Induction of nitric oxide synthase and heme oxygenase activities by endotoxin in the rat adrenal cortex: involvement of both signaling systems in the modulation of ACTH-dependent steroid production

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Abstract

The present study was designed to investigate the effect of lipopolysaccharide (LPS) on the expression levels and activities of the nitric oxide synthase (NOS) and heme oxygenase (HO) systems in the rat adrenal gland. Both enzymatic activities were significantly increased in this tissue after *in vivo* treatment with LPS. The concurrent induction of the *HO-1*, *NOS-1*, and *NOS-2* gene products was also detected as both mRNAs and protein levels were augmented by this treatment in a time-dependent way. A significant interaction between both signaling systems was also demonstrated as *in vivo* blockage of NOS activity with N(G)-nitro-L-arginine methyl ester (L-NAME) resulted in a significant reduction in HO expression and activity levels,

while an increase in NOS activity was observed when HO was inhibited by Sn-protoporphyrin IX (Sn-PPIX). As both NOS and HO activities have been previously involved in the modulation of adrenal steroidogenesis, we investigated the participation of these signaling systems in the adrenal response to LPS. Our results showed that acute stimulation of steroid production by ACTH was significantly increased when either NOS or HO activities were inhibited. We conclude that adrenal NOS and HO can be induced by a non-lethal dose of endotoxin supporting a modulatory role for these activities in the adrenal response to immune challenges.

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Introduction

Nitric oxide (NO) and carbon monoxide (CO) are involved in a wide array of biological processes (Moncada *et al.* 1991, Ryter *et al.* 2002). The biochemical pathways leading to NO formation from L-arginine have been associated with three NO synthase (NOS) isoforms differing in subcellular localization, regulation, and biological function (Palmer *et al.* 1988). Two constitutively expressed types first characterized in neurons and vascular endothelium are calcium- and calmodulin-dependent NOS (NOS-1 and NOS-3, respectively), whereas the inducible type (NOS-2) binds calmodulin tightly at normal intracellular Ca^{2+} concentrations. Its activity is thus generally considered to be Ca^{2+} independent and its expression is induced by a multiplicity of effectors like endotoxin, interferon (IFN)- γ , tumor necrosis factor- α , hypoxia, stress, and tissue damage (Michel & Feron 1997).

In mammals, microsomal heme oxygenase (HO) is the rate-controlling enzyme for heme degradation. Its activity results in the production of CO, free iron, and biliverdin. In

the presence of NADPH-cytochrome P450 reductase and NADPH, biliverdin is subsequently converted to bilirubin by biliverdin reductase (Tenhunen *et al.* 1969). To date three HO isoforms have been identified: the inducible enzyme HO-1, a second isoform, HO-2 that is constitutively active (Maines *et al.* 1986), and a third isozyme with low enzymatic activity (HO-3), which is predicted to play a role in either heme binding or sensing (McCoubrey *et al.* 1997). Immunolocalization techniques have shown basal expression of HO-2 in the brain, spleen, liver, testis, and vasculature (Maines 1997). HO-1, also known as the stress protein heat shock protein 32 (HSP32), is present in normal conditions in the spleen; however its augmented expression can be observed in various tissues following stressful stimuli, such as ultraviolet radiation, endotoxin, NO donors, heavy metals and hyperoxia/hypoxia, thus being associated with defense mechanisms against stressful situations (Immenschuh & Ramadori 2000).

The HO and NOS families bear strong similarities. Both present constitutive and inducible isoforms. They share

common stimulators, such as cytokines, endotoxin, and reactive oxygen species. In fact, regulatory mechanisms simultaneously controlling the activity of both HO-1 and NOS-2 have been described (Choi & Alam 1996, Maines 1997). However, important differences, such as chemical reactivity, stability and binding properties, suggest that both mediator systems are not redundant. In this sense, it has been proposed that these systems act coordinately and that any one might modulate the other's activity and/or expression levels (Takahashi *et al.* 1996, Foresti & Motterlini 1999). It has been well established that NO donors can stimulate HO-1 gene expression and activity in a variety of cell types (Yee *et al.* 1996, Foresti *et al.* 1997), although the mechanisms underlying the modulation of HO-1 by NO remain unclear. On the other side, it has been suggested that both inducible and constitutive HOs modulate the expression of NOS by depleting intracellular heme groups (Chakder *et al.* 1996, Turcanu *et al.* 1998). These findings suggest that in certain physiological or pathological situations there is a significant interaction between the HO and NOS systems. In these conditions, one enzymatic activity may counter regulate, compensate or prevail over the other.

Previous studies from our laboratory demonstrated the expression of both constitutive NOS isoforms and the induction of NOS-2 by lipopolysaccharide (LPS) in adrenal cells (Cymeryng *et al.* 2000, 2002). We have also presented evidence supporting a modulatory role for NO on adrenal steroidogenesis: NO donors (Cymeryng *et al.* 1998) and L-arginine (Cymeryng *et al.* 1999) significantly inhibit steroid synthesis by a mechanism that probably involves a direct interaction of NO with CYP11A1 (cytochrome P450_{scc}). Based on these results we postulated NO to be an autocrine/paracrine modulator of adrenal physiology (Cymeryng *et al.* 2002). As regards HO activity in the adrenal gland, recent studies from our laboratory indicate that, while HO-2 is constitutively expressed in adrenal cells, HO-1 expression is induced by adrenocorticotrophin (ACTH). An inhibitory effect of HO activity on steroidogenesis could be inferred from our results (Pomeraniec *et al.* 2004).

Present *in vivo* experiments were designed to evaluate the interaction(s) between these gaseous signaling systems in the rat adrenal cortex. We first measured the impact of immune stimulation with LPS on NOS and HO expression and activity. Then, in a second series of experiments, we examined the effects of NOS or HO inhibitors (L-NAME or Sn-PPIX) on these enzymatic parameters, as well as on basal and ACTH-stimulated corticosterone release in animals pretreated with LPS or saline.

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). AG 50W-X8 cation exchange resin and peroxidase-conjugated anti-IgG antibodies were purchased from Bio-Rad Laboratories Inc. Enhanced chemiluminescence (ECL) reagent came from Amersham Pharmacia Biotech. HO-1 and HO-2 antibodies were from StressGen Biotechnologies Corp. (Victoria, Canada).

LPS (*Escherichia coli* serotype O111:B4, lot 78H4086) and L-NAME were purchased from Sigma Chemical Co. Sn(IV)-protoporphyrin IX dichloride was from Porphyrin Products Inc. (Logan, Utah, USA). All other reagents were commercial products of the highest grade available.

Animals

Adult male Wistar rats (*Rattus norvegicus*) weighting 200–250 g were used in the present study. The animals were kept in cages with controlled temperature (23 ± 2 °C) and lighting (12 h light:12 h darkness cycles) with free access to water and Purina chow. In order to minimize the effects of circadian fluctuation rats were killed in the morning. Animals were killed by decapitation according to protocols approved by the animal care and use committee from the University of Buenos Aires. Trunk blood was collected for corticosterone measurements, and adrenal tissues were excised immediately thereafter.

Experimental procedures

In the first set of experiments the rats were randomly assigned into control and treated groups, injected (i.p.) with either 200 µl pyrogen-free saline alone or saline containing LPS (500 µg/kg rat) and killed after 3, 6, 12, and 18 h.

In the second set of experiments the animals were randomly assigned to six groups that were injected (i.p.) with pyrogen-free saline alone or with LPS (500 µg/kg) or L-NAME (50 mg/kg) or SnPPIX (20 mg/kg) or LPS + L-NAME or LPS + SnPPIX. The rats were killed after 18 h. In addition, in a third set of experiments selected groups of animals were treated with ACTH (7.5 IU/kg, i.p.) 60 min before killing.

Corticosterone was extracted from serum with dichloromethane and its levels were assessed by radioimmunoassay. Corticosterone antisera were kindly provided by Dr A Bélanger, Laval University, Quebec, Canada.

Zona fasciculata-reticularis (ZF-R) 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 together with the adhering zona glomerulosa cells was carefully dissected away. Finally, the medulla was excised under a dissecting microscope.

ZF-R adrenal tissue was homogenized in 50 mM KPO₄ pH 7.4, 0.2 mM EDTA, 0.25 M sucrose, 10 µM pepstatin, 10 µM leupeptin, and 0.8 µM aprotinin in a final volume of

0.2 ml per gland. The homogenate was first centrifuged at 2000 *g* for 10 min, then at 9000 *g* for 20 min and finally at 105 000 *g* for 60 min to obtain cytosolic and microsomal fractions. In some experiments a portion of ZF-R adrenal gland tissue was homogenized in TRIzol reagent in order to obtain RNA.

RNA isolation and RT-PCR

Total RNA was obtained from ZF-R adrenal tissue with Trizol reagent according to the manufacturer's instructions. RNA (2 µg) was pretreated with RNase-free DNase, heated at 70 °C for 10 min, placed on ice for 1 min and then incubated with a mixture containing 0.5 mM dNTPs mix, 25 ng/µl (8 µM) random primers, 1× first strand buffer, 25 units rRNase inhibitor, 200 units Moloney murine leukemia virus (MMLV) reverse transcriptase, and water to a final volume of 25 µl for 1 h at 42 °C. The reaction was stopped by heating at 90 °C for 5 min. The reaction mixture was brought to 100 µl with diethylpyrocarbonate-treated water and stored at -70 °C. In selected tubes the reverse transcriptase was omitted as a control of amplification from contaminating genomic DNA.

PCRs were carried out in a Tpersonal Thermocycler (Biometra Biomedizinische Analytik, Göttingen, Germany) and were performed using 2 µl cDNA for the amplification of specific gene products. The PCR was performed in a final volume of 20 µl of the following reaction mixture: 1×PCR buffer, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 500 nM of each specific oligonucleotide primer, and 0.625U Taq polymerase (Life Technologies). The sequence for the oligonucleotide primers were as follows: for HO-1: forward: 5'-ACTTTCA-GAAGGGTCAGGTGTCC-3' reverse: 5'-TTGAGCAG-GAAGGCGGTCTTAG-3' (522 bp fragment); for HO-2: forward primer 5'- CCACCACTGCACTTTACTTC-3'; reverse primers 5'-GGTCTTCATACTCAGGTCCA-3' (436 bp fragment), for NOS1: forward: 5'-TTTCTG-TCCGTCTCTTCAAACGCAAAGTGG-3', reverse: 5'-GCGGGAGACTGTTCTGTTCTCTGAATACGGG-3', for NOS2: forward: 5'- CACGGAGAACAGCAGAGTTGG-3', reverse: 5'-GGAACACAGTAATGGCCGACC-3'. For NOS3: forward: 5'-CTGTGTCCAACATGCTGCTA-GAAATTG-3', reverse: 5'-TAAAGGTCTTCTTCCTGGT-GATGCC-3'. GAPDH; forward primer: 5'-TCCCT-CAAGATTGTCAGCAA-3', reverse primer: 5'-AGATCCA-CAACGGATACATT-3' (309 bp fragment). The identity of the amplified products was confirmed by direct sequencing.

Immunoblot analysis

Samples were boiled for 5 min in SDS-PAGE loading buffer and electrophoresed on 7.5% or 12% polyacrylamide gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes for 1 h at 15 V in a Trans-Blot SD system (Bio-Rad Laboratories Inc.) in 48 mM Tris-HCl pH 9.2, 39 mM glycine and 1.3 mM SDS. PVDF

membranes were blocked in 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.25% Tween 20 and 5% non-fat milk for 60 min at room temperature and then incubated overnight with a 1:500 dilution of the respective NOS (Santa Cruz Biotechnology Inc.), HO (StressGen Biotechnologies Corp.) or γ -tubuline (Sigma-Aldrich) antiserum at 4 °C. Membranes were washed with 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, and 0.25% Tween 20 and then incubated for 2 h with a 1:5000 dilution of a goat anti-rabbit IgG antibody-horseradish peroxidase conjugate. The filters were washed, and the bands were visualized by chemiluminescence (ECL Western Blotting Analysis System, Amersham Pharmacia Biotech) and autoradiography.

NOS activity

NOS activity was determined in cytosolic fractions from rat adrenal ZF-R by monitoring the conversion of L-[³H]arginine into L-[³H]citrulline as previously described (Cymeryng *et al.* 2002). Fifty micro liter protein fractions were incubated with 50 µl reaction buffer (20 mM HEPES, 20 µM L-arginine, 1 mM dithiothreitol, 1 mM NADPH, 187 nM L-[2,3-³H]arginine, and 2.4 mM CaCl₂) at 37 °C for 15 min. The reaction was terminated by the addition of 800 µl ice cold suspension of the cation exchange resin AG 50W-X8 in stop buffer (10 mM EGTA, 10 mM EDTA, and 50 mM HEPES, pH 5). L-[³H]citrulline was quantified in the supernatants by liquid scintillation spectroscopy. NOS activity is shown as picomoles of L-[³H]citrulline formed/mg protein per minute.

HO activity

HO activity was determined in the microsomal fractions by monitoring the conversion of heme into bilirubin as previously described (Pomeranec *et al.* 2004). In brief, 50 µl microsomal proteins were incubated with 100 µl reaction mixture containing 0.33 mM hemin, 0.5 mg/ml rat liver cytosol, 0.2 mM MgCl₂, 2 mM glucose 6 phosphate, 1.62 units/ml glucose 6 phosphate dehydrogenase, 0.5 mM NADPH, and 25 mM potassium phosphate buffer (pH 7.4) at 37 °C for 2 h. The reaction mixture was then extracted with 0.6 ml chloroform and the bilirubin concentration of the chloroform layer was spectrophotometrically measured by the difference in absorbance between 464 and 530 nm (ξ = 40 mM/cm). HO enzyme activity is indicated as nmoles of bilirubin formed/mg protein per hour.

Statistical analysis

All values are expressed as mean $\pm$ S.E.M. of *n* experiments. Differences between groups were analyzed by factorial one-way ANOVA. When the ANOVA was significant (*P* < 0.05) *post hoc* comparisons (Dunnet's or Tuckey's test) were made to determine the statistical levels of difference between groups

using GraphPad InStat version 3.06 for Windows (GraphPad Software, San Diego, CA, USA).

Results

We first examined the effect of LPS on HO activity and expression levels in the rat adrenal cortex at different time points. As depicted in Fig. 1A, HO-1 mRNA levels were elevated 3 and 6 h post-treatment throughout the duration of the experiment. No changes were detected in HO-2 mRNA levels after LPS challenge. Accordingly, western blot analysis also showed an LPS-dependent increase in HO-1 levels 6 h after injection that was still evident at 18 h. In contrast, the expression of HO-2 did not change at the different time points examined (Fig. 1B). HO activity was significantly increased 12 and 18 h after the immune challenge (Fig. 1C).

The temporal pattern of NOS activity and expression was also analyzed in this model. As shown in Fig. 2A, no NOS-2 mRNA was detected by RT-PCR analysis in the adrenal

cortex of untreated animals, while a strong signal was observed at 3 and 6 h post-LPS injection, disappearing thereafter. NOS-1 and NOS-3 transcripts were also increased 6 h post-injection. As expected, LPS treatment caused a marked rise in NOS-2 and NOS-1 protein levels, while NOS-3 protein levels were not affected. NOS activity was significantly elevated 6 h after LPS injection and remained elevated up to 18 h (Fig. 2C).

In order to investigate the interaction between both gaseous signaling systems in the adrenal cortex of the rat, the effects of *in vivo* treatment with the inhibitors for NOS and HO (L-NAME and Sn-PPIX) were studied both in control and LPS-treated animals. Determinations were performed 18 h after injection of LPS, a time point at which the activity of both enzymes was significantly elevated. As illustrated in Fig. 3, *in vivo* inhibition of NOS activity with L-NAME inhibited the increase in HO-1 mRNA levels produced by LPS (Fig. 3A), while it had no effect on the levels of the HO-2 transcript. L-NAME treatment partially blocked

Figure 1 Effect of LPS on HO activity and expression in rat adrenal ZF-R. Animals were injected with saline alone or with LPS (i.p., 500 µg/kg) and killed 3, 6, 12 and 18 h after. Adrenal glands were rapidly excised and homogenized as described under Materials and Methods and HO expression levels and activity were determined. (A) RT-PCR products of HO-1 and HO-2. Semiquantitative analysis was performed on equal amounts of total RNA (2 µg) with specific primers pairs. The histogram shows the densitometric analysis of the relative abundance of HO-1 (solid bar) and HO-2 (open bar) mRNAs normalized with the corresponding GAPDH. Each bar represents means $\pm$ S.E.M. ($n=3$); *** $P<0.001$; ** $P<0.01$, and * $P<0.05$ versus control by Tuckey's test. (B) Western blot analysis for HO-1, HO-2 and γ -tubuline proteins obtained from the same experiment as in (A). (C) HO activity was determined spectrophotometrically as described before. Each point represents means $\pm$ S.E.M. of three experiments performed independently, ** $P<0.01$ versus control by Dunnett's test.

Figure 2 Effect of LPS on NOS activity and expression in rat adrenal ZF-R. Animals were treated as described in the legend of Fig. 1. NOS activity and expression levels were determined at different time points after LPS treatment. (A) Semiquantitative RT-PCR analysis of NOS-1, NOS-2 and NOS-3 expression levels. PCRs were performed as described in Materials and Methods with the inclusion of tracer quantities of [α - 32 P]dCTP in the reaction mixture and PCR products were then separated by SDS-PAGE. The densitometric analysis shows the relative abundance of gene transcripts compared with the housekeeping gene *GAPDH*. Each bar, NOS-1 (solid bar), NOS-2 (open bar) and NOS-3 (grey bar) represents means $\pm$ S.E.M., $n=3$. *** $P<0.001$ and ** $P<0.01$ versus respect control by Tukey's test. (B) Western blot analysis of NOS-1, NOS-2, NOS-3 and γ -tubuline proteins obtained in the same experiment as in A). (C) NOS activity was determined by the radiochemical conversion of L-[3 H]arginine to L-[3 H]citrulline. Each point represents the means $\pm$ S.E.M. of four experiments performed independently, ** $P<0.01$, *** $P<0.001$ versus control by Dunnett's test.

the stimulation of HO activity induced by LPS (Fig. 3B, left panel). Figure 3B (right panel) also shows the marked inhibition of NOS activity attained after L-NAME treatment in both control and LPS-injected animals.

Similar experiments were performed in order to assess the effect of HO inhibition on the NO generator system, animals being injected with saline, LPS, Sn-PPIX or LPS + Sn-PPIX. NOS-1 mRNA levels were significantly increased in LPS + Sn-PPIX-treated rats, while NOS-3 mRNA levels were increased by Sn-PPIX and LPS + Sn-PPIX (Fig. 4A). NOS-2 mRNA levels were undetectable 18 h after LPS + Sn-PPIX treatment (data not shown). NOS activity was significantly increased by Sn-PPIX treatment, both in control and LPS-stimulated animals (Fig. 4B, left panel). HO activity in these experimental conditions is shown as a control (Fig. 4B, right panel).

Blood corticosterone levels were significantly elevated after LPS treatment, remaining above control levels throughout the sampling period (Fig. 5A). ACTH-stimulated corticosterone secretion was evaluated 18 h after treatment initiation in saline or in LPS-, LPS + L-NAME-, or LPS + Sn-PPIX-injected animals. Systemic inhibition of either NOS or HO activities induced a significant increase in ACTH-related corticosterone release (Fig. 5B).

Discussion

The results presented here demonstrate that *in vivo* treatment with LPS increases both NOS and HO activities in the rat adrenal cortex. In addition, a significant interaction between

Figure 3 Effect of L-NAME on HO activity and expression levels induced by LPS, in rat adrenal cortex. Rats injected with saline alone or with LPS (500 µg/kg) or L-NAME (50 mg/kg) or LPS+L-NAME were killed 18 h after injection. (A) Semiquantitative RT-PCR products of HO isoforms. Relative abundance of HO-1 and HO-2 mRNAs is compared with GAPDH. Each bar, HO-1 (solid bar) and HO-2 (open bar), represents means $\pm$ S.E.M., $n=3$, * $P<0.01$ versus its respective control, by Tukey's test. (B) Enzymatic activities of HO (left panel) and NOS (right panel) are shown. Data represents means $\pm$ S.E.M., $n=4$, *** $P<0.001$ versus control; ### $P<0.001$ and # $P<0.01$ versus LPS alone by Tukey's test.

both signaling systems and its influence on ACTH-dependent steroid production is described.

An involvement of HO activity in the LPS-dependent modulation of AVP, CRH, and ACTH release has been suggested (Kostoglou-Athanassiou *et al.* 1998, Mancuso *et al.* 1999). Since LPS treatment did not modify the expression levels of any of the HO isozymes at hypothalamic or pituitary level (Jacobs *et al.* 1997, Satta *et al.* 1998) these effects could be due to changes in enzymatic activity. In previous experiments from our laboratory, HO activity (mainly attributed to the expression of HO-2) has been detected in basal conditions and ACTH-dependent HO-1 induction was also demonstrated in adrenal cells (Pomeranec *et al.* 2004). To our knowledge, the results presented in this paper are the first report on the LPS-dependent induction of HO-1 along the hypothalamus-hypophyseal-adrenal (HHA) axis.

The stimulatory effect of inflammatory stress caused by LPS on HO activity has been widely documented, both *in vitro* and *in vivo*. Comparable results were obtained in mice liver (Rizzardini *et al.* 1993), rat Kupffer cells (Immenschuh *et al.*

1999), macrophages (Srisook & Cha 2004), and rat glial cells (Kitamura *et al.* 1998). As was also demonstrated in other tissues or cell types, the induction of this enzymatic activity is probably involved in the development of cytoprotective mechanisms triggered by both injury and infection (Ryter & Tyrrell 2000). In this sense, we have previously demonstrated that the ACTH-dependent increase in HO activity observed in adrenal cells correlated with diminished levels of TBARS and protein-carbonyls, both parameters of oxidative stress (Pomeranec *et al.* 2004). This effect is probably associated with a decrease in the pro-oxidant levels of heme or alternatively, to the endogenous antioxidant activity of bilirubin, as demonstrated in Y1 cells (Pomeranec *et al.* 2004).

LPS treatment has been described to increase levels of both NOS-1 and NOS-2 mRNAs (Harada *et al.* 1999) in the hypothalamus, and of NOS-2 mRNA at the pituitary level (Satta *et al.* 1998). However, the relevance of NO signaling to LPS-induced ACTH secretion is still controversial (Harada *et al.* 1999).

Figure 4 Effect of Sn-PPIX on NOS activity and expression levels induced by LPS in rat adrenal cortex. Rats were injected with saline alone or in combination with LPS (500 µg/kg), or Sn-PPIX (20 mg/kg) or with LPS + Sn-PPIX and killed 18 h after. (A) Semiquantitative RT-PCR of NOS-1 and NOS-3 products. The densitometric analysis shows the relative abundance of gene transcripts compared with the housekeeping gene GAPDH. Data expressed as means $\pm$ S.E.M., $n=3$, corresponds to NOS-1 (solid bar) and NOS-3 (open bar) transcripts. * $P<0.05$ versus control, *** $P<0.001$ and § $P<0.05$ versus Sn-PPIX alone, by Tukey's test. (B) Enzymatic activities of NOS (left panel) and HO (right panel) are shown. Each point represents means $\pm$ S.E.M. of four experiments performed independently, ** $P<0.01$ and *** $P<0.001$ versus control; ### $P<0.001$ versus Sn-PPIX alone by Tukey's test.

In the rat adrenal cortex both constitutive NOS isozymes were detected in basal conditions (Cymeryng *et al.* 2002). Present results show that LPS treatment significantly increased NOS activity and markedly induced the expression of NOS-1 and NOS-2 isozymes in this tissue.

Production of NO by NOS-2 is an evolutionarily conserved mechanism of host defense. Nitrosative and oxidative stress cause the death of exogenous agents but could also result in deleterious effects to host cells. In order to prevent these effects a plethora of antioxidant mechanisms came into place. Among them, induction of HO-1 by several stressful stimuli (u.v. radiation, endotoxin, NO donors, heavy metals, hyperoxia and hypoxia) has been demonstrated (Ryter & Tyrrell 2000).

As in the rat adrenal cortex, both NOS and HO enzymatic activities were increased by LPS treatment, we explored possible interactions among these signaling pathways. Many

reports have previously indicated that NO donors increase HO activity and induce the expression of HO-1 in different cell types (Otterbein & Choi 2000). In this sense, (Immenschuh *et al.* 1999) have shown that HO-1 induction by LPS is mediated by NO. The attenuation of HO activity by NOS inhibitors has also been demonstrated both *in vitro* and *in vivo* models (Datta & Lianos 1999, Datta *et al.* 2002). In our experiments, the increase in HO activity was preceded by the activation of the nitridergic system, and the blockade of this system by L-NAME significantly inhibited the LPS-dependent increase in HO activity. L-NAME treatment also blunted the LPS-associated increase in HO-1 mRNA levels. According to these results it could be speculated that an increase in NO generation is involved in LPS-dependent stimulation of HO activity.

Analysis of the effect of HO activity on the nitridergic system showed that blockage with Sn-PPIX further

Figure 5 Corticosterone production in LPS-treated animals. (A) Time course of serum corticosterone levels after i.p. injection of LPS (500 µg/kg). Data are means $\pm$ S.E.M. for three independent experiments. ** $P < 0.01$ versus control by Dunnett's test. (B) Effect of NOS or HO inhibition on serum corticosterone levels acutely induced by ACTH on LPS treated rats. Rats were i.p. injected with saline or LPS (500 µg/kg) alone or in combination with L-NAME (50 mg/kg) or with Sn-PPIX (20 mg/kg) 18 h before ACTH stimulation (7.5 IU/kg, i.p.). Corticosterone levels were determined one hour later. Data are expressed as means $\pm$ S.E.M., $n = 3$, *** $P < 0.001$ versus control, # $P < 0.05$ and ### $P < 0.001$ versus LPS + ACTH by Tukey's test.

augmented LPS-induced NOS activity. These results could be explained by the following hypothesis: (1) as heme is involved in the catalytic activity of NOS, this enzyme is susceptible to inhibition by CO (White & Marletta 1992); (2) HO activity could also regulate NO generation as NOS could provide heme as a substrate for HO; and (3) HO products could inhibit gene transcription, as was demonstrated for bilirubin (Wang *et al.* 2004).

Previous results from our laboratory demonstrate that both NOS and HO activities are involved in the modulation of adrenal steroidogenesis (Cymeryng *et al.* 1998, 1999, Pomeranec *et al.* 2004). As shown previously in rats, systemic administration of LPS elicits a prompt release of ACTH and corticosterone: peak values were attained around 1 or 2 h after injection, and basal levels were regained 3–4 h post-injection

(Uribe *et al.* 1999, Ma *et al.* 2000). In our experimental setting LPS also induced a rapid increase in serum corticosterone levels but a significant effect of LPS on NOS and HO activities was evident only 6 h after treatment. It is thus suggested that these activities might be involved in later stages of the adrenal response to LPS. In fact, 18 h after LPS injection both HO and NOS activities appear to negatively modulate the adrenal response to exogenous ACTH as corticosterone levels were significantly increased when either of these activities was blocked. This effect might be attributed to the interaction of NO or CO with the heme moiety of cytochrome p450 enzymes, as was previously described (Morgan *et al.* 2001).

The endotoxic activity of LPS appears to be mediated by the interaction with specific cell surface receptor(s). In this sense, the expression of Toll-like receptors 4 and 2 in human adrenal cells (Bornstein *et al.* 2004) and a direct effect of LPS on corticosterone production in rat fasciculata-reticularis cells have been demonstrated (Enriquez de Salamanca & Garcia 2005). According to these results the observed effects of LPS on the adrenal cortex could be attributed to LPS alone, or to a combination of LPS with enhanced cytokine production and/or ACTH release resulting from LPS injection as was also suggested (Beishuizen & Thijs 2003). The exact nature of the adrenal stimulant remains to be established.

Steroid-producing cells within the adrenal cortex constitutively express NOS-1, NOS-3, and HO-2 enzymes, and respond to an immune challenge by increasing NOS-2 expression and activity (Cymeryng *et al.* 2000, 2002, Pomeranec *et al.* 2004). As discussed in previous reports from our laboratory (Cymeryng *et al.* 2002), the obtained ZF-R fragments necessarily contain different proportions of endothelial, neuronal, and immune cells displaying NOS and/or HO activity. The contribution of these inevitable 'contaminants' to the observed changes in enzyme messenger/protein/activity cannot be ascertained in the present experiments and is beyond the scope of this study. However, the *in vivo* approach employed in this study allows a comprehensive evaluation of the steroidogenic response to LPS and its possible regulation by gaseous messenger systems.

The adrenal gland is involved in the complex set of reactions evoked by tissue trauma, chemical irritants, or infection. Corticosteroids produced in the zona fasciculata act as a major feedback control limiting inflammatory responses and preventing the immune system from over-reacting and causing tissue damage or autoimmunity, and in this sense they are essential for survival in critical illness. Furthermore, glucocorticoids are also involved in numerous aspects of metabolism, growth and physiological functioning (Beishuizen & Thijs 2003). All the above considered, the existence of modulatory mechanisms controlling glucocorticoid production, such as the NOS and HO systems, acquires the utmost importance, as they enable the gland to provide a finely tuned response that is essential in illness and other stressful situations.

In conclusion, the results presented above demonstrate the induction of both NOS and HO systems in the adrenal gland and the participation of both signaling systems in the modulation of ACTH-dependent steroid production in the context of an immune response. A significant interaction between both enzymatic systems is also shown. It is suggested that NO derived from LPS-induced NOS entails an increase in HO activity and that this activity, in turn, is involved in the consequent inhibition of NOS.

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