Endocrine
DOI 10.1007/s12020-013-0104-y

ORIGINAL ARTICLE

Crosstalk between nitric oxide synthases and cyclooxygenase 2 in the adrenal cortex of rats under lipopolysaccharide treatment

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Received: 23 August 2013 / Accepted: 26 October 2013

Abstract The effect of lipopolysaccharide on the modulation of steroid production by adrenal cells has been recently acknowledged. The purpose of this study was to determine the in vivo effects of LPS on adrenal cyclooxygenase 2 (COX-2) expression, analyze its crosstalk with the nitric oxide synthase (NOS) system, and assess its involvement on the modulation of glucocorticoid production. Male Wistar rats were injected with LPS and with specific inhibitors for NOS and COX activities. PGE_2 and corticosterone levels were determined by RIA. Protein levels were analyzed by immunoprecipitation and western blotting. Transfection assays were performed in murine adrenocortical Y1 cells. Results show that LPS treatment increases PGE_2 production and COX-2 protein levels in the rat adrenal cortex. Systemic inhibition of COX-2 blunted the glucocorticoid response to ACTH, as well as the increase in NOS activity and the NOS-2 expression levels induced by LPS. Conversely, NOS inhibition prevented the LPS-dependent increase in PGE_2 production, COX-2 protein levels, and the nitrotyrosine modification of COX-2 protein. Treatment of adrenocortical cells with a NO-donor significantly potentiated the LPS-dependent increase in NFκB activity and COX-2 expression levels. In conclusion, our results show a significant crosstalk between COX-2 and NOS in the adrenal cortex upon LPS stimulation, in which each activity has a positive impact on the other. In particular, as both the activities differently affect adrenal steroid production, we hypothesize that this kind of fine modulation enables the gland to adjust steroidogenesis to prevent either an excessive or an insufficient response to the endotoxin challenge.

Keywords Glucocorticoids · Adrenal cortex · Nitric oxide synthase · Cyclooxygenase · Lipopolysaccharide · Prostaglandins

Introduction

Glucocorticoids (GCs) generated by lipopolysaccharide (LPS) within the adrenal cortex play a key role in the inflammatory response to infections, impacting a plethora of signaling pathways. Among their main targets, the production of immunologic mediators accounts for their anti-inflammatory and immunomodulatory effects. However, high and sustained GC levels could be harmful to cells and tissues. In this sense, GC response is considered a "double-edged sword" setting a fine balance between protective and detrimental effects. In this context, studies on the modulation of adrenal steroidogenesis by endogenously generated mediators become relevant.

It is well known that among other stimuli, treatment with the bacterial cell wall-derived LPS stimulates the hypothalamus pituitary adrenal (HPA) axis resulting in increased levels of plasma GC [1–3]. In addition, adrenal steroidogenesis is also regulated by mediators generated by LPS-activated immune cells (among them IL-1α and IL-1β, and TNF-α) [4, 5] and by autocrine/paracrine

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Published online: 23 November 2013

modulators [6–8]. The products of nitric oxide synthase and heme oxygenase activities have been included in this latter category [9–13].

Prostaglandins generated by the activity of cyclooxygenase-2 (COX-2) belong to another group of local modulators known to affect adrenal steroid production in different stressful situations [14]. In that sense, we have recently demonstrated that LPS treatment of murine adrenocortical cells stimulates steroid production by a mechanism involving the induction of COX-2 activity and the activation of the NFκB transduction pathway [15].

Previous studies from our laboratory have shown that in vivo treatment with LPS results in a significant increase in both the heme oxygenase (HO) and nitric oxide synthase (NOS) activities in the adrenal cortex of the rat and demonstrated the involvement of both the modulatory systems in the negative regulation of steroid production induced by LPS [12]. As a variety of reciprocal effects of COX-2 and NOS activities have been demonstrated in different cell types or experimental conditions [16, 17], we sought to analyze the in vivo effects of LPS on the activity of these modulatory systems at the adrenal level and the possible crosstalk between them, an issue that has not been addressed until now.

Materials and methods

Chemicals

ACTH was obtained from ELEA Laboratories (Buenos Aires, Argentina). Anti-NOS antibodies were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Antibodies raised against COX-2 and COX-1 were acquired from Cayman Chemical Company (Ann Arbor, MI, USA) and those against β-actin were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Peroxidase-conjugated goat anti-rabbit IgG, AG, and 50 W-X8 cation exchange resin were obtained from Bio-Rad (Hercules, CA, USA). Corticosterone antiserum was kindly provided by Dr. A. Bélanger (Laval University, QC, Canada). Rabbit anti-nitrotyrosine antibody, LPS (*Escherichia coli* endotoxin serotype O111:B4, 1,000 EU/μg), indomethacin (COX1/2 inhibitor), Nγ-nitro L-arginine methyl ester (L-NAME), and the protease and phosphatase inhibitor cocktails were purchased from Sigma–Aldrich (Buenos Aires, Argentina). Parecoxib was provided by Pfizer Ltd. (UK). Protein A-agarose was obtained from Pierce Biotechnology (Rockford, IL, USA). All the other chemicals were of the highest quality available.

Animals

Male Wistar rats weighing 150–190 g were used in the present study. The animals were kept under controlled conditions of temperature (23 ± 2 °C) and lighting (12 h light:12 h darkness cycles) with free access to water and standard chow. In order to avoid circadian effects, all the animals were euthanized in the morning by decapitation, according to the protocols approved by the Animal Care and Use Committee from School of Medicine, Universidad de Buenos Aires (CICUAL). Trunk blood was collected for corticosterone measurements, and adrenal glands were excised immediately after sacrifice and kept on ice.

"In vivo" experimental procedures

In the first set of experiments, rats were randomly assigned into control and treatment groups, injected (i.p.) with 200 μL LPS (500 μg/kg rat), and sacrificed 3, 6, 12, 15, 18, and 24 h later.

In a second set of experiments, selected groups of animals were treated with 500 μg/kg LPS and the cyclooxygenase inhibitors parecoxib (30 mg/kg) or indomethacin (12.5 mg/kg) and sacrificed 15 h later. A group of animals was further stimulated with ACTH (7.5 IU/kg, i.p.) 15 h after LPS (±inhibitors) treatment and sacrificed 60 min after ACTH injection. Corticosterone concentration was determined in serum, after dichloromethane extraction, by radioimmunoassay as described elsewhere [10].

In another set of experiments, rats were simultaneously injected with LPS and the NOS inhibitor, L-NAME, and sacrificed 15 h later. We chose this time point taking into account the increase in NOS activity we have previously observed in the adrenal cortex of LPS-treated rats [12].

Adrenocortical tissue preparation

Adrenal glands were rapidly dissected and placed on a chilled plate. The surrounding fat was removed and the glands were halved. The capsule and the medulla were carefully dissected. Adrenocortical tissue was homogenized in 50 mM Na_2HPO_4 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 homogenate was centrifuged at 2,000×*g* for 10 min at 4 °C, and the supernatants were collected.

Immunoblot analysis

Homogenate samples were analyzed by SDS-PAGE on 7.5 or 10 % polyacrylamide gels, and after electrophoresis, proteins were transferred to polyvinylidine difluoride

(PVDF) membranes for 1 h at 15 V in a Trans-Blot SD system (Bio-Rad Laboratories Inc.) in 25 mM Tris–HCl pH 8.3, 39 mM glycine, and 20 % methanol. PVDF membranes were processed as described elsewhere [12] and assayed with specific primary antibodies (NOS-1 1:500; NOS-2 1:200; NOS-3 1:300; COX-1 1:1000; COX-2 1:200; nitrotyrosine 1:200; actin 1:500). Goat anti-rabbit IgG antibody–horseradish peroxidase conjugate (1:5,000) was used as a secondary antibody. Chemiluminescence signals were visualized and registered with an ImageQuant Imaging System (GE Healthcare) and processed with the AlphaEase Fluorchem software (V. 4.1.0, Alpha Innotech Corporation).

Immunoprecipitation (IP)

Adrenocortical tissues were homogenized in IP buffer (25 mM Tris–HCl pH 7.2, 150 mM NaCl, 1× protease inhibitor cocktail, and 1× phosphatase inhibitor cocktail) in a final volume of 0.5 ml/gland. Adrenal homogenates (500 µg) were incubated under agitation overnight at 4 °C, in the presence of 4 µg of rabbit anti-nitrotyrosine antibody. 100 µL of protein A-agarose slurry was added to each sample, and mixtures were incubated at room temperature for 2 h, under agitation. After centrifugation (2,500 g × 2.5 min), the beads were washed three times with 500 µL of IP buffer. Supernatants were removed; 20 µL 5× loading buffer was added to the beads, and the samples were boiled at 95–100 °C for 5 min. After SDS-PAGE and transference, membranes were probed with rabbit anti-COX-2 or anti-nitrotyrosine antibodies and analyzed following the above-mentioned procedures.

NOS activity

NOS activity was determined by monitoring the conversion from L-[^{3}H]arginine to L-[^{3}H]citrulline as previously described [12]. In brief, protein fractions were brought to 100 µl in a reaction mixture that contained 10 mM HEPES (pH 7.4), 0.75 mM β-NADPH, 1.25 mM CaCl2, 20 µM L-arginine, 187 nM L-[2,3-^{3}H]arginine, and 0.5 mM dithiothreitol. The mixture was incubated for 15 min, and the reaction was terminated by the addition of 300 µl ice-cold stop buffer (10 mM EGTA, 10 mM EDTA, and 50 mM HEPES pH 5). L-[^{3}H]Citrulline was separated from the reaction mixture by cation exchange chromatography and quantified by liquid scintillation spectroscopy. NOS enzyme activity is indicated as picomoles of L-[^{3}H]citrulline formed per mg protein/min.

PGE_2 production

Control, L-NAME, LPS, and LPS + L-NAME-treated rats were sacrificed at the corresponding periods after the initiation of treatments, and their adrenal glands were excised, decapsulated, and dissected in quarters. Adrenal tissues were incubated in 140 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose for 30 min at 37 °C. Supernatants were collected and stored at −20 °C until measured. PGE_2 levels were determined directly in duplicate samples by RIA with anti-PGE_2 from Sigma-Aldrich as reported elsewhere [17]. The sensitivity of the assay was 10 pg per tube.

Determination of lipid peroxides (TBARS)

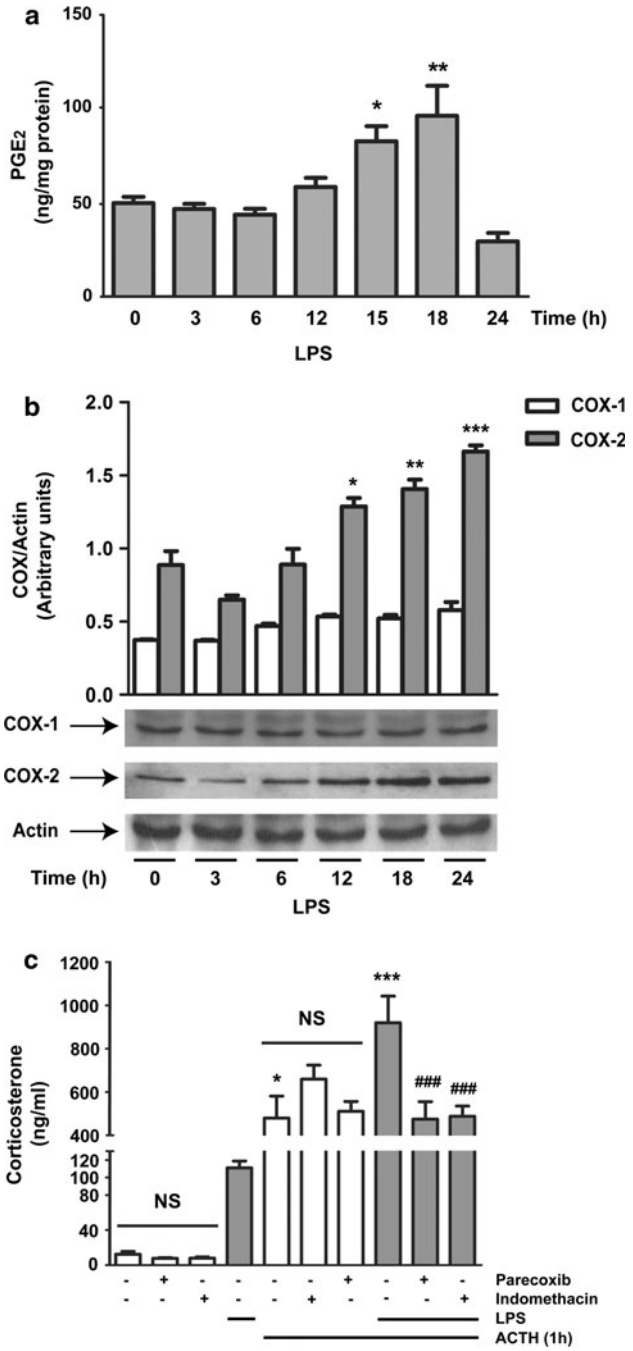
Determination of malondialdehyde levels as thiobarbituric acid reactive, a parameter of oxidative stress generation, was performed as described previously [18]. In brief, 50 µl of SDS 10 % was mixed with 250 µl of the homogenate in 12 mM potassium phosphate buffer, 48 mM KCl. 930 µl of 8 mg/ml thiobarbituric acid was added, and the samples were heated in a boiling water bath for 60 min. TBARS were measured by the fluorescence emission at 555 nm after excitation at 515 nm. A calibration curve was performed using malondialdehyde as a standard prepared from 1,1,3,3-tetramethoxypropane, and each curve point was subjected to the same treatment as the supernatants. TBARS were calculated as the concentration of malondialdehyde in mM formed per milligram of protein [19].

Cell culture and treatments

Y1 is a mouse adrenocortical tumor cell line isolated by Yasumura et al. [20]. Cells were grown as monolayers in plastic culture dishes in Ham's F-10 medium (Sigma-Aldrich, Argentina) containing heat-inactivated 10 % fetal calf serum (Natocor, Cordoba, Argentina), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen Argentina, Buenos Aires, Argentina) in a humidified incubator at 37 °C and 5 % CO_2. Treatments were initiated by replacing the growth medium with fresh Ham's F-10 without serum for 24 h and then treating with 10 µg/ml LPS or 1 mM DETA-NO (t½:22 h) or both for 24 h. Cells were then harvested with lysis buffer containing 1 % Triton X-100, 1 % phosphatase inhibitor cocktail (Sigma-Aldrich, Argentina), and 1× protease inhibitor cocktail (Sigma-Aldrich, Argentina) in 1× PBS buffer.

Transfections and luciferase 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 NF-κB-reporter plasmid and 0.02 µg of pCMV-βgal (β-galactosidase expression plasmid) in serum-free



◄ **Fig. 1** Effect of LPS on the activity and expression levels of COX-1 and COX-2 in the rat adrenal cortex and its involvement in the modulation of ACTH-dependent corticosterone production in rats. Male Wistar rats were injected with saline or with LPS (500 μg/kg rat, i.p.) and sacrificed 3, 6, 12, 15, 18, and 24 h later. Adrenal glands were rapidly excised and processed as described under Materials and Methods, and cyclooxygenase activity and expression levels were determined. (**a**) Adrenal quarters obtained from control and LPS-treated rats were incubated for 30 min at 37 °C, and PGE_2 levels were assessed in the incubation media by radioimmunoassay; (**b**) representative western blot analysis for COX-1, COX-2, and actin proteins in the adrenal cortex of LPS-treated rats. A densitometric analysis of COX-1 and COX-2 expression levels normalized to actin is shown below. Results in (**a**) and (**b**) are expressed as mean ± SEM, $n = 6$, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus the corresponding control; (**c**) rats were treated with saline or with LPS (500 μg/kg) and with or without parecoxib (30 mg/kg) or indomethacin (12.5 mg/kg) and sacrificed 15 h later. Selected groups of rats were injected with ACTH (7.5 IU/kg, i.p.) 60 min before sacrifice. Trunk blood was collected, and corticosterone concentration was determined in the serum by radioimmunoassay. Results are expressed as mean ± SEM ($n = 6$); $^{***}p < 0.001$ versus LPS; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus LPS + ACTH, by Tukey's test. *NS*: not significant

Opti-MEM medium (Invitrogen Argentina, Buenos Aires, Argentina). After 3 h, the medium was replaced with fresh Ham's F10 containing 10 % of fetal calf serum, and incubations were continued for another 12 h. The medium was replaced with fresh Ham's F10 media without serum, and cells were incubated with 10 μg/ml LPS or 1 mM DETA-NO or both for 24 h. Luciferase activity was determined with the Steady-Glo Luciferase Assay System (Promega Corporation, USA), and results were normalized to β-galactosidase activity. The NFκB-reporter plasmid was a kind gift from Dr Adali Pecci (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires).

Statistical analysis

All the values are expressed as mean ± SEM of n replicates. After testing data distribution for normality (Kolmogorov–Smirnoff test), differences between groups were analyzed by ANOVA, followed by Tukey's post hoc test or using Student's t test for nonpaired data, as considered necessary. A p value <0.05 was considered significant.

Results

Effect of LPS on the activity and expression levels of COX-2 and its participation in the modulation of GC production

We first examined the effects of in vivo treatment with LPS (500 μg/kg i.p.) on the activity and expression levels of COX-1 and COX-2 in the adrenal cortex of rats. Animals were injected with LPS and sacrificed after the indicated periods of time. Adrenal glands were excised, and PGE_2 production from adrenal quarters was determined in the incubation media. Results showed significantly higher levels of PGE_2 in the incubation media of adrenal quarters obtained from rats sacrificed 15 and 18 h after the immune challenge (Fig. 1a). As shown in Fig. 1b, COX-2 protein levels were elevated 12 h after LPS treatment and remained elevated through the end of the experiment. No

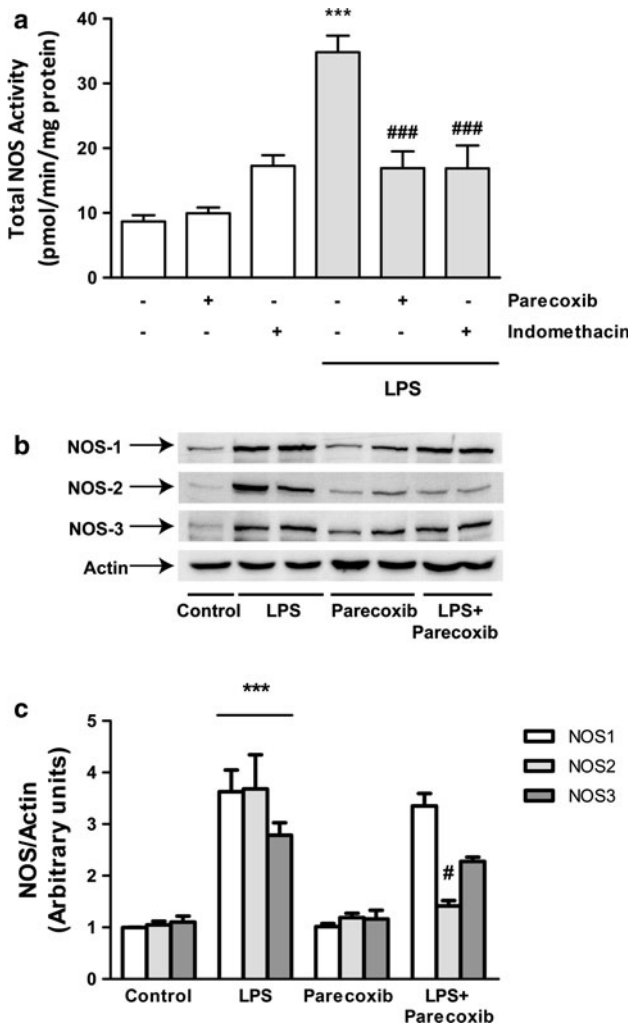


Fig. 2 Effect of COX inhibitors on the LPS-induced increase in nitric oxide synthase (NOS) activity and expression levels in the adrenal cortex of the rat. Male Wistar rats were injected (i.p.) with saline, indomethacin (12.5 mg/kg), or parecoxib (30 mg/kg) alone or with LPS (500 μg/kg) and sacrificed 15 h later. (**a**) NOS activity was determined by radiochemical conversion of L-[^{3}H] arginine to L-[^{3}H]citrulline as described in Materials and Methods. Each *bar* represents mean ± SEM; ($n = 6$); $^{***}p < 0.001$ versus control; $^{###}p < 0.001$ versus LPS alone by ANOVA followed by Tukey's test. (**b**) Representative western blot showing protein levels of NOS isoforms determined in the adrenal cortex of the following groups of animals: Control, LPS, parecoxib, and LPS + parecoxib, treated as described in a. (**c**) The histogram shows the densitometric analysis of the relative abundance of NOS protein, normalized to the corresponding actin levels. Each bar represents mean ± SEM, $n = 6$; $^{***}p < 0.001$ versus control; $^{\#}p < 0.05$ versus LPS alone by Tukey's test

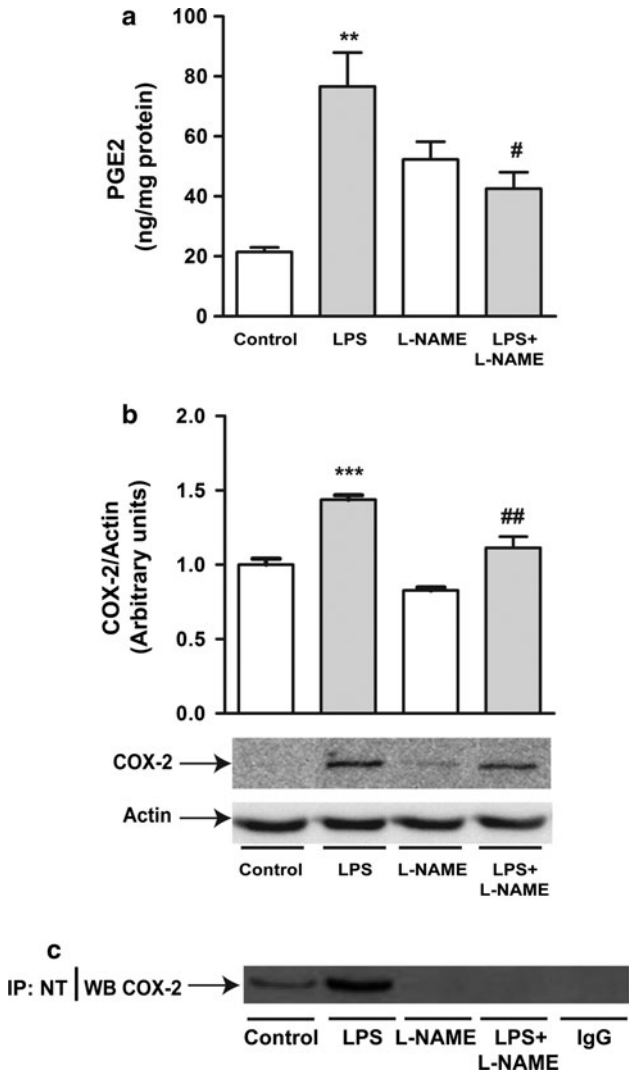


Fig. 3 Effect of a NOS inhibitor on the activity and expression levels of COX-2 in LPS-treated rats. Animals were injected with saline or with LPS (500 μg/kg) with or without L-NAME (50 mg/kg) and sacrificed 15 h after. (**a**) PGE_2 production was determined in the incubation media of adrenal quarters processed as described in the legend of Fig. 1. Results are shown as mean ± SEM ($n = 5$); $^{**}p < 0.01$ versus control, $^{\#}p < 0.05$ versus LPS alone by Tukey's test; (**b**) representative western blot showing the protein levels of COX-2 and actin. The histogram shows the densitometric analysis of the relative abundance of COX-2 protein normalized to the corresponding actin levels. Each *bar* represents mean ± SEM, $n = 6$; $^{***}p < 0.001$ versus control, $^{\#\#}p < 0,01$ versus LPS alone by Tukey's test; (**c**) adrenocortical homogenates were immunoprecipitated with an anti-nitrotyrosine antibody, and samples were analyzed by western blot with anti-COX-2 antibody, as described in Materials and Methods. A mixture of adrenal homogenates from control and LPS-treated animals incubated with nonimmune rabbit serum (IgG) is included as a control

changes were detected in COX-1 expression levels (Fig. 1b).

In order to assess the participation of COX-2 activity in the modulation of the GC production in rats under LPS treatment, we analyzed the adrenal response to an acute stimulation with ACTH. In these experiments, rats were simultaneously injected with LPS or saline and with or without a selective (parecoxib, 30 mg/kg i.p) or nonselective (indomethacin, 12 mg/kg i.p.) cyclooxygenase

inhibitor. After 15 h, ACTH (7.5 IU/ml i.p.) was injected to a selected group of animals. Corticosterone levels were determined in serum samples obtained 1 h later. Results showed that parecoxib or indomethacin treatment significantly decreased ACTH-dependent corticosterone production in LPS-treated animals (Fig. 1c). None of the inhibitors had any effect on basal corticosterone production.

Effect of COX inhibitors on the increase in NOS activity and expression levels by LPS

We have previously demonstrated that LPS treatment increases NOS activity and the expression levels of all NOS isoforms in the adrenal cortex of rats [12]. Present experiments were designed to analyze the effect of COX inhibition on NOS signaling in control and LPS-treated rats. As depicted in Fig. 2a, simultaneous treatment with LPS and either parecoxib or indomethacin prevented the increase in NOS activity determined 15 h after the injection of LPS. In addition, parecoxib also prevented the LPS-dependent increase in NOS2 protein levels, while no significant changes were detected on constitutive NOS isoforms (Fig. 2b). As for the mechanism involved, parecoxib treatment had no effect on the activity of NFκB as it did not affect p65 translocation to the nucleus triggered by LPS (data not shown).

Effect of a NOS inhibitor on the activity and the expression levels of COX-2 in LPS-treated rats

In order to analyze the effect of NOS inhibition on the LPS-dependent induction of COX-2, animals were randomly assigned to four groups and treated as follows: Control (saline i.p.), LPS (500 μg/kg i.p.), L-NAME (50 mg/kg i.p.), or L-NAME + LPS. Both the COX-2 protein levels and PGE_2 production were determined in adrenocortical tissues obtained 15 h later. Results presented in Fig. 3a show that L-NAME significantly inhibited the LPS-dependent increase in PGE_2 generation. As L-NAME treatment resulted in a significant but slighter decrease in COX-2 protein levels in LPS-treated rats ($\sim$22 %) (Fig. 3b), we hypothesized that COX-2 activity could be modulated by iNOS-derived NO. In this sense, higher levels of NO_2-Tyr-COX-2 were detected in the LPS-treated group as compared to controls, while no signal (nitrated COX-2 proteins) was observed in the LPS + L-NAME or L-NAME groups (Fig. 3c). In addition, oxidative stress generation by LPS treatment is also suggested, as an increase in lipid peroxide levels was determined in adrenocortical tissues (Control 265.4 ± 17.5 vs. LPS 333.7 ± 22.5 mM MDA/mg prot, $n = 6$, $p < 0.05$ by Student's t test).

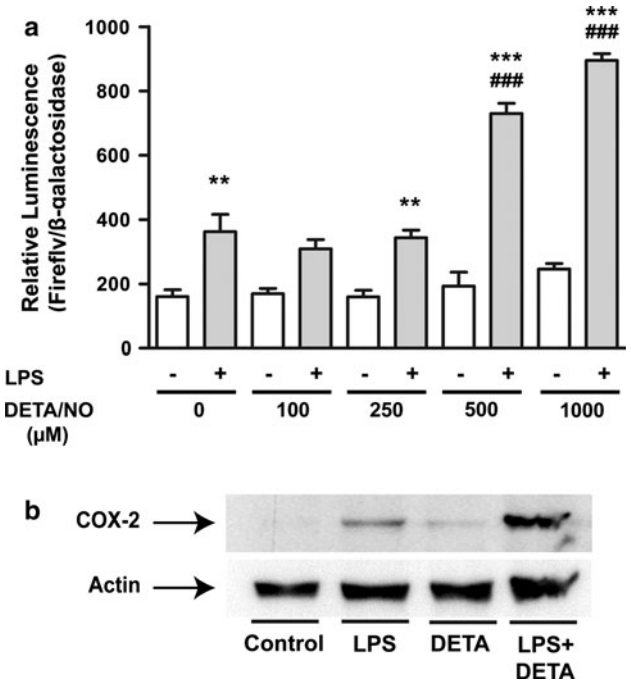


Fig. 4 Effect of a NO-donor on the LPS-dependent induction of COX-2 in murine adrenocortical Y1 cells. (**a**) Murine adrenocortical Y1 cells were transfected with a reporter plasmid for NFκB (pκB-LUC) and with pCMV-β-gal as described in Materials and Methods. Cells were treated with 10 μg/ml LPS and/or increasing doses of DETA-NO for 24 h, and relative luciferase activity was calculated as the ratio of luciferase to β-galactosidase activity. Data are presented as mean ± SEM. All transfection assays were performed in triplicate and repeated at least three times; $^{**}p < 0.01$ and $^{***}p < 0.001$ versus respective control without LPS, and $^{\#\#\#}p < 0.001$ versus LPS alone by Tukey's test. (**b**) Y1 cells were incubated with 10 μg/ml LPS and/or 1 mM DETA-NO for 24 h and COX-2, and actin protein levels were determined by Western blot analysis

Effect of the co-treatment with LPS and a NO-donor on the expression levels of COX-2 and the activity of an NFκB-reporter plasmid in a mouse adrenocortical cell line

Murine adrenocortical Y1 cells transfected with an NFκB-reporter plasmid were incubated in the absence or presence of LPS and increasing doses of a NO-donor (DETA-NO), and luciferase activity was determined after cell lysis. Results showed that although DETA-NO had no effect on luciferase activity, a significant increase in NFκB activity was determined in LPS + DETA-NO-treated cells (Fig. 4a).

In another group of experiments, Y1 cells were incubated with LPS and/or DETA-NO, and protein levels of COX-2 were determined. Results showed that DETA-NO had no effect on COX-2 protein levels, while increasing the stimulatory effect of LPS on this parameter (Fig. 4b).

Discussion

Results presented in this article demonstrate the induction of COX-2 activity in the adrenal cortex of rats under in vivo treatment with LPS and its involvement in increase in the GC response to ACTH observed in these animals. In addition, we demonstrated a positive interaction between COX-2 and NOS activities within the adrenal cortex sustaining the effects of LPS.

We have recently shown that murine adrenocortical cells express TLR-4, CD-14, and MD-2, components of the LPS receptor binding and transducing complex, and reported a significant increase in COX-2 protein levels and steroidogenesis in a murine adrenal cell line incubated with LPS, by a mechanism involving the stimulation of the transcription factor NFκB [15].

In this study, we detected an increase in COX-2 protein levels and the generation of PGE_2 in the adrenal cortex of rats after in vivo treatment with LPS. A time course indicated that both parameters were elevated several hours after LPS injection. Other studies have reported a rapid and transient increase in COX-2 protein in the adrenal cortex of rats after intravenous LPS administration [8, 21]. In this sense, we did not study the effects of LPS at earlier time points (before 3 h), but a biphasic effect of LPS on COX-2 expression levels has been detected in murine macrophages and other cell lines [22]. In addition, differences in the administration route, LPS strain, dose, etc., could also account for this apparent discrepancy.

We focused our study on this "delayed" effect of LPS on the expression of COX-2, as we have previously established the activity of two autocrine/paracrine modulatory systems of adrenal steroidogenesis, e.g., NOS and HO, that are also affected within this time frame. In particular, we demonstrated a significant increase in NOS activity in the rat adrenal cortex between 6 and 18 h after LPS treatment [12].

Serum corticosterone levels in rats are significantly increased after LPS injection, and this parameter remains significantly elevated for many hours (8-fold increase over basal after 15 h in present experiments, [12]). As we and others have previously demonstrated the involvement of COX-2 activity in LPS-stimulated steroid production in adrenocortical cells [14, 15], we speculate that among other effects (vascular, inflammatory, etc.), COX-2 activity is also involved in sustaining the GC response to LPS. Experiments performed with cyclooxygenase inhibitors support this assertion. On the other side, we have previously shown that adrenal steroidogenesis is negatively regulated by NO derived from NOS activity [12]. In this sense, it could be suggested that the combinatorial response to both the stimulatory and inhibitory modulators enables the gland to adjust steroidogenesis to prevent an excessive or an insufficient GC output.

In agreement with the previous reports, the present results demonstrate that systemic LPS treatment also increases NOS activity in the adrenal cortex of the rat [12, 23]. Experiments performed with cyclooxygenase inhibitors led us to suggest that COX-2 is involved in the LPS-dependent stimulation of NOS activity and in the increased expression of iNOS. The underlying mechanism remains elusive, although stimulation of NFκB does not seem to be affected by COX-2 activity, as parecoxib did not inhibit LPS-induced p65 translocation to the nucleus (data not shown) or the activity of an NFκB-reporter plasmid in adrenal cells under LPS treatment, as we have previously shown [15]. The effects of prostanoids on NOS activity and expression levels may depend on the tissue or cell type under investigation, as both the stimulatory and inhibitory effects have been reported in different cellular systems [24–27]. To our knowledge, this is the first study addressing the participation of COX-2 activity in the LPS-dependent induction of NOS-2 in the adrenal cortex.

In order to analyze the other side of the interaction, we used L-NAME, a NOS inhibitor, administered simultaneously with LPS. Our results suggest that endogenously generated NO is involved in the stimulation of COX-2 expression and activity (PGE_2 generation) in the adrenal cortex of rats treated with LPS, as both parameters were significantly lower in the LPS + L-NAME group (as compared to LPS alone). In support of the proposed effect of NO, we showed that the simultaneous addition of a NO-generator resulted in a further increase in the LPS-dependent stimulation of the transcriptional activity of NFκB. Accordingly, NO triggered a significant increase in COX-2 protein levels in LPS-treated adrenal cells. Different effects of NO on COX-2 activity and expression levels have been reported, suggesting that the dose of LPS used, the tissue under study, or the time elapsed after LPS treatment should also be taken into consideration [28–31].

Our results indicate that NO generated within the adrenal gland is also involved in a post-translational modification of COX-2 protein, as higher NO_2-Tyr-COX-2 levels were detected in this tissue. In this sense, the effects of NO or peroxynitrite on COX-2 activity have been previously reported [32, 33]. The peroxynitrite anion is a short-lived oxidant species that is produced by the reaction of nitric oxide (•NO) and superoxide ($O2^{\bullet -}$) radicals at diffusion-controlled rates. In our experiments, LPS treatment triggered an increase in oxidative stress in the adrenal cortex of rats (higher lipoperoxide levels), and we have previously detected the stimulation of ROS generation in adrenal cells incubated with LPS (Astort, personal communication). Accordingly, several studies have demonstrated that LPS induces the generation of superoxide in

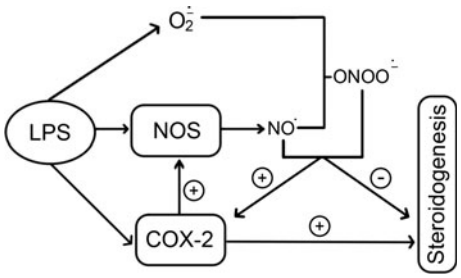


Fig. 5 Regulatory circuit triggered by LPS in the adrenal cortex. Systemic treatment with LPS results in an increase in NO and PGE_2 synthesis by NOS and COX-2, respectively, in the adrenal cortex of the rat. A reciprocal interaction between these two systems is established, where each activity exerts a stimulatory effect on the other. An increase in oxidative stress is also detected in the adrenal cortex, probably contributing to the generation of the highly reactive peroxynitrite anion that could also modulate COX-2 activity. In summary, both modulatory systems are involved in the fine tuning of adrenal steroid production under LPS treatment

mitochondria [34, 35] and/or by the activation of membrane-associated NAPDH oxidase [36, 37]. Based on the above results, we hypothesized that peroxynitrite could be the nitrating agent of COX-2 in the adrenal cortex of LPS-treated rats. In agreement with our results, Tsatsanis et al. [38] showed that peroxynitrite anions can enhance COX-2 catalytic activity. In summary, NO generated within the adrenal cortex in LPS-treated rats appears to impact on COX-2 at two levels: first, by promoting an increase in COX-2 protein levels and, second, by modulating COX-2 activity by post-transductional modifications.

In conclusion, our results demonstrate that induction of COX-2 by systemic treatment with LPS contributes to the amplification of the GC response to ACTH. In addition, a stimulatory crosstalk between the NOS and COX systems sets up a regulatory circuit where each activity appears to be upregulated by the other (see diagram in Fig. 5). In particular, LPS-dependent increase in NO generation stimulates COX-2 protein levels and PGE_2 generation, sustaining the effects of LPS at adrenal level.

Acknowledgments This work was supported by grants from Universidad de Buenos Aires (UBACYT M014-M021), CONICET (PIP5525), and ANPCyT (PICT 2005 N1 38283). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work. The authors thank Mr. Mark Stetina for critical reading of this manuscript.

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