et al. **Figure 7**









Díaz-Muñoz et al. **Figure 8**

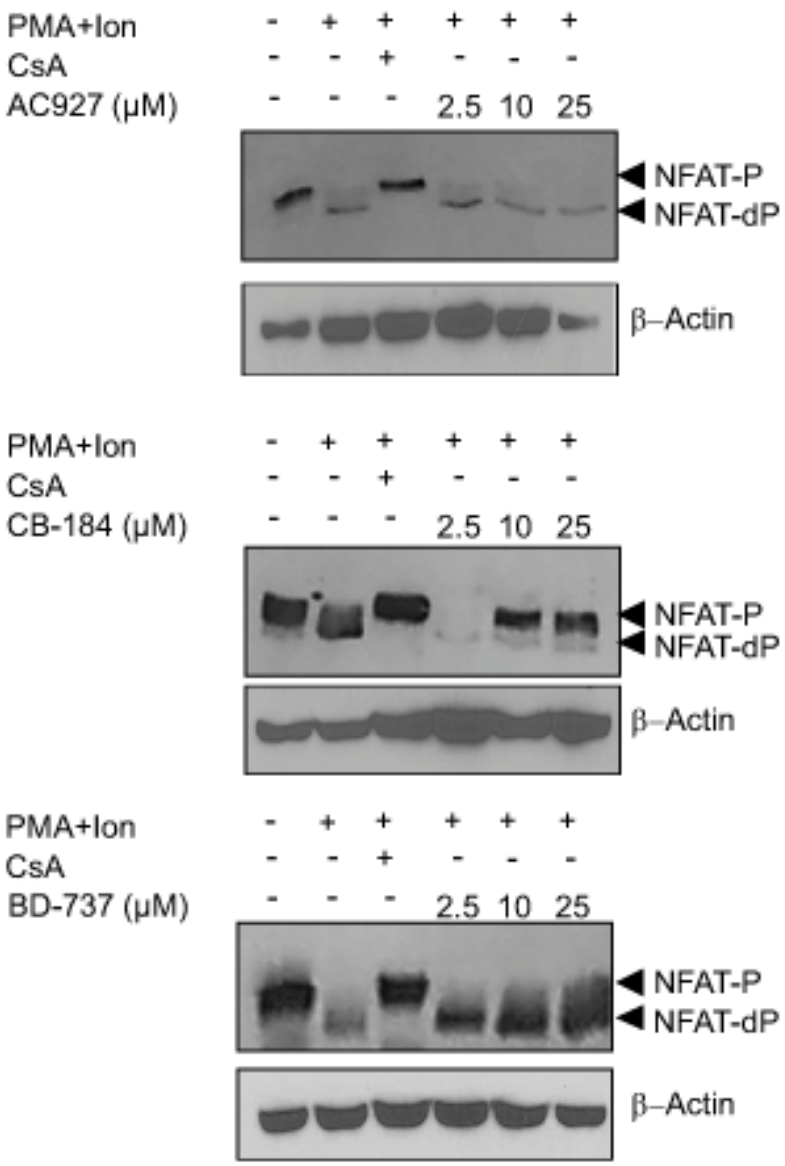


Figure 9. Iñiguez et al.