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Coordinate regulation between the nuclear receptor peroxisome proliferator-activated receptor-γ and cyclooxygenase-2 in renal epithelial cells

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ABSTRACT

The peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors involved in lipid metabolism and glucose utilization, in cell growth, differentiation and apoptosis, and in the regulation of pro-inflammatory genes expression such as cyclooxygenase-2 (COX-2). PPARγ is the main isoform in the renal inner medulla where it is believed to possess nephroprotective actions. In this kidney zone, COX-2 acts as an osmoprotective gene and its expression is modulated by changes in interstitial osmolarity. In the present work we evaluated whether hyperosmolar-induced COX-2 expression is modulated by PPARγ in renal epithelial cells MDCK subjected to high NaCl medium. The results presented herein show that ligand-activated PPARγ repressed COX-2 expression. But more important, the present findings show that hyperosmolar medium decreased PPARγ protein and increases the PPARγ phosphorylated form, which is inactive. ERK1/2 and p38 activation precedes PPARγ disappearance and induced-COX-2 expression. Therefore, the decrease in PPARγ expression is required for hyperosmotic induction of COX-2. We also found that PGE_2, the main product of COX-2 in MDCK cells, induced these changes in PPARγ protein. Our results may alert on the long term use of thiazolidinediones (TZD) since they could affect renal medullary function that depends on COX-2 for cellular protection against osmotic stress.

1. Introduction

The peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors belonging to the steroid hormone nuclear receptor superfamily. These receptors can be activated by natural fatty acids, leukotrienes, prostaglandins, and some synthetic agonist, including antidiabetic drugs such as rosiglitazone, and pioglitazone [1]. There are three isoforms of PPARs: α, β/δ and γ and they can all activate and inhibit gene expression by different mechanisms. PPARs isoform expression varies widely from tissue to tissue. In the kidney each isoform exhibits a distribution pattern being PPARγ the main isoform in the renal inner medulla [2,3]. PPARγ is involved in lipid and lipoprotein metabolism and glucose utilization, in cell growth, differentiation and apoptosis, and in the regulation of pro-inflammatory genes expression such as IL-6, IL-1β, TNF, and inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), among others [1]. Besides, it has been reported that PPARγ has nephroprotective actions in the renal medullary tissue but the mechanism is unknown.

COX-2 is the inducible isoform of cyclooxygenase family, and its expression is usually associated with physiological and pathological stimuli. This enzyme catalyzes the first step in the formation of prostaglandins from arachidonic acid [4,5]. In mammalian kidney, prostaglandins play a critical role in maintaining renal medullary blood flow and salt and water homeostasis [6]. Despite the inducible feature of COX-2 protein, its constitutive expression and activity has been demonstrated in the renal medulla [7]. In vivo [8,9] and in vitro [10] experiments showed that the increase of NaCl enhances the expression of COX-2 in renal medullary cells. Moreover, it has been proposed that COX-2 constitutive expression is a consequence of the constant hyperosmotic stimulus on

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medullary epithelial cells [11]. We and others previously demonstrated that in the renal medullary cell line MDCK, hyperosmolarity induces COX-2 mRNA and protein expression [7]. Hyperosmotic-induced COX-2 has been proposed to act in renal cytoprotection against changes in environmental osmolarity and is considered a survival gene. It has been demonstrated that PPARγ is involved in the regulation of COX-2 expression in various tissues [1]. Scoditti et al. found that PPARγ activation by rosiglitazone or GW1929 suppresses COX-2 expression in human endothelial cells [12]. COX-2 expression is also regulated by a negative feedback loop mediated through PPARγ in the macrophage-like-differentiated U937 cells [13]. In contrast, the NSAIDs-enhanced COX-2 expression could be mediated through PPARγ activation [14,15]. Therefore, COX-2 expression is likely to be regulated in a cell specific manner by different PPAR activators.

Considering that COX-2 is a survival protein that catalyzes prostaglandin synthesis from arachidonic acid, that PPARγ, which is expressed in the renal medulla, could act as a nephroprotective molecule and that PPARγ is involved in the regulation of genes related to lipid metabolism as is COX-2, we examined the relationship between PPARγ activation and COX-2 expression in renal epithelial cells. We also determined which signaling pathway is involved in this process. The results demonstrated that PPARγ activation represses hyperosmolarity-induced COX-2 expression. Also, they showed that hyperosmolarity downregulates PPARγ while it increases the phosphorylated form, pPPARγ, which is inactive. The phosphorylation of this nuclear receptor requires the participation of ERK1/2 and p38 MAP kinases and seems to be mediated by COX-2 product, PGE_2. Our results suggest that a decrease in PPARγ is necessary for COX-2 expression in MDCK cells under hyperosmotic stress.

2. Materials and methods

2.1. Materials

Madin–Darby canine kidney (MDCK) cells were purchased from American Type Culture Collection, passages 45–50 (Manassas, VA, USA). MDCK cells were grown in Dulbecco's modified Eagle's medium Ham'-F12 (1:1) (GIBCO®-Life Technologies Corporation, NY, USA), 10% fetal bovine serum (FBS) (Natocor, Córdoba, Argentina) and 1% antibiotic mixture (GIBCO®-Life Technologies Corporation, NY, USA). The PPARγ antagonist GW9662, rosiglitazone (Rosi), 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), U0126, PD98059, SB203580, SP600125, prostaglandin E2 (PGE_2) and indomethacin (Indo) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-COX-2 polyclonal antibody and Anti-PPARγ polyclonal antibody were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). Anti-β-tubuline polyclonal antibody was purchased from Abcam (Cambridge, MA, USA). The PVDF membranes, the secondary antibody anti-rabbit horseradish peroxidase conjugate and the ECL Plus detection system were supplied by GE Healthcare Life-science (Buckinghamshire, UK). The Vectastain ABC Kit was purchased from Vector Laboratories (Burlingame, CA, USA). All the reagents for the RT-PCR and the SV total RNA isolation System were supplied by Promega (Madison, MI, USA) and the primers, by Alpha DNA (Montreal, Quebec, Canada). The [1-^{14}C]-arachidonic acid was purchased from Perkin Elmer® (Massachusetts, USA). The thin layer chromatography (TLC) plates and the isooctane, the ethylacetate and the acetic acid were supplied by MERK (Darmstadt, Germany). General reagents were from Mallinckrodt or Merck and purchased from local suppliers.

2.2. Cell culture conditions

Madin–Darby canine kidney (MDCK) cells (American Type Culture Collection, passages 45–50) were grown in a mixture containing Dulbecco' modified Eagle' medium and Ham'-F12 (1:1), 10% fetal bovine serum (FBS) (Natocor, Córdoba, Argentina) and 1% antibiotic mixture (GIBCO®-Life Technologies Corporation, NY, USA). After reaching 70–80% of confluence, cells were placed in low serum medium (0.5% FBS) for 24 h and then subjected to hyperosmolarity for different periods of time. Hyperosmolar media were made by adding an aliquot of sterile 5 M NaCl to commercial medium to achieve desired final concentrations (250 mM NaCl, 512 ± 12 mOsm/kg H_2O). No changes in final osmolarities of the media were detected due to the different serum concentration.

In those experiments where specific PPARγ antagonist as GW 9662 (0.01, 0.1 and 1 μM) and agonists as rosiglitazone (Rosi) and 15-deoxy-Δ12,14-prostaglandin J_2 (15d-PGJ_2) (50, 100 and 500 nM, and 0.1, 1 and 10 μM for Rosi and 15d-PGJ_2, respectively), and MAPK inhibitors as U0126 (5 μM), PD98059 (5 μM), SB203580 (7.5 μM), SP600125 (7.5 μM) were used, they were added 30 min before the addition of NaCl to the medium. All the drugs used in these experiments were from Sigma-Aldrich (St. Louis, MO, USA). In other set of experiments cells were treated with PGE_2 (0.1 μM) or indomethacin (1 μM) or both 30 min before the addition of NaCl to the incubation media. After treatments, the culture medium that contained dead cells and debris was discarded, and attached cells were collected by trypsin-EDTA (GIBCO®-Life Technologies Corporation, NY, USA) digestion. Collected cells were counted in a hemocytometer chamber (Neubauer' chamber) in the presence of trypan blue to obtain the number of total and viable cells. The viability was calculated from these data as the percentage of non-trypan blue stained cells of total counted cells. Aliquots of cell suspensions containing an adequate number of cells or protein concentration were used for the different experimental protocols. Despite non-viable cells were included in cell population used in the experiments, the number of trypan blue-stained cells was always lower than 5%. When it was required cells were resuspended in lysis buffer (0.089% NaCl–phosphate buffer, pH 7.2, containing 0.05% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, 1 mM leupeptin and 1 mM sodium orthovanadate), and protein concentration was determined by the method of Lowry et al. [16].

2.3. Western blot analysis

Samples from different treatments containing 50 μg of protein from cell lysates were incubated with 4× Laemmli buffer at 100 °C for 5 min, resolved in a 10% SDS-polyacrylamide gel and blotted to PVDF membranes (GE Healthcare Life-science, Buckinghamshire, UK). Blots were blocked with 10% non-fat milk in TBS-Tween and incubated overnight at 4 °C with primary antibodies: rabbit polyclonal COX-2 antibody, 1:250 (Cayman Chemical Co, Ann Arbor, Michigan, USA), rabbit polyclonal PPARγ antibody; 1:450 (Cayman Chemical Co, Ann Arbor, Michigan, USA) and rabbit polyclonal β-tubulin antibody, 1:5000 (AbCam, Cambridge, MA, USA). After washing, blots were incubated with secondary antibody, donkey anti-rabbit horseradish peroxidase conjugate 1:6000 (GE Healthcare Life-science, Buckinghamshire, UK), and bands were visualized using the ECL Plus detection system (GE Healthcare Life-science, Buckinghamshire, UK). In the experiments where chromogenic detection was used, blots were incubated with byotinylated anti-rabbit secondary antibody (1:200) and bands were visualized with Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA). The intensity of each band was estimated by optical densitometry with Gel-Pro Analyzer 3.1.

2.4. RT-PCR

MDCK cells were grown, treated and collected as described above, and then 2×10^6 cells were used for total RNA extraction by using SV total RNA isolation system (Promega, Madison, MI, USA) in accordance with the manufacturer's instructions. The first-strand cDNA was synthesized by using the Reverse Transcription System (Promega, Madison, MI, USA). After that, PCRs for PPARγ and COX-2 gene-expression products were performed. The primers used were: PPARγ, For: 5′CAGAAAATGACAGACCTCAGA3′; Rev: 5′TGTAGGAGTGGGTG-TGTAGGAGTGGGTGAAGA3′; COX-2: For: 5′TCAGCCATACAGCAAA-TCAGCCATACAGCAAATCCTT3′, Rev: 5′GTGCACTGTGTTTGGAGTGG3′; and GAPDH, For: 5′TCCACCACCCTGTTGCTGTA3′, Rev: 5′ACCACAGTC-ACCACAGTCCATGCCATCAC3′.

2.5. COX activity

MDCK cells were grown and subjected to the treatments described above. Previous to harvesting, culture medium was replaced by FBS-free DMEM/F-12 containing 1 μl [1-^{14}C]-arachidonic acid (S.A. 51 mCi/mmol, A.C. 0.05 mCi/0.5 ml, Perkin Elmer®, Massachusetts, USA). After 30 min at 37 °C, the incubation medium was recovered, acidified up to pH 3 by adding 1 M citric acid and mixed with 2 vol of chloroform. Radiolabeled prostaglandins were extracted in the lower organic phase and separated by TLC using the upper phase of a mixture containing isooctane/ethylacetate/acetic acid/water (66:30:12:60, v/v/v/v) as solvent system [17] (MERK, Darmstadt, Germany). Each prostaglandin was identified by the corresponding standard, and specific area on TLC was visualized by autoradiography, scrapped off and quantified by liquid scintillation counting.

2.6. Statistic analysis

The results are expressed as the mean ± SEM. Data from controls and different treatments were analyzed by ANOVA and significant differences were assessed by "a posteriori" Scheffé test ($p < 0.05$).

3. Results

3.1. Effect of GW9662, rosiglitazone and 15-deoxy-Δ12,14-prostaglandin J_2 (15d-PGJ_2) on COX-2 expression in the renal epithelial cell line MDCK

All the experiments in this work were performed in Madin–Darby canine kidney (MDCK) cell line. MDCK cell line has been typified as a transporting-polarized epithelium that possesses both morphological and enzymatic properties of cells from distal tubules and medullary collecting ducts of the kidney [18,19]. As MDCK cells subjected to high sodium hyperosmotic medium (512 ± 12 mOsm/kg H_2O) behave as inner medullary collecting duct cells, they are usually used as a model system for studying medullary cell physiology and adaptation to osmotic stress [20]. Numerous in vivo and in vitro experiments have demonstrated that changes in environmental osmolarity regulate the expression of COX-2 in renal medullary tissue and in renal cell lines [10,21,22]. The treatment with high NaCl-hyperosmotic medium increased both COX-2 mRNA and protein in MDCK cells. Such increase was dependent on the incubation time and showed a biphasic kinetic for mRNA expression (Fig. 1A). Short-time incubations with NaCl (up to 3 h) decreased mRNA levels. Its expression was recovered after 6 h, being maximal at 24 of treatment. COX-2 protein expression remained constant until 6 h, and after 12 and 24 h of treatment it was significantly increased respect to the control. Even though COX-2 isoform expression was

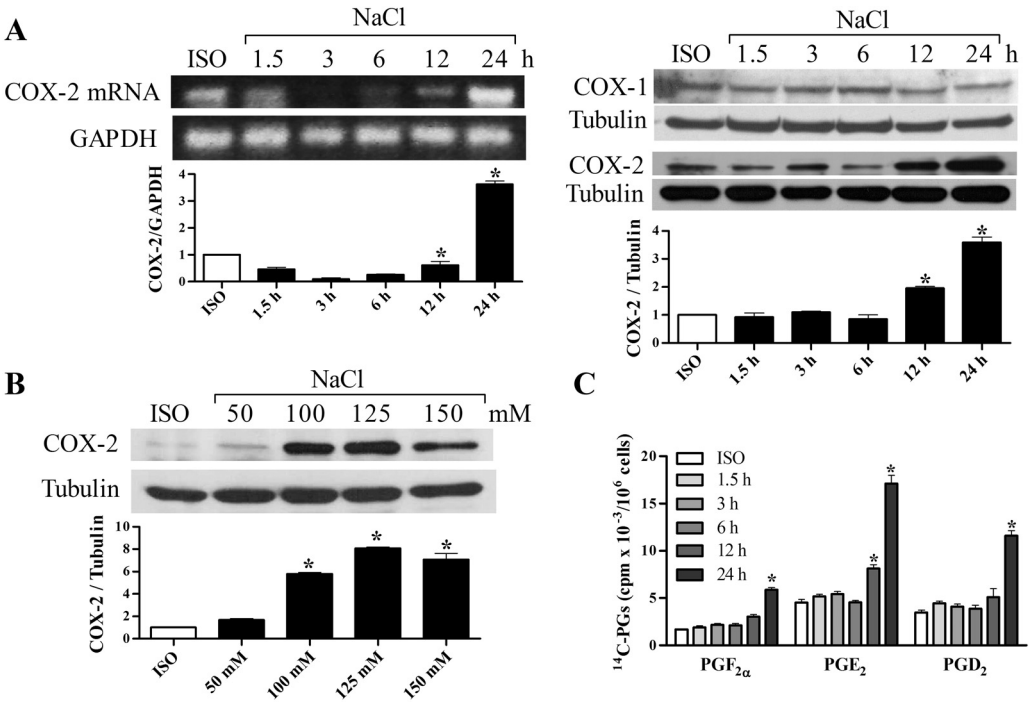

Fig. 1. *NaCl-hyperosmotic medium increases the mRNA, protein and activity of COX-2.* MDCK cells were grown in a mixture containing DMEM/Ham'-F12 (1:1), 10% FBS and 1% antibiotic mixture. After reaching 70–80% of confluence, cells were placed in low serum medium (0.5% FBS) for 24 h and then subjected to NaCl-hyperosmotic medium for different periods of time. After treatment, cells were harvested and COX-2 mRNA, COX-2 and COX-1 protein, and COX activity were evaluated as described in Section 2. The treatment with hyperosmotic medium (125 mM NaCl added to commercial medium) increases COX-2 mRNA and protein levels as a function of the incubation time but it does not affect the COX-1 protein (A). Hyperosmotic media also increases COX-2 protein as function of the NaCl concentration (B). COX activity increases as a function of the incubation time following COX-2 expression (C). PGE_2 was the main COX product in both conditions; the relative amount of PGE_2, PGD_2 and $PGF_{2\alpha}$ was around 50%, 35% and 15%, respectively. The images are representative of three independent experiments. * Significantly different from hyperosmolar control value, $p < 0.05$.

enhanced by hyperosmolarity, the constitutive isoform, COX-1, was not affected by the treatment (Fig. 1A). COX-2 protein increase was also dependent on the NaCl concentration (Fig. 1B). As we expected, the increase in COX-2 protein was linked to an increase in COX activity (Fig. 1C). Under hyperosmotic conditions, the most abundant prostaglandin in MDCK cells was PGE_2 followed by PGD_2 and to a much lesser extent by $PGF_{2\alpha}$ (Fig. 1C). This profile is similar to the prostaglandin profile of the renal medulla [23]. PGs synthesis varied following the kinetic of COX-2 protein expression. Both PGE_2 and PGD_2 begun to increase after 12 h of treatment coinciding with the increase of COX-2 protein while $PGF_{2\alpha}$ only showed a slight increase at 24 h. Considering that COX-1 protein did not change with hyperosmolarity, we can speculate that the increase in COX activity was exclusively due to COX-2 protein. In order to evaluate whether PPARγ activation mediates hyperosmotic-COX-2 induction, cells were treated with 0.01, 0.1 and 1 μM GW9662, a specific antagonist of PPARγ, before sodium addition to the culture. After 24 h of treatment, COX-2 protein level was evaluated by Western blot analysis. The treatment with GW9662 did not affect hyperosmotic-induced COX-2 expression at any concentration assayed (Fig. 2A). This result could indicate either that PPARγ does not operate on COX-2 gene or that PPARγ is not activated by hyperosmolarity and in consequence the effect of the transcription factor on gene expression is not detectable. To determine which of these possibilities was occurring, in the next experiment the cultures were treated with selective PPARγ agonists to ensure its activation. Afterwards, COX-2 expression was evaluated (Fig. 2B and C). Rosiglitazone (Rosi), a synthetic ligand of PPARγ, did not affect osmotic induction of COX-2 protein at any concentration assayed. However, 15-deoxy-Δ12, 14-prostaglandin J_2 (15d-PGJ_2), which is the main natural ligand of PPARγ nuclear receptor, decreased COX-2 expression in a concentration dependent manner (Fig. 2C). This result shows that high NaCl stimulus does not activate PPARγ in MDCK cells, and also shows how the activation of this transcription factor represses COX-2 gene expression under hyperosmotic conditions. Fig. 2D shows that the decrease in COX-2 expression caused by 10 μM 15d-PGJ_2 was prevented by the treatment with the specific PPARγ antagonist GW9662. This result confirms the fact that PPARγ activation impairs COX-2 expression in the renal epithelial cell line MDCK.

The repression of COX-2 gene by PPARγ activation was more evident in MDCK cultures grown under normal isosmotic conditions (Fig. 3). In these experiments cells were incubated in isotonic commercial medium (298 ± 12 mOsm/kg H_2O) and treated with 0.01, 0.1 and 1 μM of GW9662 for 24 h. Similar to that found in hyperosmolarity, GW9662 did not affect COX-2 expression at any concentration assayed (Fig. 3A). However, the treatment with 50, 100

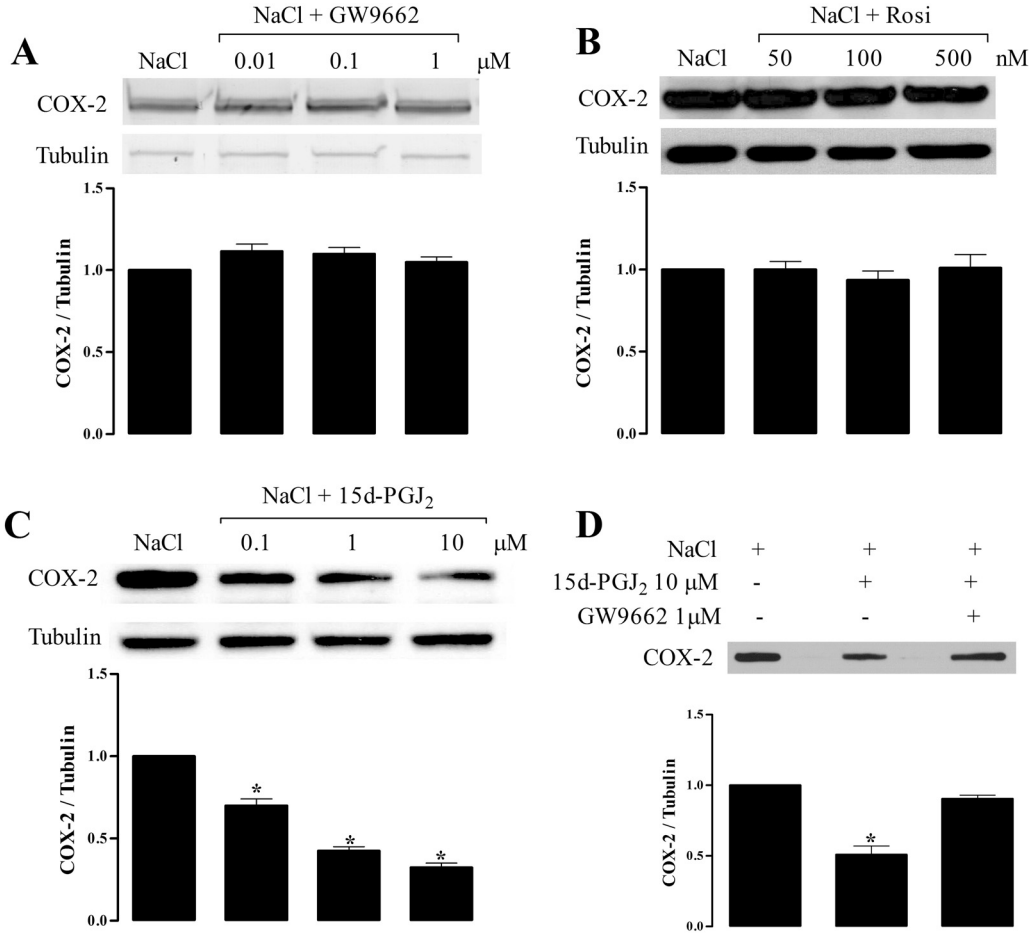

Fig. 2. *Effect of GW9662, rosiglitazone and 15d-PGJ_2 on hyperosmotic-induced COX-2 expression in MDCK cells.* MDCK cells were grown in a mixture containing DMEM/Ham'-F12 (1:1), 10% FBS and 1% antibiotic mixture. After reaching 70–80% of confluence, cells were placed in low serum medium (0.5% FBS) for 24 h and then treated with PPARγ antagonist (0.01, 0.1 and 1 μM GW9662) or ligands (50, 100 and 500 nM rosiglitazone and 0.1, 1 and 10 μM 15d-PGJ_2) 30 min before NaCl addition. After 24 h, cells were collected and lysates were subjected to 10% SDS-PAGE and transferred to PVDF membranes. Immunoblots were probed with 1:250 polyclonal COX-2 antibody (Cayman) and reprobed with 1:5000 polyclonal β-tubulin (AbCam). Bands were visualized by Vectastain ABC Kit (Vector Lab) or ECL (GE, Healthcare) as described in Section 2. Each image is representative of three independent experiments. GW9662 (A) and Rosi (B) did not affect osmotic induction of COX-2 protein whereas 15d-PGJ_2 (C) decreased COX-2 expression in a concentration dependent manner. The antagonist GW9662 reverted the effect of 15d-PGJ_2 on COX-2 expression (D). Results represent the mean ± ESM of three independent experiments. * Significantly different from hyperosmolar control value, $p < 0.05$.

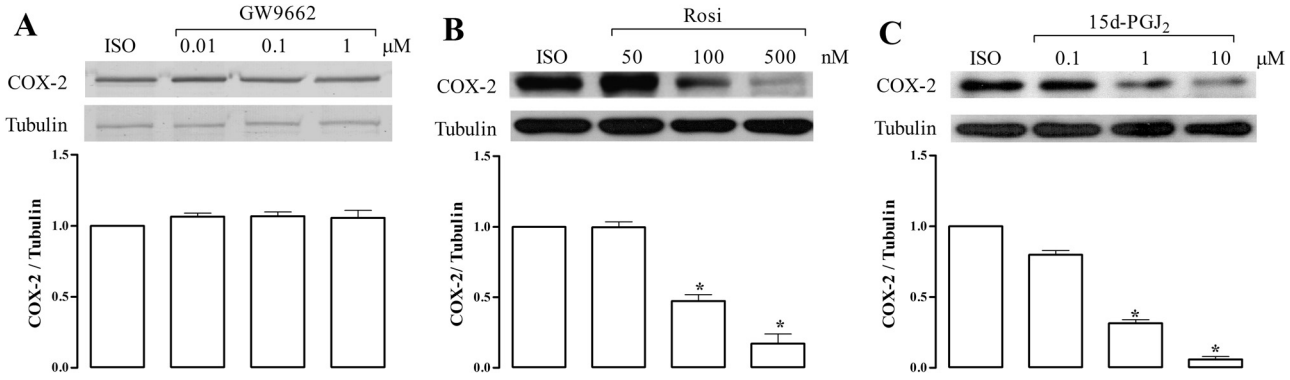

Fig. 3. *Effect of GW9662, rosiglitazone and 15d-PGJ$_2$ on COX-2 expression under isosmotic conditions.* MDCK cells were grown in a mixture containing DMEM/Ham'-F12 (1:1), 10% FBS and 1% antibiotic mixture. After reaching 70–80% of confluence, cells were placed in low serum medium (0.5% FBS) for 24 h and then treated with PPARγ antagonist (0.01, 0.1 and 1 μM GW9662) or ligands (50, 100 and 500 nM rosiglitazone and 0.1, 1 and 10 μM 15d-PGJ$_2$). After 24 h of treatment, cells were collected and lysates were subjected to 10% SDS-PAGE and transferred to PVDF membranes. Immunoblots were probed with 1:250 polyclonal COX-2 antibody (Cayman) and reprobed with 1:5000 polyclonal β-tubulin (AbCam). Bands were visualized by Vectastain ABC Kit (Vector Lab) or ECL (GE, Healthcare) as described in Section 2. Each image is representative of three independent experiments. GW9662 did not affect COX-2 expression at any concentration evaluated (A). However, Rosi (B) and 15d-PGJ$_2$ (C) decreased COX-2 protein levels in a concentration dependent manner under isosmotic conditions. Results represent the mean ± ESM of three independent experiments. * Significantly different from isosmolar control value, $p < 0.05$.

and 500 nM of Rosi, and 0.1, 1 and 10 μM of 15d-PGJ$_2$ significantly decreased COX-2 protein levels (Fig. 3B and C). Thus, the activation of PPARγ also represses the basal level of COX-2 expression in MDCK cells.

3.2. High NaCl downregulates PPARγ in MDCK cells

It is well established that changes in environmental osmolarity modulate the expression of several renal medullary osmoprotective proteins such as COX-2 [8–10]. Although PPARγ is expressed in renal medulla where it is believed to exert nephroprotective actions [24–26], no reports are available on how osmolarity affects the expression of this protein [2,3]. Thus, we studied the effect of hyperosmotic treatment on PPARγ expression in the cell line MDCK. Fig. 4 shows that hyperosmotic medium did not significantly change PPARγ mRNA expression at any experimental time assayed. In contrast, Western blot analysis revealed that hyperosmotic treatment caused a significant decrease in PPARγ expression (Fig. 4B). Moreover, hyperosmolarity increased the levels of the phosphorylated form of PPARγ (pPPARγ), which was also detected by the antibody (Fig. 4B). The decrease in PPARγ and the concomitant increase in pPPARγ were evident from the beginning of the stimulation (Fig. 4C). Together, the present results indicate that changes in the medium osmolarity do not activate PPARγ, decrease PPARγ levels and induce PPARγ phosphorylation. Several groups have reported that PPARγ could be phosphorylated and that such post-translational modification could modulate the transcriptional activity of this receptor [27–29]. Interestingly, the phosphorylation of PPARγ may inhibit or stimulate its transcriptional activity depending on the cellular background and the kinase involved [1,28]. In addition, the PPAR phosphorylation status may regulate PPARγ ubiquitination and its subsequent degradation [27].

Taken together these results strongly suggest that the expression of COX-2 protein, which is absolutely necessary for cell survival when the cells are exposed to abrupt changes in environmental osmolarity, occurs only if PPARγ is down-regulated.

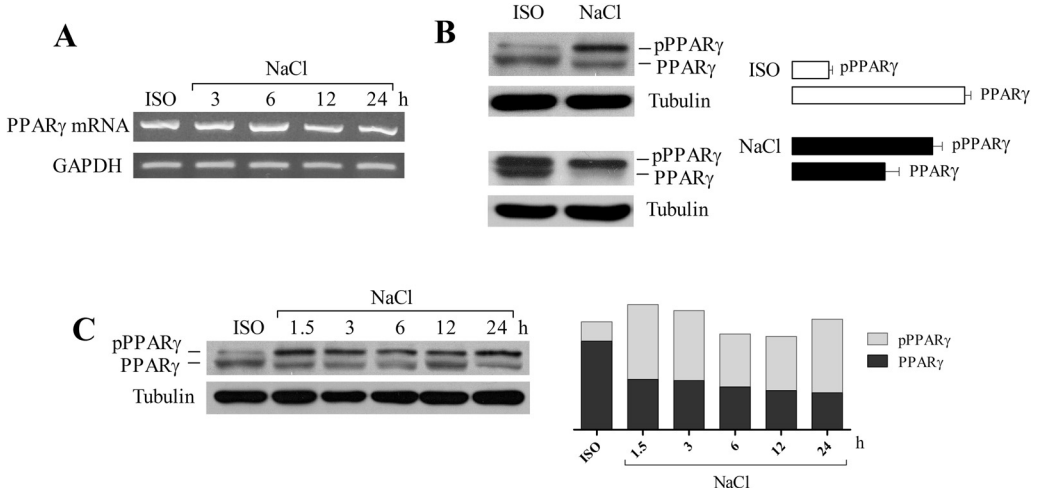

Fig. 4. *High NaCl downregulates PPARγ in MDCK cells.* MDCK cells were grown in a mixture containing DMEM/Ham'-F12 (1:1), 10% FBS and 1% antibiotic mixture. After reaching 70–80% of confluence, cells were placed in low serum medium (0.5% FBS) for 24 h and then subjected to isosmotic or NaCl-hyperosmotic media. After treatments, cells were collected and PPARγ mRNA or protein levels were evaluated as described in Section 2. The antibody recognizes PPARγ and its phosphorylated form (pPPARγ). The treatment did not affect PPARγ mRNA levels (A). The main form under isosmolar conditions was PPARγ whereas after NaCl treatment was the phosphorylated form (B). The addition of NaCl decreased PPARγ and increased the phosphorylated form pPPARγ (C). The increase in pPPARγ was already observed after 1.5 h treatment. Each image is representative of three independent experiments. Results represent the mean ± ESM of three independent experiments. * Significantly different from isosmolar control value, $p < 0.05$.

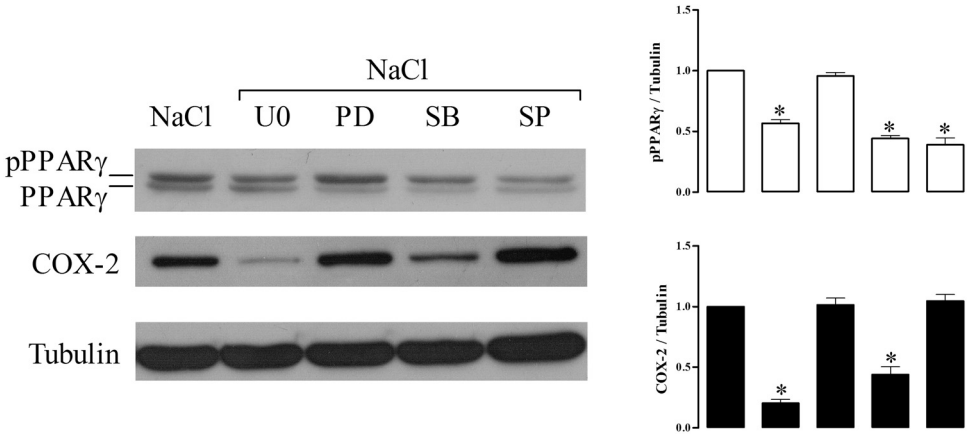

Fig. 5. *Effect of MAPK inhibitors on hyperosmotic-induced PPARγ phosphorylation in MDCK cells.* MDCK cells were grown in a mixture containing DMEM/Ham'-F12 (1:1), 10% FBS and 1% antibiotic mixture. After reaching 70–80% of confluence, cells were placed in low serum medium (0.5% FBS) for 24 h. Before NaCl addition, cells were treated with 5 μM U0126, 7.5 μM SP600125, 7.5 μM SB230580 and 5 μM PD98059 for 30 min and later subjected to hyperosmotic medium for 24 h. After treatment, cells were collected and lysates were subjected to Western blot analysis by probing the membrane with 1:450 polyclonal PPARγ antibody (Cayman), 1:250 polyclonal COX-2 (Cayman) antibody, and 1:5000 polyclonal β-tubulin (AbCam) antibody. Each image is representative of three independent experiments eliciting similar pattern. U0126, SB230580 and PD98059 decreased pPPARγ but only U0126 and SB230580 impaired COX-2 expression. * Significantly different from hiperosmolar control value, $p < 0.05$.

3.3. ERK1/2 and p38 kinases are required for hyperosmotic PPARγ phosphorylation in MDCK cells

Various kinases are known to be activated by hyperosmotic stress such as MEKK3, MKK3, PI3K, JNK, PKA, p38, ATM and Fyn [30,31]. Several researchers have demonstrated that PPARγ can be phosphorylated via ERK [32,33] and p38 MAPK [34].

In addition, the expression of COX-2 requires the activation of the mitogen-activated protein kinases ERK1/2, p38 and JNK [35]. Thus, we evaluated whether MAPKs pathways could be involved in the phosphorylation of PPARγ. To do this, we treated MDCK cultures with specific MAPKs inhibitors previous to NaCl addition. After 24 h of treatment, COX-2 and PPARγ expression was evaluated by Western blot. The treatment with U0126 (impedes ERK1/2 activation through the inhibition of MEK), SB203580 (p38 inhibitor) and PD98059 (MAKK inhibitor) decreased the levels of the phosphorylated form of the nuclear receptor PPARγ (Fig. 5). Concomitantly, U0126 and SB203580, but not PD98059, impaired COX-2 protein expression. On the basis of these results, obtained with MAPK specific inhibitors, we suggest that the phosphorylation of PPARγ is mediated by MAPKs ERK1/2 and p38. Such modification could inactivate or label PPARγ protein for its degradation. This experiment also suggests the negative modulation of COX-2 by PPARγ.

3.4. Effect of PGE_2 on PPARγ phosphorylation in MDCK cells

The treatment of MDCK cells with high-NaCl hyperosmotic media induces the expression of COX-2 (Fig. 1), which is necessary for osmoprotection and, as we previously discussed, the COX-2 expression only occurs if PPARγ is inactivated by hyperosmolarity. Thus, the question arising was how hyperosmolarity induces PPARγ inactivation. Previously, Reginato et al. demonstrated that $PGF_{2\alpha}$ blocks adipogenesis by inducing PPARγ-phosphorylation through MAPK activation [36]. In MDCK, PGE_2 is the major COX-2 prostaglandin synthesized (Fig. 1C). It has been published that PGE_2 suppresses PPARγ actions in adipocytes [37,38]. The interaction of PGE_2 with EP2 and/or EP4 receptors can lead to the activation of several kinases such as ERK1/2 and p38. By using selective inhibitors of these pathways we can infer that ERK1/2 and p38 are involved in PPAR phosphorylation (Fig. 5). Thus, we wanted to evaluate whether PGE_2 affects PPARγ phosphorylation in MDCK cells. To do this, cells were treated with 0.1 μM PGE_2 for 24 h and then PPARγ protein level was evaluated by Western blot analysis. In order to avoid the endogenous PGs synthesis which can interfere with the action of the added PGE_2, the experiments were performed in the presence indomethacin that is a potent inhibitor of both, COX-1 and COX-2. The treatment with PGE_2 decreased total PPARγ protein respect to control value (Fig. 6). In the presence of indomethacin, the effect of PGE_2 was even higher since the level of no-phospho PPARγ dropped to a half of PGE_2 level while phospho-PPARγ level was significantly increased. Unexpectedly, indomethacin alone caused a similar effect to PGE_2. All together, these results lead us to suggest that the inactivation of PPARγ could be mediated by the action of PGE_2, which is a main product of COX activity in renal cells.

4. Discussion

The present results clearly show that PPARγ agonists negatively modulate COX-2 protein expression in renal epithelial cells. The natural PPARγ ligand15d-PGJ_2 inhibited the hyperosmotic-induced COX-2 protein. Such an effect was observed even under isosmolar conditions but in this case, the repression of COX-2 protein was also observed with the synthetic ligand rosiglitazone. COX-2 is a key enzyme in prostaglandin synthesis. Despite COX-2 is an inducible protein in many tissues, it has been reported its constitutive expression in some brain, ovary and kidney cells [5,11]. In renal tissue, COX-2 is considered an osmoprotective gene since it is involved in the adaptation of medullary interstitial [39] and epithelial cells [7,22,40] to hyperosmotic stress. Thus, the fully understanding of the regulation of COX-2 expression [7] and/or activity [40] is an important issue to address for renal physiology.

The expression of PPARs nuclear receptors in the kidney has been previously reported [2]. Each kidney zone and structure differentially expresses a particular type of PPAR. PPARα is predominantly expressed in the renal cortex. PPARγ is dominantly expressed in the inner renal medulla mainly in the collecting system, including connective renal tubules and collecting ducts [2,41]. It has been reported that PPARγ activation by specific agonists, such as glitazones, exerts beneficial effects on the kidney. Some of these nephroprotective actions take place at hemodynamic level since PPARγ agonists reduce blood pressure, increase NO production and cause vasodilatation [42]. Also, PPARγ agonists exert anti-proliferative and anti-inflammatory effects in renal tissue [43]. Despite these beneficial effects of PPARγ on the kidney,

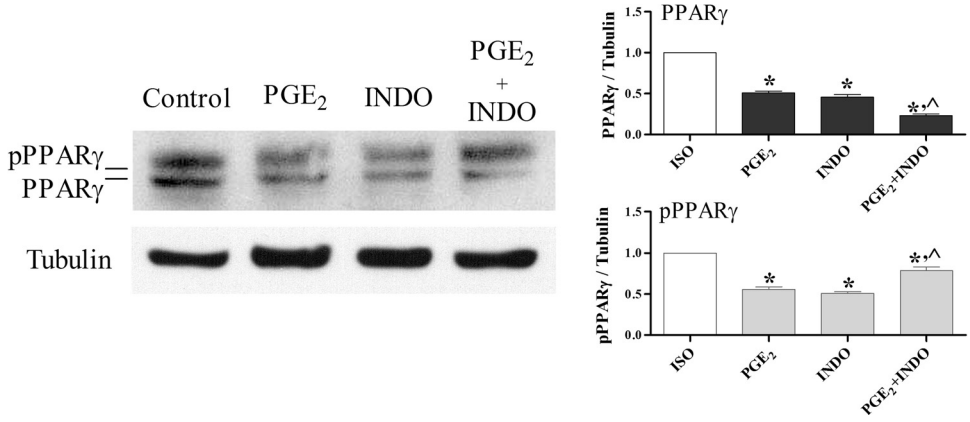

Fig. 6. *Effect of PGE_2 on PPARγ phosphorylation.* MDCK cells were grown in a mixture containing DMEM/Ham'-F12 (1:1), 10% FBS and 1% antibiotic mixture. After reaching 70–80% of confluence, cells were placed in low serum medium (0.5% FBS) for 24 h and then treated with 0.1 μM PGE_2 and/or 1 μM indomethacin 30 min before NaCl addition. After 24 h of treatment, cells were collected and lysates were subjected to 10% SDS-PAGE and transferred to PVDF membranes. Immunoblots were probed with 1:450 polyclonal PPARγ antibody (Cayman) and reprobed with 1:5000 polyclonal β-tubulin (AbCam). Bands were visualized by ECL (GE, Healthcare) as described in Section 2. Each image is representative of three independent experiments. The treatment with PGE_2 increased PPARγ phosphorylation and decreased the levels of PPARγ. * Significantly different from isosmolar control value, $p < 0.05$. ^ Significantly different from INDO value, $p < 0.05$.

the exact mechanism is not well understood. The control region of COX-2 gene possesses one response element for PPAR (PPRE) [5] and, the participation of PPARγ in the transcriptional activation of COX-2 gene in other tissues has been reported [44–46]. Thus, we first hypothesized that PPARγ beneficial actions in renal structures could occur through the induction of COX-2. However, the activation of PPARγ by its agonists decreased COX-2 protein expression in a concentration dependent manner (Figs. 2 and 3). This result agrees with a previous report from Sawano et al. who demonstrated that 15d-PGJ_2 inhibits IL-1β induced COX-2 expression in renal mesangial cells. In that work, the repression of COX-2 by PPARγ is a beneficial effect since blocks the inflammatory increase of COX-2 in renal cortical cells [47]. This is not the case of renal medullary tissue where COX-2 has a protective action. It is worth to mention that PPARγ is inactive either in isosmotic or hyperosmotic conditions, as was shown when GW9662 was used (Figs. 2A and 3A). However, PPARγ activation by thiazolidinediones (TZD) causes the repression of COX-2. Then, the constant activation of PPARγ by TZD could counteract the nephroprotective actions reported for this nuclear receptor. The different TZD operate with different effectiveness in the diverse cell systems and organisms [48]. Besides, TZD are used in type II diabetes treatment, and it has been reported that at renal level TZD induce fluid retention through a decrease in Na^+ excretion [49,50], which could it be related to COX-2 expression inhibition (since PGE_2 is a natriuretic and diuretic hormone in renal medulla), these results may alert on the long term use of TZD. However, such hypothesis has to be further proven.

Thus, when the medullary interstitium is affected by an abrupt increase in the concentration of solutes, and renal cells need to express COX-2 to face it up, PPARγ activity would have to be abolished. In this sense, we found that PPARγ expression is negatively regulated by the COX-2 activity product, PGE_2 (Fig. 6). We found that hyperosmotic treatment decreased PPARγ protein expression and increased the phosphorylated form of PPARγ in renal epithelial MDCK cells (Fig. 4). In this condition, both COX-2 expression and PGE_2 synthesis are high (Fig. 1). It has been reported that PPAR activity can be modulated via phosphorylation [28], and it is well established that hyperosmolarity activates different kinase-signaling pathways such as p38, ERK1/2 and JNK, among others [51]. Fig. 5 shows that the use of SB203580 and UO126, which are selective inhibitors of p38 and ERK1/2, respectively, decreased the phosphorylated form of PPARγ, suggesting the participation of both kinases in PPAR phosphorylation. These experiments clearly show that kinases inhibition, that decreases pPPARγ levels, concomitantly reduces hyperosmotic-induced COX-2 protein, and reinforce the repressive action of PPARγ on COX-2 expression. ERK- and JNK-MAPKs have been implicated in PPARγ phosphorylation and, in consequence, in its inactivation [27,36]. The phosphorylation-mediated inhibition of PPARγ has been proposed as an important switch-off of its activity [52]. The phosphorylation represses PPARγ activity by different molecular mechanisms that include the inhibition of ligand binding and PPAR protein sumoylation [27]. Independently of the mechanism, the present results lead us to suggest that hyperosmotic-induced phosphorylation of PPARγ might repress the activity of the nuclear factor to ensure the expression of the cytoprotective protein COX-2 under osmotic stress. Moreover, we found that PGE_2, the main prostaglandin synthesized in MDCK cells under hyperosmotic conditions (Fig. 1), decreases PPARγ total protein level and induces an increase in the phosphorylated form (Fig. 6). This result is in agreement with previous report from Chen et al. that demonstrates how the high production of PGE_2 and the elevated levels of COX-2 in LPS-stimulated old mice macrophages occurs only when the nuclear receptor RXRα, a main partner of PPAR, is down-regulated [53]. The fact that COX-2-synthesized PGE_2 suppresses PPARγ actions was also demonstrated in adipocytes [37,38]. It has been reported that PGE_2 can activate MAPK pathways [54,55]. Thus, it could be possible that hyperosmotic-induced PGE_2 represses PPARγ through the activation of ERK1/2 and p38. However, such hypothesis has to be proven.

Summarizing, the present results demonstrate a coordinate action between COX-2 and PPARγ exists in renal cells subjected to hyperosmolar stress. When osmolarity abruptly increases, renal cells induce the expression of the osmoprotective gene COX-2. PGE_2, the main product of COX-2 activity, could activate ERK1/2 and p38, which in turn could phosphorylate and inactivate PPARγ thus preventing its repressive action on COX-2 gene (see Graphical abstract).

Conflict of interest statement

The authors state no conflict of interest.

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