Molecular and Cellular Endocrinology 384 (2014) 43–51

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Involvement of PI3K/Akt and p38 MAPK in the induction of COX-2 expression by bacterial lipopolysaccharide in murine adrenocortical cells

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ARTICLE INFO

Article history:
Received 21 December 2013
Accepted 8 January 2014
Available online 11 January 2014

Keywords:
Cyclooxygenase-2
Lipopolysaccharide
p38 MAPK
AKT/PKB
Adrenocortical cells

ABSTRACT

Previous studies from our laboratory demonstrated the involvement of COX-2 in the stimulation of steroid production by LPS in murine adrenocortical Y1 cells, as well as in the adrenal cortex of male Wistar rats. In this paper we analyzed signaling pathways involved in the induction of this key regulatory enzyme in adrenocortical cells and demonstrated that LPS triggers an increase in COX-2 mRNA levels by mechanisms involving the stimulation of reactive oxygen species (ROS) generation and the activation of p38 MAPK and Akt, in addition to the previously demonstrated increase in NFκB activity. In this sense we showed that: (1) inhibition of p38 MAPK or PI3K/Akt (pharmacological or molecular) prevented the increase in COX-2 protein levels by LPS, (2) LPS induced p38 MAPK and Akt phosphorylation, (3) antioxidant treatment blocked the effect of LPS on p38 MAPK phosphorylation and in COX-2 protein levels, (4) PI3K inhibition with LY294002 prevented p38 MAPK phosphorylation and, (5) the activity of an NFκB reporter was decreased by p38 MAPK or PI3K inhibition.

These results suggest that activation of both p38 MAPK and PI3K/Akt pathways promote the stimulation of NFκB activity and that PI3K/Akt activity might regulate both p38 MAPK and NFκB signaling pathways.

In summary, in this study we showed that in adrenal cells, LPS induces COX-2 expression by activating p38 MAPK and PI3K/Akt signaling pathways and that both pathways converge in the modulation of NFκB transcriptional activity.

1. Introduction

Although glucocorticoid production is mainly regulated by the pituitary hormone adrenocorticotropin, adrenal steroidogenesis is also modulated in an autocrine/paracrine way (Hanke et al., 1998; Bornstein et al., 1997, 2004; Engstrom et al., 2008; Cymeryng et al., 1999, 1998; Grion et al., 2007; Pomeraniec et al., 2004). In this sense, we have recently demonstrated the involvement of COX-2 activity in the modulation steroidogenesis induced by LPS in Y1 murine adrenocortical cells (Martinez Calejman et al., 2011), as well as in the adrenal cortex of male Wistar rats (Sanchez et al., 2013).

COX enzymes catalyze the rate-limiting step in prostaglandin, prostacyclin and thromboxane synthesis that is the conversion of arachidonic acid to PGH_2. Two isoforms of COX have important physiological functions: the constitutively expressed COX-1 and the inducible COX-2. Under physiological conditions, COX-2 levels are normally very low in most tissues but are rapidly induced by cytokines, pro-inflammatory agents, tumor promoters, and certain hormones (Herschman, 1994; Smith et al., 1996). In particular, induction of COX-2 is part of the inflammatory response elicited by LPS (Smyth et al., 2009; Hata and Breyer, 2004).

Interaction of LPS with TLR4 in target cells prompts the recruitment of one or more adaptor proteins such as MyD88, Toll-interleukin 1 receptor domain containing adaptor protein Mal, TRIF, or TRAM (Bode et al., 2012). Several downstream effects of LPS involve the stimulation of the NFκB signaling pathway and result in increased transcription of multiple target genes including IL-6, KC, and COX-2. Indeed, this mechanism is involved in the induction of COX-2 by LPS in adrenal cells, as we have previously demonstrated (Martinez Calejman et al., 2011). Other signal transduction pathways have also been shown to participate in the induction of COX-2 by LPS in different cell types: the transcription factor AP-1, surface receptors and their adapter proteins, protein kinases of

Abbreviations: COX-2, cyclooxygenase 2; LPS, lipopolysaccharide; ROS, reactive oxygen species.

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http://dx.doi.org/10.1016/j.mce.2014.01.007

PKC and Src families, PI3K and mitogen-activated protein kinases (MAPKs) Chun and Surh, 2004; Tsatsanis et al., 2006. Regarding this last group of proteins, it is well known that inflammatory stimuli may elicit cellular responses through the activation of mitogen-activated protein kinases (MAPKs). Three MAPK families have been well-characterized: extracellular-signal-regulated kinases, p42/44 (ERK1/2), c-jun amino terminal kinases (JNKs) and p38 MAPKs (Herschman, 1994; Smith et al., 1996) and MAPK family members are involved in the induction of COX-2 gene expression in various cell types, such as macrophages (Bode et al., 2012), cardiomyocytes (Degousee et al., 2003), HUVECs (Kuldo et al., 2005) and smooth muscle cells (Shi et al., 2012).

Among them, p38-MAPK plays a crucial role in inflammation (Schieven, 2005). Activation of p38 MAPK by an increase in its kinase activity, involves its dual phosphorylation on Tyr and Thr by MKK3 and MKK6 (Cuenda and Rousseau, 2007). Activated p38 MAPK upregulates the production of key inflammatory mediators, including TNF-α, IL-1β and COX-2, by several independent mechanisms, including direct phosphorylation of transcription factors, direct or indirect mRNA stabilization and enhanced translation of mRNAs (Cuenda and Rousseau, 2007; Ridley et al., 1998; Molina-Holgado et al., 2000; Mifflin et al., 2002).

Several studies have demonstrated increased mitochondrial activity and ROS formation in macrophages and other cells types treated with LPS (Hsu and Wen, 2002; Simon and Fernandez, 2009). In turn, oxidative stress has been shown to mediate the activation of MAPK pathways (Runchel et al., 2011). In particular, LPS/ROS dependent stimulation of p38 MAPK, a stress-activated serine/threonine protein kinase has been widely acknowledged (Runchel et al., 2011; Emre et al., 2007; Haddad and Land, 2002).

LPS also stimulate the PI3K/Akt pathway, a cell survival regulatory pathway, in different cell types (Lai et al., 2009; Schabbauer et al., 2008). Activation of PI3K promotes the binding of Akt to membrane phospholipids facilitating its phosphorylation and activation by phosphatidylinositol-dependent kinases (Hemmings and Restuccia, 2012). pAkt, in turn, induces changes in the catalytic activity of downstream targets, such as glycogen synthase kinase-3(GSK-3) and the mammalian target of rapamycin (mTOR) Hemmings and Restuccia, 2012. Akt signaling is also known to be involved in the production of inflammatory mediators such as NO, PGE2, and TNF-α through the activation of NFκB (Rajaram et al., 2006).

Considering that possible interactions among different signaling pathways vary from cell to cell and in different physiological/pathological conditions, and that their relative contribution to the LPS-dependent increase in COX-2 levels in adrenal cells has not been analyzed, in the present study we assessed the involvement of ROS/p38 MAPK and Akt signaling pathways in the induction of COX-2 by LPS treatment in murine adrenal cells.

2. Materials and methods

2.1. Chemicals

Antibodies raised against COX-2 were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). Antibodies against phosphorylated p38 MAPK, α-p38 MAPK and against β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phosphorylated Akt and total Akt were purchased from New England Biolabs (Ipswich, MA, USA). Peroxidase-conjugated goat anti rabbit-IgG was obtained from Bio-Rad (Hercules, CA, USA). Fetal calf serum was from Natocor (Cordoba, Argentina). Penicillin and streptomycin were from Invitrogen (Life Technologies, Buenos Aires, Argentina). TRI Reagent was from Genbiotech (Buenos Aires, Argentina). Wortmannin, Ly 294002, SB203580, LPS (*Escherichia coli* endotoxins serotype O111:B4), Ham's F10 cell culture media and the protease and phosphatase inhibitor cocktails were purchased from Sigma–Aldrich (Buenos Aires, Argentina). All other chemicals were of the highest quality available.

2.2. Cell culture and treatments

Y1 is a mouse adrenocortical tumor cell line isolated by Yasumura et al. (1966). Cells were grown as monolayers in plastic culture dishes in Ham's F-10 medium containing heat-inactivated 10% fetal calf serum and 100 U/ml penicillin, 100 μg/ml streptomycin in a humidified incubator at 37 °C and 5% CO_2. Treatments were initiated 24 h after replacing the growth medium with fresh Ham's F-10 without serum. After the treatments, incubation media were collected and cells were washed twice in PBS and lysed in 20 moll/l Tris–HCl pH 7.4, 250 mmol/l NaCl, 1%Triton X-100, 1× protease inhibitor cocktail and 1× phosphatase inhibitor cocktail.

2.3. In vitro studies in rat adrenocortical tissue

Control naïve rats were sacrificed at approximately 10 AM and their adrenal glands were excised, decapsulated, demedullated and dissected in quarters on ice. Adrenal tissues were then transferred to Ham's F10 medium at 37 °C, and incubated with or without the addition of 10 μg/ml LPS for 0, 30 or 60 min. At the end of the incubation period the tissues were homogenized in 50 mM Na_2HPO4 pH 7.4, 0.2 mM EDTA, 100 mM KCl, 1× protease inhibitor cocktail, and 1× phosphatase inhibitor cocktail in a final volume of 0.5 ml per gland. The homogenates were centrifuged at 2000*g* for 10 min at 4 °C, and the supernatants were collected. The expression of total and phosphorylated isoforms of either p38 MAPK or Akt was analyzed by Western Blot.

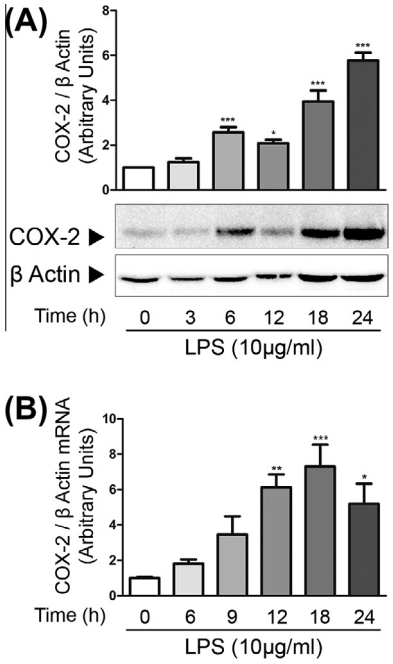

Fig. 1. Time course of cyclooxygenase-2 (COX-2) induction by LPS in murine adrenocortical cells. Y1 cells were incubated with 10 μg/ml LPS for the times indicated and total RNA and proteins were obtained as described in Section 2. (A) A representative western blot of COX-2 and β-actin protein levels is shown below a densitometric analysis of normalized data from three independent experiments. (B) COX-2 mRNA levels were determined by reverse transcription of total RNA and Real Time-PCR. COX-2 mRNA values were normalized to β-actin mRNA levels. Data are presented as means ± SEM, $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. 0 h by ANOVA followed by Tukey's post hoc test.

2.4. Western blotting

Protein samples from cells or adrenal tissue (80 μg) were boiled for 5 min in SDS–PAGE loading buffer with 0.1 mol/l dithiothreitol and electrophoresed on 10% polyacrylamide gels. After electrophoresis, proteins were transferred to polyvinylidenefluoride membranes (PVDF) for 1 h at 15 V in a semi-dry transfer system (Bio-Rad Trans-Blot SD, CA, USA) in 25 mmol/l Tris–HCl pH 9.2, 192 mmol/l glycine and 20% methanol buffer. PVDF membranes were blocked in TBS-Tween (50 mmol/l Tris–HCl pH 7.4, 0.15 M NaCl, 0.05% Tween-20) containing 5% skimmed milk for 60 min at room temperature and then incubated overnight at 4 °C with specific rabbit antibodies against COX-2 (1:200), phospho-p38 MAPK (1:500), p38α (1:1000), phospho-Akt, total Akt or β-actin (1:1000). Anti-rabbit IgG linked to horseradish peroxidase was used as a secondary antibody and incubations were conducted for 1 h at room temperature. Immunoreactivity was detected by chemiluminescence and quantitative data was obtained with the ImageQuant Imaging System (GE Healthcare, Piscataway NJ, USA) and AlphaEase Fluorchem software (V. 4.1.0, Alpha Innotech Corporation).

2.5. RNA isolation and RT-PCR

Total RNA was obtained from Y1 cells using TRI reagent. RNA (2 μg) was treated with RNase-free DNase I (Promega Corporation, Madison, WI, USA) for 30 min at 37 °C. 1 μl Stop buffer was added, the mixture was heated at 65 °C for 10 min and then placed on ice. Total RNA samples were incubated with a mixture containing 0.5 mmol/l dNTPs mix, 12.5 ng/μl random primers, 12.5 ng/μl oligodT, 1× first-strand buffer, 2 U/μl of RNAse inhibitor, 10 U/μl MMLV reverse transcriptase and water in a final volume of 20 μl, for 50 min at 37 °C. The reaction was stopped by heating at 70 °C for 15 min and the mixture was brought to 100 μl with diethylpyrocarbonate-treated water and stored at −70 °C. In selected tubes reverse transcriptase was omitted as a contamination control. PCR reactions were performed on the Corbett Research Rotor-Gene Real Time Amplificationsystem (RG-3000, Corbett Research,

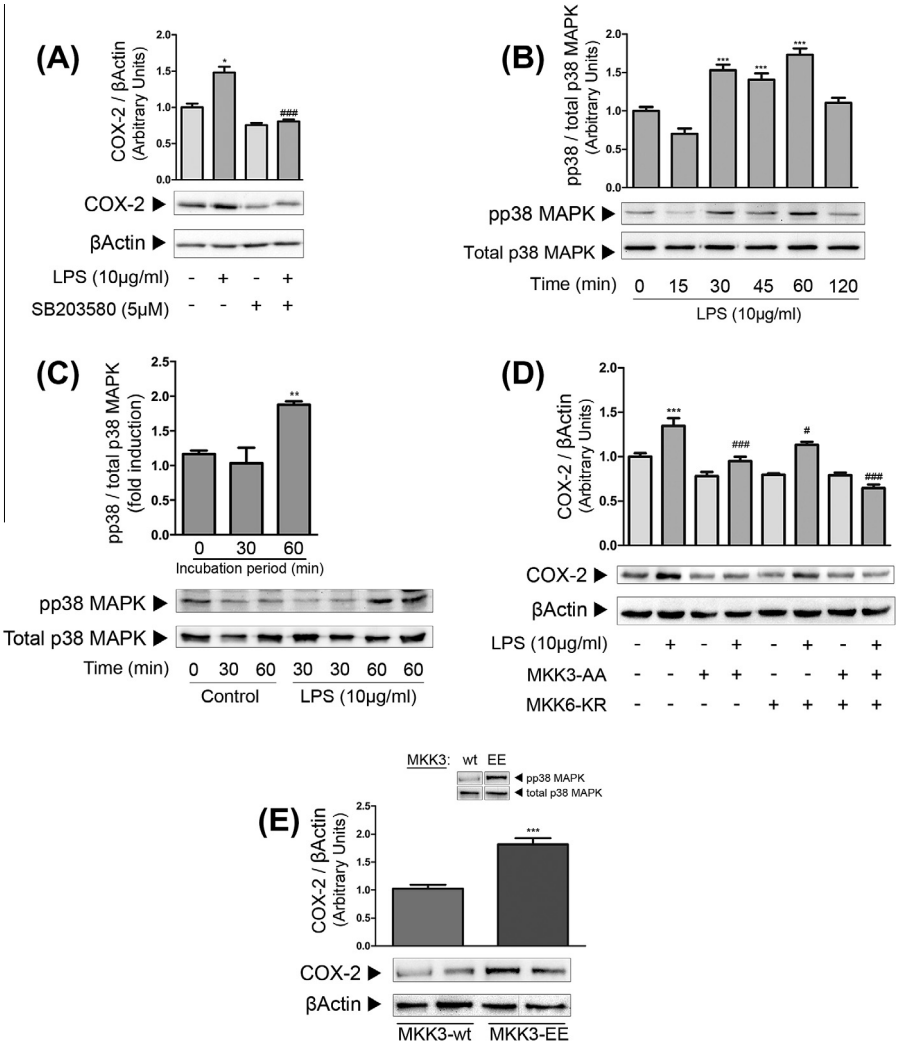

Fig. 2. Activation of p38 MAPK is involved in LPS-stimulated COX-2 expression. (A) Y1 cells were incubated in the presence or absence of 5 μM SB203580 (p38 MAPK inhibitor) for 30 min, then 10 μg/ml LPS were added to the culture medium and incubations were continued for 24 h. Sample proteins were analyzed by Western blotting with anti-COX-2 and anti-β-actin antisera. (B and C) Murine Y1 cells or rat adrenal quarters were incubated with 10 μg/ml LPS for the indicated time intervals. Phosphorylated and total p38 MAPK levels were detected with specific antibodies. (D) Y1 cells were transfected with expression plasmids containing the dominant negative isoforms MKK3-AA and/or MKK6-KR as described in Section 2 and then stimulated with 10 μg/ml LPS for 24 h. COX-2 and β-actin protein levels were determined by western blot in whole cell proteins. (E) Y1 cells were transfected with an expression plasmid for the constitutively active isoform MKK3-EE as described in Section 2, and COX-2 and β-actin protein levels were determined by Western blotting. Phosphorylated and total p38 MAPK levels are shown below as a control. Representative immunoblots are shown below the corresponding densitometric analysis of normalized data from three independent experiments. Data are shown as means ± SEM, $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.001$ and $^{***}p < 0.001$ vs. respective control, and $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs. LPS alone by ANOVA followed by Turkey's post hoc test (A–E).

Mortlake, NSW, Australia) using TaqGo polymerase (Promega, Madison, WI, USA) and Eva Green fluorescent dye (Genbiotech, Buenos Aires, Argentina). The sequence for the oligonucleotide primers were as follows: COX-2 forward: 5′-ATGAGTACCGCAA ACGCTTC-3′, reverse: 5′-CCCCAAAGATAGCATCTGGA-3′; β-Actin: forward: 5′-CCACACCCGCCACCAGTTC-3′, reverse: 5′-GAC-CCATTCCCACCATCACACC-3′. Expression of COX-2 and β-Actin was analyzed using RotorGene 6000 Series Software (version 1.7 Build 40) and mRNA levels were normalized to the housekeeper gene β-Actin.

2.6. Transfections and luciferase reporter assays

Y1 cells (10^4 cells per well) were seeded in 96-well plates and transfected on the following day with 0.5 μl of Lipofectamine 2000 Transfection Reagent (Invitrogen Argentina, Buenos Aires, Argentina) containing 0.18 μg of pκB-LUC and 0.02 μg of pCMV-βgal (β-galactosidase expression plasmid) according to the manufacturer instructions in serum-free OptiMEM medium. Incubations were performed for 3 h, and the media was aspirated and replaced with fresh Ham's F10 containing 10% FCS. Cells were incubated under specified conditions for another 24 h. Luciferase activity was determined with the Steady-Glow Luciferase Assay System (Promega Corporation, Madison, WI, USA) and values were normalized to β-galactosidase activity. The NFκB reporter plasmid (pκB-Luc) was supplied by Dr. Adali Pecci (School of Sciences, University of Buenos Aires, Argentina).

In other set of experiments, Y1 cells (5×10^5 cells per well) were seeded in 12-well plates and transfected on the following day with Lipofectamine 2000 Transfection Reagent. Plasmids expressing either wild type or dominant negative isoforms of MAP-Ks or constitutively active MAPKs were used throughout: MKK3 AA, unable to be activated due to the replacement of serine and threonine residues in its phosphorylation motif by alanine, and MKK6 KR, a kinase-deficient mutant of MKK6 in which a lysine residue that is critical for ATP binding, Lys 82, was mutated to arginine (Raingeaud et al., 1996). Wild type and dominant negative isoforms (pCEFL-akt DK) of AKT were also used (Montaner et al., 2001). These plasmids were kindly provided by Dr. Gutkind (NIC-DR, NIH, MD, USA).

2.7. Determination of intracellular ROS generation

At the end of the treatments, the cells were washed twice in 1× PBS and further incubated in 1× PBS containing 10 μmol/l of the fluorophore 2′, 7′-dichlorodihydrofluorescein diacetate (DCF-DA) for 1 h at 37 °C. Then the cells were rapidly washed twice with 1× PBS and fluorescence (excitation 485 nm, emission 535 nm) was determined in a Multi-mode microplate reader, FLUOstar Omega (BMG Labtech, Ortemberg, Germany). ROS levels are expressed in arbitrary units normalized to total protein content.

3. Results

Y1 cells were incubated with 10 μg/ml LPS and the time course of COX-2 protein accumulation was determined in total cell lysates. As shown in Fig. 1, increased COX-2 protein levels were already detected 6 h after the addition of LPS, while a strong induction was observed 18 to 24 h later. In turn, COX-2 mRNA levels were significantly increased between 12 and 24 h after LPS addition to the incubation media (Fig. 1B). LPS did not stabilize COX-2 mRNA as concluded from experiments on the rates of mRNA degradation in the presence of actinomycin D (data not shown).

In order to determine the involvement of mitogen-activated protein kinases (MAPKs) in COX-2 induction, we first analyzed the effect of specific inhibitors of each signaling pathway. Our

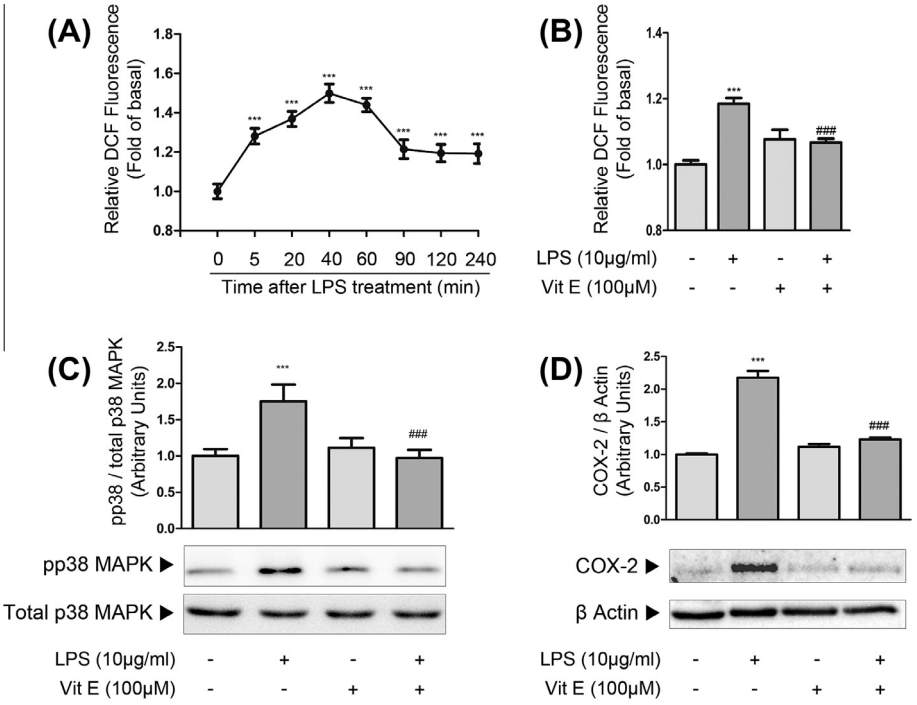

Fig. 3. ROS generation induced by LPS is involved in the stimulation of COX-2. (A) Y1 cells were treated with 10 μg/ml LPS for the indicated time periods and incubated with 10 μM 2′, 7′-dichlorodihydrofluorescein diacetate (DCF-DA) for 30 min. After rinsing twice with 1× PBS, ROS generation was determined as described in Section 2. (B) ROS generation was determined in Y1 cells treated with 100 μM vitamin E (Vit E) for 30 min then with 10 μg/ml LPS for 1 h. (C) Y1 cells were treated with 100 μM Vit E for 30 min and then with 10 μg/ml LPS for another 30 min. Cell extracts were analyzed by western blotting using antibodies raised against phosphorylated and total isoforms of p38 MAPK. (D) Y1 cells were treated with 100 μM Vit E for 30 min and then with 10 μg/ml LPS for another 24 h. Western blot analysis with antibodies raised against COX-2 and β-actin is shown below the corresponding histogram of normalized data from three independent experiments. Data are shown as means ± SEM, $n = 3$, $^{***}p < 0.001$ vs. respective control, and $^{###}p < 0.001$ vs. LPS alone by ANOVA followed by Tukey's post hoc test.

results showed that incubation of Y1 cells with a p38 MAPK inhibitor (SB 203580) blocked the effect of LPS on COX-2 protein levels (Fig. 2A), while inhibitors of JNK (SP 600125) or ERK (PD 98059) had no effect on this parameter (data not shown). LPS induced a transient increase in the levels of pp38 MAPK, readily detected between 30 and 60 min after its addition to the culture media (Fig. 2B). In order to confirm the activation of this pathway in rat adrenal tissue, we incubated adrenal quarters with LPS and analyzed p38 MAPK phosphorylation levels by western blotting. Our results showed that in adrenal quarters, LPS was able to induce a significant increase in the ratio of pp38MAPK/p38 MAPK after 60 min (Fig. 2C). The participation of p38 MAPK in the mechanism of COX-2 induction by LPS was also analyzed through a molecular approach. Our results showed that LPS failed to induce COX-2 in Y1 cells transfected with dominant negative isoforms of two MAP kinase kinases that phosphorylate and activate p38 MAP (MKK3 and MKK6) (Fig. 2D). In addition, overexpression of a constitutively active MKK3 increased p38 MAPK phosphorylation and COX-2 accumulation in Y1 cells (Fig. 2E). These results show the involvement of p38 MAPK in the induction of COX-2 by LPS in adrenal cells.

We next investigated whether oxidative stress participates in COX-2 induction by p38 MAPK stimulation in Y1 cells. Our results showed that LPS treatment triggered a rapid increase in the generation of ROS, already detected 5 min after the addition of LPS (Fig. 3A). Antioxidant treatment with vitamin E prevented ROS generation (Fig. 3B) and blocked the effect of LPS both on p38 MAPK phosphorylation and COX-2 protein levels (Fig. 3C and D) suggesting that in this cell line, ROS generation, induced by LPS is involved in the activation of p38 MAPK and in the induction of COX-2.

Incubation of Y1 cells with LPS in the presence of either LY294002 or Wortmannin blocked the increase in COX-2 protein levels (Fig. 4A), suggesting that COX-2 induction by LPS is also mediated by the PI3K/PKB pathway. As shown in Fig. 4B, an increase in pAkt levels was determined between 30 and 60 min after the addition of LPS to the cells. Similar results were obtained when adrenal quarters from control rats were incubated in the presence of LPS (Fig. 4C).

In other experiments, Y1 cells were transfected with expression plasmids for either the wild type (AKT-wt) or the dominant negative (AKT-DN) isoform of AKT and then treated with LPS for 24 h. COX-2 induction by LPS was significantly decreased in Y1 cells transfected with the AKT-DN (Fig. 4D).

In another set of experiments, a cross talk between p38 MAPK and Akt signaling pathways was examined. We first showed that a PI3K inhibitor (Ly 294002) prevented the increase in pp38MAPK levels triggered by LPS (Fig. 5A). Similar results were obtained when cells were preincubated with Wortmannin (data not shown). On the other side, the increase in Akt phosphorylation levels by LPS was not affected by SB 203580, thus suggesting that stimulation of PI3K by LPS occurs upstream to the activation of p38 MAPK (Fig. 5B).

Finally we analyzed the involvement of p38 MAPK stimulation by LPS on the activation of NFκB, as we have previously demonstrated that COX-2 accumulation induced by LPS entails the activation of the NFκB pathway (Martinez Calejman et al., 2011). To this end, adrenal cells transfected with the κB-LUC reporter plasmid were preincubated with SB203580 to attenuate p38 MAPK activity and then with LPS. According to our previous results, LPS treatment of Y1 cells resulted in the activation of NFκB while preincubation in the presence of SB203580, or transfection with the dominant negative isoform MEK3, significantly prevented NFκB stimulation

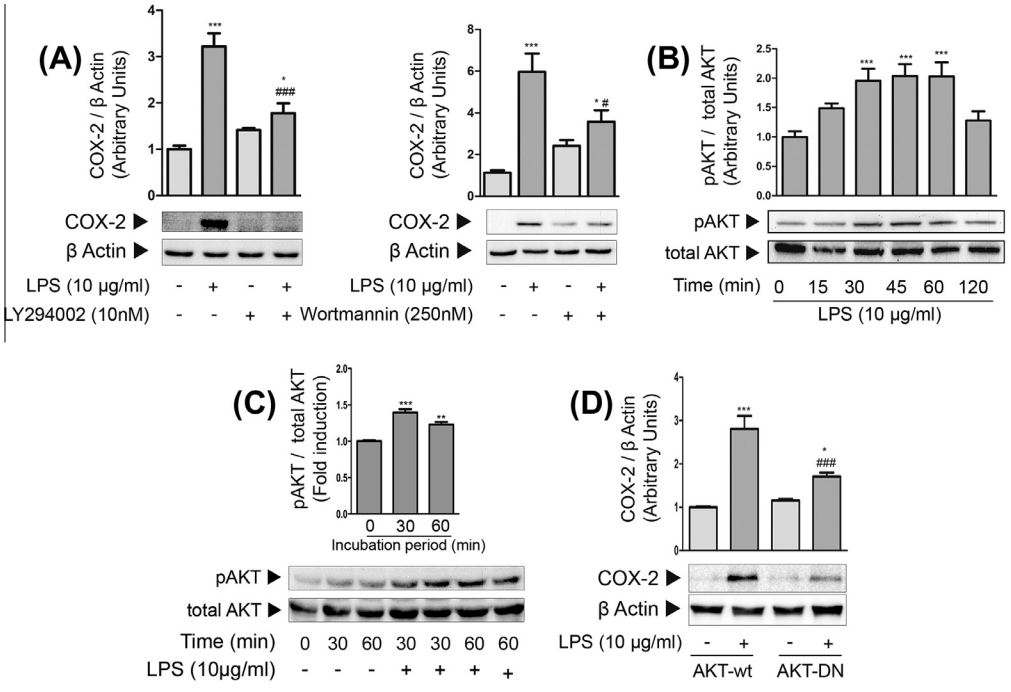

Fig. 4. Involvement of PI3K/Akt pathway in LPS-stimulated COX-2 expression. (A) Y1 cells were treated with PI3K inhibitors (Wortmannin (W) or LY294002) for 30 min then 10 μg/ml LPS was added to the culture medium and incubations were continued for 24 h. Cell lysates were analyzed by Western blotting with anti-COX-2 and anti-β-actin. (B) Cells were incubated with 10 μg/ml LPS for the indicated time intervals. Protein extracts were separated by SDS–PAGE and the levels of phosphorylated and total Akt were detected by immunoblot with specific antibodies. (C) Adrenal glands were excised from control rats and adrenal quarters were incubated in Ham's F10 medium with 10 μg/ml LPS, as described in Section 2, for the indicated time intervals. Phosphorylated and total Akt levels were detected with specific antibodies. (D) Y1 cells were transfected with expression plasmids harboring the wild type (Akt-wt) and a dominant negative isoform (Akt-DN) of Akt as described in Section 2. Transfected cells were stimulated with 10 μg/ml LPS for 24 h. COX-2 and β-actin protein levels were determined by western blot in cell lysates. (A–D) Representative western blots are displayed below the corresponding densitometric analysis of normalized data obtained in three independent experiments. Data is shown as means ± SEM, $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. respective control, and $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs. LPS alone by ANOVA followed by Tukey's test.

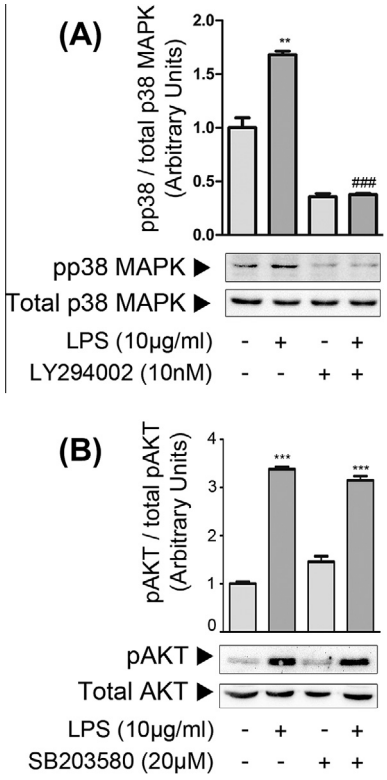

Fig. 5. Crosstalk between PI3K/Akt and p38 MAPK pathways in Y1 cells treated with LPS. Y1 cells were treated with different concentrations of the PI3K inhibitor LY294002 for 30 min, then 10 μg/ml LPS was added to the culture medium and incubations were continued for another 30 min. Total p38 and p38 MAPK phosphorylation levels were assessed by western blot. (B) Cells were treated with 20 μM SB203580 for 30 min, 10 μg/ml LPS was added to the medium and incubations were continued for another 30 min. Cell extracts were analyzed by western blot for phosphorylated and total Akt isoforms. Representative western blots are shown below the corresponding densitometric analysis of normalized data obtained from three independent experiments. Data are shown as means ± SEM, $n = 3$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. respective control, and $^{\#\#\#}p < 0.001$ vs. LPS alone by ANOVA followed by Tukey's test.

by LPS (Fig. 6A and B). Inhibition of the PI3K/Akt pathway with Wortmannin also blocked the stimulation of the κB-LUC reporter plasmid (Fig. 6C). These results suggested that stimulation of p38 MAPK and Akt signaling pathways also impacts on the activity of NFκB.

4. Discussion

Previous studies from our and other groups have demonstrated that *in vivo* treatment with LPS results in an increase in COX-2 protein levels and PGE2 production in the adrenal cortex of the rat (Sanchez et al., 2013; Mohn et al., 2011). The involvement of adrenocortical COX-2 activity in the increase in serum corticosterone levels by LPS was also suggested based on the effect of specific inhibitors (Sanchez et al., 2013).

In this sense, we have also demonstrated that LPS treatment of murine adrenocortical cells results in the stimulation of steroid production by mechanisms involving an increase in COX-2 protein levels and the activation of the NFκB signaling pathway (Martinez Calejman et al., 2011).

In the present study we demonstrate that the effect of LPS entails the transcriptional activation of COX-2 gene by mechanisms involving both Akt/PKB and p38 MAPK signaling pathways upstream the activation of NFκB. Time course of kinase activation by our *in vitro* treatments with LPS in Y1 cells (as measured by an increase in the phosphorylation levels of both proteins) correlates with kinase activation in ex vivo adrenal tissue as demonstrated.

We hereby showed that LPS increased the expression of COX-2 at both protein and mRNA levels. COX-2 protein levels were significantly elevated from 6 h after LPS addition to the culture media up to 24 h (and even for 36 h, data not shown), while its mRNA levels were significantly elevated from 12 h onwards. A similar time course was observed in human monocytes (Maloney et al., 1998), macrophages (Hinz et al., 2000) and in human retinal pigment epithelial cells (Chin et al., 2001) among other cell types. We have previously suggested that LPS increases COX-2 expression levels by stimulating its transcription (Martinez Calejman et al., 2011) while several studies have also demonstrated a posttranscriptional regulation of COX-2 gene expression, involving the stabilization of its mRNA. Nonetheless, the relative contribution of transcriptional and posttranscriptional mechanisms appears to be cell type and stimulus specific. In our experimental conditions, the LPS-dependent increase in COX-2 mRNA levels does not appear to involve stabilizing effects, as LPS treatment did not increase COX-2 mRNA half-life.

A significant role of p38 MAPK in the induction of COX-2 by LPS in adrenal cells is inferred from our results: First, COX-2 induction was abrogated by the specific inhibitor of p38 but not by inhibitors of ERK, or JNK activities. Second, constitutively active MKK3, a selective activator of p38 causes COX-2 up-regulation in the absence of external stimuli. Third, transfections with dominant

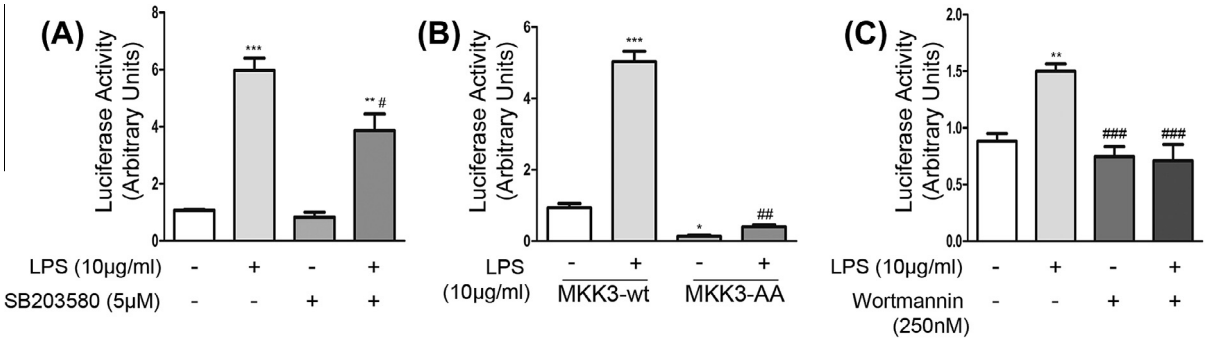

Fig. 6. Effect of p38 MAPK and Akt on NFκB activation in Y1 cells treated with LPS. Y1 cells were transfected with NFκB reporter plasmid pκB-LUC and then treated with 10 μg/ml LPS in the presence of 20 μM SB203580 (A) or 0.25 μM Wortmannin (C) for 24 h. (B) Y1 cells were co-transfected with pκB-LUC and either the wild type isoform of MKK3, MKK3-wt or the dominant negative MKK3-AA, and then treated with 10 μg/ml LPS for 24 h. Luciferase activity was determined in the cell extracts as described in Section 2. An expression plasmid for β-galactosidase was used as a transfection control and luciferase activity was normalized to β-galactosidase activity as described in Section 2. Data is expressed as means ± SEM, $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. respective control and $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. LPS alone or LPS + MKK3-wt, by ANOVA followed by Tukey's post hoc test.

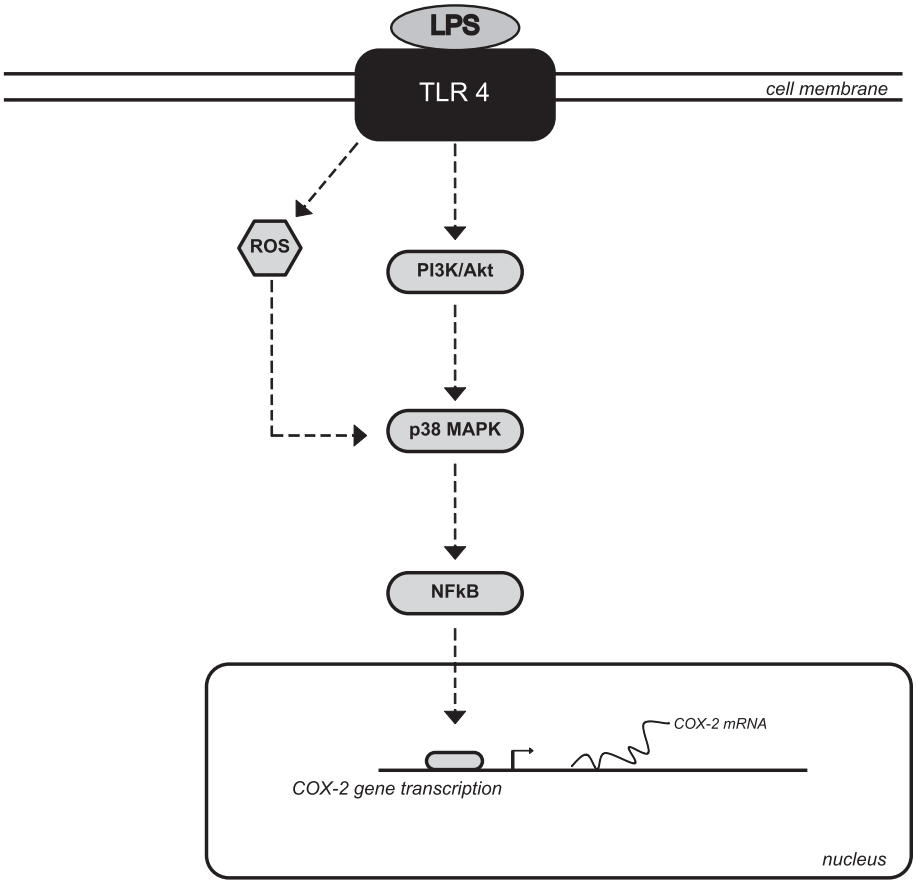

Fig. 7. Signaling pathways involved in LPS-dependent COX-2 induction. We hypothesize that binding of LPS to TLR-4 in adrenocortical cells, triggers the sequential activation of PI3K/Akt and p38 MAPK converging in the stimulation of NFκB translocation to the nucleus and increasing transcription of COX-2 gene. LPS also increases the generation of ROS in Y1 cells, which may contribute to p38 MAPK activation and its downstream effects.

negative isoforms of MKK3 or MKK6 blocked the effect of LPS on COX-2 protein levels. Several reports indicate a role of p38 in COX-2 expression in intestinal myofibroblasts, enterocyte cell lines, macrophages, and other cell types (Walton et al., 2009; Chen et al., 1999; Grishin et al., 2006). Our experiments suggest that p38 activation in adrenal cells is not only necessary but also sufficient to account for the effect of LPS on COX-2 expression.

LPS is known to enhance the formation of reactive oxygen species and lipid peroxidation products in different tissues and cell types (Giralt et al., 1993; Nowak et al., 1993) mainly by promoting mitochondrial dysfunction (Noble et al., 2007) and/or the activation of NADPH oxidase (Lee et al., 2012). In turn, increase formation of free radicals has been linked to the activation of the stress-sensitive p38 MAPK (McCubrey et al., 2006; Torres and Forman, 2003). In agreement, our results showed that LPS treatment induces ROS generation in adrenal cells leading to p38 MAPK activation and COX-2 induction. In this sense, antioxidant treatment prevented both effects, as was also demonstrated in other studies (De Stefano et al., 2007; Jin et al., 2008; Kim et al., 2008).

LPS has been also shown to activate the PI3K/Akt signaling pathway in several cell types e.g. adrenal *zona glomerulosa* (Huang et al., 2010) and murine mesangial cells (Sheu et al., 2005). Our results demonstrate that LPS also induces the activation of Akt/PKB in murine adrenocortical cells. We also demonstrated that COX-2 induction by LPS is mediated by Akt/PKB activation as both pharmacological inhibition of PI3K, as well as transfections with a dominant negative Akt isoform, prevented the induction of COX-2 by LPS. In addition, we determined that PI3K/Akt is involved in the activation of p38 MAPK, as inhibition of PI3K prevented LPS-dependent p38 MAPK phosphorylation. This interaction is not reciprocal, since p38 MAPK inhibition had no effect on the phosphorylation levels of Akt.

Finally, we analyzed the effects of p38 MAPK and PI3K/Akt on the activation of NFκB as others and we have demonstrated the activation of this transcription factor and its involvement in COX-2 induction in cells treated with LPS (Martinez Calejman et al., 2011; Walton et al., 2009). Our results showed that activation of NFκB by LPS was blunted by a specific inhibitor of p38 MAPK and in cells transfected with the dominant negative isoform of MKK3. We thus hypothesize that NFκB could be a target of p38 MAPK activity in adrenal cells, as was previously demonstrated in monocytes (O'Sullivan et al., 2009) and astrocytes (Gorina et al., 2011). In addition, Akt/PKB inhibition also effectively blocked NFκB activation. Accordingly, activation of NFκB target genes by Akt has also been demonstrated (Jijon et al., 2004).

Whether NFκB is a direct substrate for p38 MAPK or Akt/PKB phosphorylation in adrenal cells remains to be established. In this sense, phosphorylation of RelA (NFκB subunit) by several kinases (Casein Kinase II, Akt, PKA and p38 MAPK, among others, (Schmitz et al., 2001) has been shown to increase the transcriptional activity of NFκB by mechanisms still not fully determined. In any case, our results suggest that LPS treatment increases Akt/PKB and p38 MAPK activities and that both pathways promote NFκB activation. Similar TLR4 downstream effects were reported in macrophages stimulated with saturated fatty acids (Lee et al., 2003).

5. Conclusions

In summary, our results suggest that LPS stimulates COX-2 expression levels in adrenal cells by increasing the transcription

of COX-2 gene. This mechanism involves the sequential activation of Akt/PKB, p38 MAPK and NFκB (see Fig. 7). The participation of NFκB-independent effects seems unlikely, as inhibition of NFκB completely abrogates the induction of COX-2 by LPS, as we have previously shown (Martinez Calejman et al., 2011).

Given that glucocorticoid production is positively modulated by COX-2 activity, unveiling the mechanism involved in its induction becomes relevant to the development of therapeutic strategies designed to modulate the inflammatory response.

Acknowledgements

This work was supported by the following grants: PICT 2008 No. 1034 (Agencies de Promoción Científica y Técnica) and UBACyT (2011–2013) No. 20020100100301 (Universidad de Buenos Aires). We would like to dedicate this work to our former president Dr. N. Kirchner (1950–2010) for the support given to all scientists in Argentina.

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