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***In vivo* Angiotensin II AT₁ receptor blockade selectively inhibits LPS-induced innate immune response and ACTH release in rat pituitary gland**

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Abstract

Systemic lipopolysaccharide (LPS) administration induces an innate immune response and stimulates the hypothalamic-pituitary-adrenal axis. We studied Angiotensin II AT₁ receptor participation in the LPS effects with focus on the pituitary gland. LPS (50 µg/kg, i.p.) enhanced, 3 hours after administration, gene expression of pituitary CD14 and that of Angiotensin II AT_{1A} receptors in pituitary and hypothalamic paraventricular nucleus (PVN); stimulated ACTH and corticosterone release; decreased pituitary CRF₁ receptor mRNA and increased all plasma and pituitary pro-inflammatory factors studied.

The AT₁ receptor blocker (ARB) candesartan (1 mg/kg/day, s.c. daily for 3 days before LPS) blocked pituitary and PVN AT₁ receptors, inhibited LPS-induced ACTH but not corticosterone secretion and decreased LPS-induced release of TNF-α, IL-1β and IL-6 to the circulation. The ARB reduced LPS-induced pituitary gene expression of IL-6, LIF, iNOS, COX-2 and IκB-α; and prevented LPS-induced increase of nNOS/eNOS activity. The ARB did not affect LPS-induced TNF-α and IL-1β gene expression, IL-6 or IL-1β protein content or LPS-induced decrease of CRF₁ receptors. When administered alone, the ARB increased basal plasma corticosterone levels and basal PGE₂ mRNA in pituitary.

Our results demonstrate that the pituitary gland is a target for systemically administered LPS. AT₁ receptor activity is necessary for the complete pituitary response to LPS and is limited to specific pro-inflammatory pathways. There is a complementary and complex influence of the PVN and circulating cytokines on the initial pituitary response to LPS. Our findings support the proposal that ARBs may be considered for the treatment of inflammatory conditions.

Keywords

Renin-Angiotensin System; inflammation; IL-6; LIF; NF-κB; COX-2; iNOS; NOS activity; HPA axis; paraventricular nucleus

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INTRODUCTION

The response to infection is a combination of immune, hormonal and behavioral adaptive responses; these include increased production of pro-inflammatory cytokines, with effects in the periphery and the brain, HPA axis stimulation and sickness behavior (Dantzer and Kelley, 2007; Dantzer et al., 2008). The balance between both systems helps to fight the infection and restore homeostasis (Beishuizen and Thijs, 2004; Han and Ulevitch, 2005; Hopkins, 2007). However, when they become unrestricted or maladaptive, homeostasis is not achieved and pathological conditions arise (Lawrence and Gilroy, 2007).

A useful experimental model and an excellent example of the immune-endocrine relationship is the systemic administration of the bacterial endotoxin lipopolysaccharide (LPS). LPS induces an innate immune response with a major upregulation of pro-inflammatory factors including enhanced pro-inflammatory cytokine production (Han and Ulevitch, 2005; Rivest, 2003). This is accompanied by a classical stress response characterized by activation of the hypothalamic-pituitary-adrenal (HPA) axis (Beishuizen and Thijs, 2004; Hopkins, 2007). During HPA axis activation, there is enhanced synthesis and release of hypothalamic corticotrophin-releasing factor (CRF) followed by an increase in pituitary ACTH formation and release, leading to augmented adrenal pro-inflammatory mineralocorticoid and anti-inflammatory glucocorticoid secretion (Elenkov and Chrousos, 2006; Fernandes et al., 2008; Grinevich et al., 2001; Moncek et al., 2003).

The pituitary gland is a critical component for the endocrine-immune response to infection, and is a target organ for LPS (Denef, 2008; Lohrer et al., 2000; Quan et al., 1998; Ray and Melmed, 1997; Tierney et al., 2005; Wang et al., 1996; Whiteside et al., 1999; Wong et al., 1996). The pituitary ACTH release in response to infection and stress is regulated at three levels: a) by integrated inputs from the central nervous system, including a major role of hypothalamic CRF; b) by an intra-pituitary network of cytokines, prostaglandins and nitric oxide; and c) by influences from peripheral target tissues such as adrenal glucocorticoid feedback (Denef, 2008; Ray and Melmed, 1997).

The complex paracrine network of pituitary cells is of special interest for the present study. Endotoxin injection increases the synthesis of local pituitary pro-inflammatory cytokines including interleukin-6 (IL-6), interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), leukemia inhibitory factor (LIF) and macrophage migration inhibitory factor (MIF) (Lohrer et al., 2000; Tierney et al., 2005; Wang et al., 1996; Whiteside et al., 1999). LPS also up-regulates the synthesis of pituitary pro-inflammatory enzymes such as cyclooxygenase-2 (COX-2) and inducible nitric oxidase synthase (iNOS) (Quan et al., 1998; Wong et al., 1996). These pro-inflammatory components strongly affect the final pituitary output. For example, while IL-6, LIF and prostaglandins increase the ACTH release (Auernhammer and Melmed, 2000; Denef, 2008; Gadek-Michalska and Bugajski, 2004; Ray and Melmed, 1997); TNF- α and NO may reduce it (Gadek-Michalska and Bugajski, 2004; Gaillard et al., 1990; Spangelo et al., 1991; Uribe et al., 1999). The properties mentioned above make the pituitary gland an ideal site for integration of the immune and hormonal responses to inflammation.

Pharmacological treatments to restore homeostasis following intense stress and inflammatory challenges are incomplete and have limitations (Gilgun-Sherki et al., 2006). For these reasons the search for novel therapies to properly regulate the response to inflammation and stress is of great therapeutic importance. One such promising mechanism is the therapeutic modulation of the Renin-Angiotensin System (RAS). Angiotensin II (Ang II) is the active principle of RAS. It is now accepted that Ang II is a multitasking factor throughout the organism beyond its well known role in blood pressure control (Saavedra et al., 2006b). Of interest here are its pro-inflammatory effects and its contribution to stress-induced activation of the HPA axis

(Castren and Saavedra, 1988, 1989; Marchesi et al., 2008; Saavedra and Benicky, 2007; Saavedra et al., 2006b).

Ang II elicits both its pro-inflammatory and pro-stress actions through stimulation of AT₁ receptors (Hunyady and Catt, 2006; Marchesi et al., 2008; Saavedra and Benicky, 2007). In rodents, the AT₁ receptor type consists of AT_{1A} and AT_{1B} isoforms, differentially regulated but pharmacologically indistinguishable (Inagami et al., 1994; Johren et al., 1995; Kakar et al., 1992; Kitami et al., 1992; Lenkei et al., 1999; Leong et al., 2002; Sanvitto et al., 1997; Shanmugam et al., 1994). The AT_{1A} receptor subtype has been closely linked with both inflammation and stress (Leong et al., 2002; Tsutsumi and Saavedra, 1991; Yang et al., 2005).

Ang II AT₁ receptor blockers (ARBs), drugs commonly used in the treatment of high blood pressure, reduce peripheral and cerebrovascular inflammation in hypertension (Ando et al., 2004; Savoia and Schiffrin, 2006; Zhou et al., 2005); prevent stimulation of the HPA axis during isolation stress (Armando et al., 2001); and reduce the central sympathetic stimulation of isolation and cold-restraint stress (Armando et al., 2001; Bregonzio et al., 2003; Saavedra et al., 2006a). The anti-inflammatory and anti-stress properties of ARBs suggested that these compounds could be effective in other inflammatory conditions. We recently found that the ARB candesartan, independently of its effects on blood pressure, reduced the LPS-induced production of inflammatory cytokines and their release to the general circulation; and it significantly reduced the inflammatory reaction to LPS in the adrenal gland (Sanchez-Lemus et al., 2008) and spleen (Sanchez-Lemus et al., 2009).

The presence of an active local RAS system suggests that the pituitary may be a major target site for the modulation of endocrine and immune responses by Ang II. Previous studies have concluded that anterior pituitary AT₁ receptors are an important factor in the regulation of the HPA axis during stress (Leong et al., 2002; Saavedra, 1992). However, the role of pituitary AT₁ receptors in the response to inflammation has not been studied.

In the present study, we focused on the effects of LPS administration and ARB pre-treatment on the pituitary gland. We hypothesized that AT₁ receptors participate in the pituitary inflammation and stress response induced by the endotoxin. To test our hypothesis we used the *in vivo* model of peripheral LPS administration to normotensive rats pre-treated with the ARB candesartan.

METHODS

Experimental animals

Male Wistar Hannover rats weighing 250 to 300 g were purchased from Taconic Farms (Germantown, NY, USA). They were kept in groups of three per cage under standard laboratory conditions with water and rat chow *ad libitum*, room temperature at 22°C, and 12 hours of light each day. The NIMH Animal Care and Use Committee approved all procedures. All efforts were made to minimize the number of animals used and their suffering (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 80-23, revised 1996).

Treatment

We performed five different experiments. For each experiment the animals were divided into four groups, 7 to 9 animals each: vehicle-saline, candesartan-saline, vehicle-LPS and candesartan-LPS. For 3 consecutive days, rats received s.c. injections of vehicle or candesartan (Astra-Zeneca, Molndal, Sweden) 1 mg/kg/day at 9:00 AM. This treatment produces a substantial increase in plasma renin activity and 50–70% decrease of AT₁ receptor binding in different tissues (Sanchez-Lemus et al., 2009; Sanchez-Lemus et al., 2008), indicating effective

blockade of AT₁ receptors. Candesartan was dissolved in 0.1 N Na₂CO₃ and further diluted in isotonic saline, at a final pH of 7.5–8.0. On day 3 starting at 10:00 AM, rats received an i.p. injection of LPS 50 µg/kg (*Escherichia coli* serotype 055:B5; Sigma Chemical Co., St Louis, MO, USA) or sterile saline. Three hours after LPS/saline injection, animals were removed from their cages and taken to a different room to be killed by fast decapitation. Trunk blood was collected in polyethylene tubes with EDTA (1 mg/ml blood). The blood was immediately centrifuged at 1800 × g, 15 min at 4°C and the plasma obtained was stored at –80°C until used. Pituitaries and brains were dissected, frozen in cold isopentane and stored at –80°C until used.

We selected a time point of three hours after LPS administration as the most convenient time for parallel determinations of circulating cytokines, gene expression and enzyme activities, based on our preliminary experiments and evidence from the literature (Sanchez-Lemus et al., 2009; Sanchez-Lemus et al., 2008; Singh and Jiang, 2004; Uribe et al., 1999).

Whole pituitaries from the first three experiments were used for real-time PCR, assay of NOS activity, and for determination of Ang II AT₁ receptor binding, respectively. Brains from the third experiment were used to determine AT₁ receptor mRNA, AT₁ receptor binding and CRF mRNA in the hypothalamic paraventricular nucleus (PVN). Pituitaries from the fourth and fifth experiments were separated into anterior and posterior lobes under a dissecting microscope control. Cytokine protein concentration, ACTH content and PGE₂ levels were measured in each lobe.

Hormones

Commercial radioimmunoassay kits were used to measure plasma corticosterone (MP Biomedicals, Orangeburg, NY, USA), and plasma ACTH (DiaSorin, Stillwater, MN, USA) following the manufactures' protocols. Anterior pituitaries were homogenized in 500 µl of acetic acid 0.1 M and then centrifuged at 10,000 × g, 15 min at 4°C. Resultant clear supernatants were diluted 1:1,000 in the radioimmunoassay buffer and used to quantify ACTH (Bachem, San Carlos, CA, USA).

PGE₂

PGE₂ in pituitary was measured by enzyme immunoassay using a commercial kit (R&D Systems, Minneapolis, MN, USA). Anterior and posterior pituitaries were separated, homogenized in 500 µl of acetic acid 0.1 M and then centrifuged at 10,000 × g, 15 min at 4°C. Clear supernatants were further diluted 1:8 in the reaction buffer for a final pH ~7.0.

Cytokine assays

Commercial ELISA kits were used to measure plasma TNF-α, IL-1β and IL-6; and pituitary IL-1β and IL-6 (R&D Systems). The manufacturer directions were followed for plasma samples. Anterior and posterior pituitaries were separated and homogenized in 500 µl of Tris-HCl pH 7.4 containing a protease inhibitors cocktail (Complete®, Roche Applied Science, Indianapolis, IN). Clear supernatants from centrifugation at 10,000 × g, 15 min at 4°C were used for analysis.

Measurement of mRNA expression by real-time PCR

Total RNA was isolated individually from homogenized pituitary samples using 500 µl TRIzol (Invitrogen, Carlsbad, CA, USA), followed by purification using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). Synthesis of complementary DNA (cDNA) was performed using 2 µg of total RNA and Super-Script III first-Strand Synthesis SuperMix (Invitrogen). Real-time PCR was performed in a 20 µl reaction mixture consisting of 10 µl SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 2 µl cDNA and 0.3 µM of each primer for

a specific target on a DNA Engine Opticon™ (Bio-Rad, Hercules, CA, USA). Specific primers used are listed in Table 1. Amplification was performed at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 56 – 60°C for 60 sec. At the end of amplification, the specificity of the PCR was confirmed by determination of the melting temperature of the PCR product. Serial dilutions of rat cDNA were used to obtain a calibration curve. The individual targets for each sample were quantified by determining the cycle threshold and by using calibration curves. The relative amount of the target RNA was normalized with the housekeeping gene for 18S ribosomal RNA (rRNA) and expressed as arbitrary units. The expression of 18S rRNA was not affected by any of the treatments used (results not shown).

NOS activity

Nitric oxide synthase (NOS) activity was determined using a commercial kit (Cayman Chemical, Ann Arbor, MI, USA) based on the conversion of L-[³H]-arginine to L-[³H]-citrulline. 50 µg of protein were incubated 45 min at 37°C in the incubation buffer enriched with NADPH 1 mM (Sigma Chemical Co.), L-[2,3,4,5 ³H]-arginine 0.2 µCi per sample (~60 nM; GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and L-Arginine 10 µM (Sigma Chemical Co.). Under these conditions the enzymatic conversion was linear. The difference of L-[³H]-citrulline generated in the presence and absence of Ca²⁺ was defined as eNOS/nNOS activity. The difference of L-[³H]-citrulline generated in Ca²⁺-free conditions and in the presence of the NOS inhibitor L-NNA 1 mM was defined as iNOS activity. NOS activity was expressed as pmol/min/mg protein.

Receptor autoradiography

Sarcosine¹-Angiotensin II (Sar¹-Ang II; Peninsula Laboratories, Belmont, CA, USA) was iodinated by the Peptide Radioiodination Service Center (School of Pharmacy, University of Mississippi, MS, USA) with a specific activity of ~2200 Ci/mmol. Consecutive 16 µm thick pituitary and brain sections containing the hypothalamic PVN were cut using a cryostat at –20°C. Sections were thaw-mounted on Superfrost/plus slides (Daigger, Vernon Hills, IL, USA), dried overnight at 4°C and then stored at –80°C until used. Sections were preincubated for 15 min at 22°C in a 10 mM sodium phosphate buffer, pH 7.4, containing 0.005% bacitracin, 5 mM Na₂EDTA, 120 mM NaCl and 0.2% proteinase-free bovine serum albumin. Sections were then incubated for 2 h at 22°C in fresh buffer containing 0.5 nM [¹²⁵I]Sar¹-Ang II. To characterize Ang II receptor subtypes, adjacent tissue sections were incubated as above in the presence of either 10 µM losartan (DuPont Merck, Wilmington, DE, USA), a selective AT₁ receptor antagonist, or 10 µM PD123319 (Parke-Davis, Ann Arbor, MI, USA), a selective AT₂ receptor ligand, or 5 µM unlabeled Ang II (Peninsula Laboratories) to assess nonspecific binding. The number of AT₁ receptors was determined as the specific binding displaced by unlabeled losartan. The number of AT₂ receptors was determined as the specific binding displaced by unlabeled PD123319. After incubation, slides were washed 4 times for 1 min in Tris-HCl, pH 7.4 at 4°C, followed by a 30-second rinse in distilled water at 4°C. Slides were then dried under a stream of cold air and exposed to Biomax MR film (Kodak, Rochester, NY, USA). Optical densities of autoradiograms generated by incubation with the ¹²⁵I ligand were normalized after comparison with [¹⁴C]microscale standards as described and quantified by computerized densitometry using the Scion Image 4.0.2 Program (Scion Corporation, Frederick, MD, USA) based on the NIH Image Program of the National Institutes of Health. Binding signal was expressed as fmol/mg of protein.

Measurement of mRNA expression by in situ hybridization

Consecutive 16 µm thick sections of hypothalamic PVN were cut using a cryostat at –20°C. Sections were thaw-mounted on Superfrost/plus slides, dried for 5 min at 50°C and then stored at –80°C.

AT_{1A} receptor—*In situ* hybridization was performed using [³⁵S]-labeled antisense and sense riboprobes for AT_{1A} receptors as described (Johren et al., 1995). Prior to use, the sections on slides were warmed in a desiccator at room temperature and then fixed in 4% formaldehyde in phosphate buffer for 10 min. After two washes in phosphate buffer, they were acetylated for 10 min in 0.1 M triethanolamine HCl, 0.9% NaCl, containing 0.25% acetic anhydride, delipidated in ethanol and chloroform, and air-dried. Each slide with sections was covered with 150 µl hybridization buffer containing 50% formamide, 0.3 M NaCl, 1 mM EDTA, 20 mM Tris, pH 7.5, 1X Denhardt's solution, 10% dextran sulfate, 100 µg/ml salmon testes DNA, 250 µg/ml yeast RNA, 250 µg/ml yeast tRNA, 150 mM DTT, 0.2% SDS, 0.2% sodium thiosulfate pentahydrate and 2×10^7 cpm/ml sense or antisense probe. After hybridization for 18 h at 54°C, the coverslips were removed and the sections were rinsed several times in 4X standard saline citrate (SSC). Non-hybridized probes were digested by incubation with 40 µg/ml RNase A (Sigma Chemical Co.) for 30 min at 37°C. After a final high stringency wash in 0.1X SSC at 65°C for 60 min, sections were dehydrated in graded ethanol containing 0.3 M ammonium acetate, air dried and exposed to Biomax MR film (Kodak) for 11 days.

CRF—42-mer sense and antisense oligonucleotides were synthesized for the rat CRF cDNA sequence localized in nucleotides 672–713 (GeneDetect, Bradenton, FL, USA, Cat#: GD2031-OP, Rat). Labeling was performed using the terminal deoxynucleotidyl transferase (New England Biolabs, Ipswich, MA, USA) to a specific activity of $3\text{--}4 \times 10^8$ cpm/µg. Each reaction was performed with 10 pmoles of oligonucleotides in the presence of 200 µCi of [α -³⁵S]ATP (NEN/Perkin Elmer, Waltham, MA, USA). The labeled oligonucleotides were separated from unincorporated nucleotides using ProbeQuant G-50 Micro columns (GE Healthcare, Buckinghamshire, UK). *In situ* hybridization of rat brain sections and post-hybridization washings were performed as described in manufacturer's protocol. Dried hybridized sections were exposed to Biomax MR film (Kodak) for 5 days.

The films generated as described were used to analyze the mRNA expression by measuring optical film densities using the Scion Image 4.0.2 Program (Scion Corporation). The intensities of the hybridization signals were expressed as nCi/g tissue equivalent after calibration with [¹⁴C]-microscales standards (Miller, 1991), and after subtraction of the values obtained in the same areas of adjacent sections hybridized with sense (control) probes, which represent nonspecific hybridization.

Statistical analysis

All results are expressed as means $\pm$ SEM, for groups of 7–9 animals measured individually. Unpaired t test was used to analyze the results of plasma cytokines, posterior pituitary IL-6 protein and pituitary iNOS activity, experiments where saline control groups were not detectable. For the rest of results, one-way ANOVA and post-hoc Newman-Keuls' test were used to assess the significance of differences. Degrees of freedom for t tests were 16 and for ANOVA were 3,28. $P < 0.05$ was considered as statistically significant. All statistics were performed with the use of Prism 3.03 software (GraphPad Software for Science, San Diego, CA, USA).

RESULTS

Cytokines in plasma

Basal TNF- α , IL-1 β and IL-6 plasma levels were not detectable in vehicle-treated rats (Fig. 1). LPS induced TNF- α , IL-1 β and IL-6 release to the circulation when determined three hours after endotoxin administration (Fig. 1). When administered alone, the ARB did not influence plasma levels of any of the cytokine studied. However, the ARB reduced the LPS-induced release of TNF- α by 66% ($p < 0.05$), IL-1 β by 45% ($p < 0.05$) and IL-6 by 83% ($p < 0.01$; Fig. 1).

AT₁ receptor transcription and expression in the hypothalamic PVN

In vehicle-treated rats, we found high AT_{1A} mRNA expression and high AT₁ receptor binding in the PVN, with a predominant concentration in the parvocellular area (Fig. 2). LPS induced an over 2-fold increase of AT_{1A} receptor mRNA in the PVN ($p<0.05$); without changes in AT₁ receptor binding (Fig. 2). Treatment with the ARB did not alter the LPS-induced increase of AT_{1A} gene expression but significantly blocked AT₁ PVN receptors by over 50% ($p<0.001$), both in vehicle-treated and LPS-treated rats (Fig. 2).

CRF transcription in the hypothalamic PVN

In vehicle-treated rats, there was high CRF mRNA expression in the PVN, localized in the parvocellular area (Fig. 3A). Neither LPS nor candesartan administration affected CRF gene expression in the PVN (Fig. 3A).

CRF₁ and CRF₂ receptor mRNA in pituitary gland

Both CRF₁ and CRF₂ receptor mRNA were expressed in the pituitary gland of vehicle-treated rats (Fig 3B). LPS administration reduced CRF₁ receptor transcription by 70% ($p<0.001$) without effect on CRF₂ receptor transcription (Fig. 3B). Candesartan treatment did not modify the LPS-induced decrease of CRF₁ receptor mRNA or CRF₂ mRNA expression (Fig. 3B).

RAS expression in pituitary

We found large numbers of AT₁ receptors in the anterior pituitary (Fig. 4) but no AT₂ receptors (data not shown). We detected mRNA for both subtypes of Ang II AT₁ receptors, AT_{1A} and AT_{1B} (Fig. 4A), and for renin, angiotensin converting enzyme (ACE) and angiotensinogen (Table 2).

LPS increased mRNA expression of AT_{1A} receptors ($p<0.05$) and did not alter AT₁ receptor binding (Fig. 4). In addition, LPS did not modify mRNA expression of AT_{1B}, renin, ACE or angiotensinogen (Fig. 4A and Table 2). Candesartan did not modify the gene expression of any of the RAS components studied either in vehicle-treated rats or after LPS administration (Fig. 4 and Table 2). AT₁ receptor binding was decreased by 70% after candesartan administration ($p<0.001$) in both saline and LPS treated animals (Fig. 4B).

Expression of LPS receptor and recognition protein in pituitary

We detected mRNA for the LPS recognition protein CD14 and the LPS receptor TLR4 (Fig. 5). LPS administration selectively stimulated CD14 mRNA expression ($p<0.01$), but did not modify the mRNA expression of TLR4 (Fig. 5). Candesartan did not change the mRNA expression of CD14 or TLR4, either under control conditions or after LPS administration (Fig. 5).

Gene expression and content of pituitary cytokines

We studied the mRNA expression of TNF- α , IL-6, IL-1 β , LIF and MIF in the whole pituitary 3 h after LPS injection (Fig. 6). LPS administration increased mRNA expression of TNF- α by 2.5-fold ($p<0.05$), IL-6 by 34-fold ($p<0.001$), IL-1 β by 17-fold ($p<0.001$), and LIF by 47-fold ($p<0.01$). Candesartan alone did not affect the basal mRNA expression of any cytokine studied (Fig. 6). The ARB pre-treatment selectively inhibited the LPS-induced gene expression of IL-6 (62%; $p<0.001$) and LIF (74%; $p<0.01$) without affecting the LPS induction of TNF- α or IL-1 β (Fig. 6). MIF mRNA expression was not affected by any of the treatments.

We measured the IL-6 and IL-1 β protein expression in pituitaries dissected into anterior and posterior lobes. LPS administration significantly increased IL-6 and IL-1 β protein content in both anterior and posterior pituitaries ($p<0.05$; Fig. 7). Relatively higher increase in IL-6

content was found in the anterior pituitary when compared to the posterior lobe, while the increase in IL-1 β content was higher in the posterior pituitary when compared to the anterior lobe (Fig. 7). The ARB did not affect cytokine content under basal or LPS-stimulated conditions (Fig. 7).

Pituitary gene expression and activity of NOS isoenzymes

LPS up-regulated iNOS mRNA expression by 37-fold ($p<0.001$; Fig. 8A), and increased Ca²⁺-independent iNOS activity from non-detectable to significant levels in the whole pituitary (Fig. 8B). LPS down-regulated eNOS and nNOS mRNA expression by 40 to 50% ($p<0.05$; Fig. 8A) and increased the Ca²⁺-dependent eNOS/nNOS activity about 5-fold ($p<0.05$; Fig. 8B). Candesartan alone did not affect the basal gene expression or enzyme activity of any of the NOS isoenzymes (Fig. 8). The ARB pretreatment significantly reduced the LPS-induced increase in iNOS gene expression ($p<0.01$), but did not affect the LPS-induced upregulation of iNOS activity (Fig. 8). Conversely, the ARB did not affect the increase in eNOS or nNOS mRNA expression produced by LPS, but completely prevented the LPS-induced increase in eNOS/nNOS activity ($p<0.05$; Fig. 8).

Pituitary COX mRNA expression and PGE₂ content

In the whole pituitary, LPS down-regulated cyclooxygenase-1 (COX-1) mRNA by 53% ($p<0.01$); and conversely, up-regulated COX-2 mRNA 2-fold ($p<0.05$; Fig. 9A). However, LPS did not change PGE₂ content, a measure of COX-2 activity, either in the anterior or in the posterior pituitary (Fig. 9B).

The ARB decreased the basal COX-1 mRNA expression by 29% ($p<0.05$) but did not significantly influence basal COX-2 mRNA (Fig. 9A). Conversely, the ARB did not significantly alter the LPS-induced decrease in COX-1 mRNA expression but significantly reduced the LPS-induced up-regulation of COX-2 by 60% ($p<0.05$). In addition, the ARB reduced anterior and posterior pituitary PGE₂ content in control rats, and the PGE₂ content in the anterior pituitary of LPS-treated rats ($p<0.05$; Fig. 9B).

Expression of early transcription factor genes in pituitary

LPS administration increased mRNA expression of I κ B- α by 3.6-fold ($p<0.001$; Fig. 10), and this was significantly reduced (54%) by the ARB ($p<0.01$; Fig. 9). Neither the LPS nor the ARB significantly affected the c-fos gene expression (Fig. 10).

Circulating and pituitary ACTH content

LPS administration markedly enhanced circulating ACTH ($p<0.001$) and corticosterone ($p<0.001$; Fig. 11A), and decreased the ACTH content in the anterior pituitary ($p<0.05$; Fig. 11B). The ARB reduced the LPS-induced increase in circulating ACTH by 40% ($p<0.05$), but was without effect on the LPS-induced enhanced circulating corticosterone or in the pituitary ACTH content (Fig. 11). In addition, the ARB significantly increased circulating corticosterone levels in control rats not treated with LPS ($p<0.05$; Fig. 11A).

DISCUSSION

Our results demonstrate that: a) the pituitary gland is a target for systemically administered LPS; b) the AT₁ receptor activity is necessary for the complete pituitary response to the endotoxin; c) the role of AT₁ receptors in the pituitary gland appears to be limited to specific LPS-responsive pro-inflammatory pathways; d) the influence of the PVN on the initial pituitary response to LPS is complex and not completely clarified; e) circulating cytokines may play a role in the regulation of the pituitary response to LPS.

Effects of LPS and the ARB on circulating pro-inflammatory cytokines

The selectivity of the ARB effects on pro-inflammatory cytokines in the pituitary contrasts with its systemic effects. We confirmed the increase in circulating cytokines as a consequence of LPS administration, and the significant reduction in cytokine levels after ARB treatment (McAleer and Vella, 2008; Sanchez-Lemus et al., 2009). The contribution of the pituitary gland to circulating levels of pro-inflammatory cytokines is very small, and candesartan effects should be interpreted as the consequence of a reduction of the systemic inflammatory response, rather than of direct effects in the pituitary (Hoch et al., 2009; Sanchez-Lemus et al., 2009).

Effects of LPS and ARB treatment on the hypothalamic PVN

The central response to endotoxin is most probably a combination of stimulation of circumventricular organs, vagal afferents, perivascular macrophages and endothelial cells, leading to increased hypothalamic CRF synthesis and release (Dantzer et al., 2008). We focused on the role of AT_{1A} receptors in the PVN, upregulated in immobilization and isolation stress, and a powerful factor regulating CRF production and release (Armando et al., 2007; Castren and Saavedra, 1988, 1989; Jezova et al., 1998; Leong et al., 2002). Increased PVN AT_{1A} receptor transcription following inflammatory stress strongly suggests that receptor upregulation is an important component of the HPA axis stimulation. The early phase of inflammatory HPA axis stimulation was not associated with increased AT₁ receptor expression, in agreement with earlier results demonstrating that enhanced receptor expression requires prolonged or repeated stress (Armando et al., 2007; Castren and Saavedra, 1988; Jezova et al., 1998; Leong et al., 2002).

In association with increased AT_{1A} receptor transcription, LPS induced pituitary CRF₁ receptor downregulation, decreased pituitary ACTH content, and increased plasma ACTH and corticosterone, unequivocal indication of CRF release (Aguilera et al., 2004; Pozzoli et al., 1996). The LPS effects occurred with no detectable influence on CRF synthesis. We interpret our results as a consequence of the subseptic dose of LPS administered, inducing CRF release with no detectable effect on its synthesis (Loum-Ribot et al., 2006), while septic doses of LPS are able to increase both CRF synthesis and release (Grinevich et al., 2001; Loum-Ribot et al., 2006; Rivest and Laflamme, 1995).

ARB treatment drastically decreased PVN AT_{1A} receptor binding, verification of central receptor blockade following peripheral ARB administration (Nishimura et al., 2000), and significantly reduced LPS-induced ACTH release from the pituitary gland, indirect evidence of blockade of CRF release. However, the ARB did not modify the selective downregulation of pituitary CRF₁ receptors. The LPS increase in corticosterone release was not reduced by the ARB, and high glucocorticoid levels can transiently reduce CRF₁ receptor expression (Aguilera et al., 2004; Pozzoli et al., 1996), counteracting a possible ARB effect. In addition, ARBs inhibit HPA axis stimulation including CRF transcription after 24 hours of isolation stress (Armando et al., 2001; Armando et al., 2007), and it is possible that these compounds might inhibit CRF transcription only under conditions of sustained, but not acute stress. We conclude that the influence of PVN AT₁ receptors on LPS-induced acute CRF release is complex and not completely clarified.

The pituitary gland as a target organ for systemically administered LPS

The present *in vivo* results support the mRNA expression of both the LPS recognition molecule CD14 and the LPS receptor TLR4 in pituitary gland, previously reported in cultured pituitary cells (Breuel et al., 2004; Iwasaki et al., 2008; Lohrer et al., 2000). LPS administration up-regulated CD14 mRNA expression in the pituitary gland, a finding that has been consistently reported for different tissues (Sanchez-Lemus et al., 2009; Sanchez-Lemus et al., 2008; Singh and Jiang, 2004). On the other hand, while LPS did not affect expression of TLR4 in the

pituitary gland or spleen (Sanchez-Lemus et al., 2009), it decreased TLR4 mRNA expression in the adrenal gland (Sanchez-Lemus et al., 2008) and it increased TLR4 in brain endothelial cells (Singh and Jiang, 2004). Thus, it appears that the endotoxin strongly affects transcription of its recognition molecules and receptors, but that the effects are selective and tissue-dependent.

LPS selectively up-regulates AT_{1A} receptors in the anterior pituitary

Pituitary AT_{1A} receptor transcription is selectively upregulated in both acute and repeated restraint stress (Leong et al., 2002), in the stress-prone Spontaneously Hypertensive Rats (Johren et al., 2003), and, as reported here, during acute inflammatory stress. Conversely, inflammation does not change pituitary AT_{1B} receptor transcription. These observations indicate that upregulation of AT_{1A} receptor mRNA, a common feature of different stress models, participates in the pituitary response to LPS. The lack of changes in AT₁ receptor binding after LPS administration in these studies is probably due to the short time after a single dose of LPS. In addition, the upregulated AT_{1A} receptor mRNA transcribes a small portion of the total AT₁ receptors in the pituitary (Kakar et al., 1992; Lenkei et al., 1999; Leong et al., 2002).

Our results provide no information on the cellular localization of the upregulated AT_{1A} receptor mRNA. While AT_{1B} receptors are localized mainly in lactotropes and corticotropes, the precise localization of AT_{1A} receptors is still uncertain (Kakar et al., 1992; Lenkei et al., 1999; Leong et al., 2002). AT₁ receptors in the anterior pituitary may respond not only to locally formed but to circulating Ang II. However, the dose of LPS administered here does not stimulate the circulating RAS (Sanchez-Lemus et al., 2008); and LPS does not alter pituitary renin, ACE or angiotensinogen mRNA. Thus, we do not have evidence of participation of higher levels of circulating or locally formed Ang II during LPS challenge.

Effects of LPS on pro-inflammatory factors in pituitary gland

LPS increased activity of the early transcription factor NF- κ B as revealed by up-regulation of I κ B- α mRNA (Ghosh and Karin, 2002; Nam, 2006), explaining the stimulation of multiple pro-inflammatory factors described here. Conversely, LPS did not affect c-fos gene expression, an effect earlier demonstrated in other organs (Lu et al., 2008; Singru et al., 2008; Wei et al., 2008) or gene expression of MIF, a pituitary factor associated with enhancement of endotoxemia (Bernhagen et al., 1993) and a counter-regulator of the anti-inflammatory effects of glucocorticoids (Bucala, 1996). LPS exerted a selective influence on NOS isoenzymes. Both iNOS gene expression and activity were increased by LPS. On the other hand, LPS stimulated nNOS/eNOS activity while reducing nNOS and eNOS transcription, perhaps the consequence of a negative feedback. In spite of the increased COX-2 gene expression following LPS, PGE₂ levels were not altered by the endotoxin at the time studied. These apparent paradoxes reveal the complexity of the LPS effects and the limitations of our approach, the determination of LPS effects with a single dose at a single time point after administration.

Effects of AT₁ receptor blockade on LPS-induced effects in pituitary

Candesartan significantly decreased the LPS-induced NF- κ B activation and IL-6, LIF, iNOS and COX-2 gene expression, factors regulated by NF- κ B (Ghosh and Karin, 2002; Nam, 2006). Conversely, the ARB did not affect IL-1 β and TNF- α gene expression, also regulated by NF- κ B (Ghosh and Karin, 2002; Nam, 2006). An attractive hypothesis to explain these complex interactions is that LPS and the ARB may predominantly target selective cells types such as the widely distributed folliculo-stellate cells (FS).

Cultured FS cells express both LPS and Ang II receptors (Lohrer et al., 2000; Sudo et al., 2001); respond to LPS increasing cytokine synthesis and release (Lohrer et al., 2000; Tierney

et al., 2005); and to Ang II increasing intracellular Ca^{2+} concentration (Sudo et al., 2001); and these cells contribute to the regulation of the neuroendocrine response to immune stress and inflammation in a paracrine fashion (Allaerts and Vankelecom, 2005; Denef, 2008; Fauquier et al., 2001; Herkenham, 2005; Lohrer et al., 2000; Vankelecom et al., 1993). In the anterior pituitary, IL-6, LIF, iNOS, nNOS and $\text{IkB-}\alpha$ genes are highly or exclusively expressed in FS cells (Allaerts and Vankelecom, 2005; Denef, 2008; Ray and Melmed, 1997; Vankelecom et al., 1993). Conversely, IL-1 β and TNF- α may be produced in macrophage-like cells (Eriksson et al., 2000; Herkenham, 2005; Nadeau and Rivest, 1999; Whiteside et al., 1999). Cell selectivity may also explain the ARB effect on NO formation. The ARB blockade of LPS-induced Ca^{2+} -dependent eNOS/nNOS activity may be very well explained by the inhibition of Ca^{2+} release from intracellular stores and capacitative Ca^{2+} entry, mechanisms that follow AT_1 receptor activation in FS cells (Suarez et al., 2002). Our results suggest that the overall effect of the ARB may be a net decrease of pro-inflammatory NO in FS cells followed by a paracrine reduction of COX-2 activation in closely associated cells (Nogawa et al., 1998). However, whether AT_{1A} receptors are predominantly located in FS cells is an open question.

In addition to its modulation of LPS effects, the ARB decreased steady-state PGE_2 production when administered alone. Since PGE_2 mediates inflammation, this may contribute to the general anti-inflammatory activity of ARBs (Marchesi et al., 2008)

ARB effects on LPS-stimulated ACTH and corticosterone

The release of ACTH from pituitary is influenced by several factors acting synchronically, including PVN activation, paracrine intra-pituitary interactions and circulating factors (Denef, 2008; Ray and Melmed, 1997). We report that the ARB significantly decreases LPS-induced ACTH release from the pituitary, an effect that may be related to a decrease in CRF release, as suggested above. However, we found strong evidence for direct ARB effects in the pituitary; the ARB decreased LPS-induced LIF and IL-6 gene expression, factors which increase ACTH release and synergize with CRF (Auernhammer and Melmed, 2000; Denef, 2008; Ray and Melmed, 1997).

While limiting the ACTH response to LPS, the ARB, paradoxically, does not decrease corticosterone release from the adrenal gland. The mechanisms of LPS increase in corticosterone secretion are complex, including not only enhancement through ACTH but also direct stimulation through splanchnic innervation of the adrenal cortex (Fleshner et al., 1998) and direct effects of the endotoxin on adrenocortical cells (Vakharia and Hinson, 2005). Failure of ARBs to decrease corticosterone release (Sanchez-Lemus et al., 2008) suggest that neither the splanchnic-mediated nor direct adrenal LPS effects are mediated through AT_1 receptor activation. This indicates the possibility that LPS releases corticosterone through Ang II-dependent (ACTH-mediated) and independent (splanchnic stimulation, direct effects on adrenal cortex) pathways. Glucocorticoids have potent anti-inflammatory properties (Elenkov and Chrousos, 2006; Fernandes et al., 2008) and preservation of glucocorticoid release may contribute to the general anti-inflammatory activity of ARBs (Marchesi et al., 2008).

In conclusion, our *in vivo* results demonstrate that the pituitary gland is a target organ for systemically administered LPS and reveal that AT_1 receptor activity is necessary for a complete response to the endotoxin. AT_1 receptor activity regulates some, but not all, the mechanisms underlying LPS effects in the pituitary, suggesting the existence of Ang II-dependent and independent regulatory mechanisms for specific pro-inflammatory factors in the gland. Our results may be best interpreted as a combination of a partial reduction on centrally-mediated HPA axis stimulation, direct effects in the pituitary gland and decreased pro-inflammatory cytokine release to the circulation. The selective effects of ARBs on pituitary mediators may be a useful tool for understanding the complex central and local regulatory mechanisms of the endotoxin effect on this gland. Our results further establish the general anti-inflammatory

effects of ARBs, and support the hypothesis that these compounds may have beneficial effects in inflammatory disorders.

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Figure 1. Circulating cytokines

Values are mean $\pm$ SEM for groups of 7–9 animals measured individually. Cytokine levels on the saline treated groups were not detectable and were omitted from the graph. # $p < 0.05$, ## $p < 0.01$ as compared to vehicle-LPS treated group.

Figure 2. Hypothalamic PVN expression of AT₁ receptors

A) Representative pictures and quantification of AT_{1A} *in situ* hybridization in PVN. **B)** Representative pictures and quantification of [¹²⁵I]Sar¹-Ang II binding to AT₁ receptors in PVN. Scale bar, 1 mm. All values are means ± SEM for groups of 7–9 animals measured individually. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to vehicle-saline treated group. ### $p < 0.001$ as compared to vehicle-LPS treated group.

Figure 3. mRNA expression of CRF in hypothalamic PVN and of CRF receptors in pituitary
A) Representative pictures and quantification of CRF *in situ* hybridization in PVN. *Scale bar*, 1 mm. **B)** Pituitary expression of CRF₁ and CRF₂ receptors. Data represent relative levels of mRNA normalized to 18S rRNA. All values are means $\pm$ SEM for groups of 7–9 animals measured individually. *** $p < 0.001$ as compared to vehicle-saline treated group.

Figure 4. Pituitary expression of AT₁ receptors

A) AT_{1A} and AT_{1B} mRNA expression in pituitary. Data represent relative levels of mRNA normalized to 18S rRNA. **B)** Representative pictures and quantification of [¹²⁵I]Sar¹-Ang II binding to anterior pituitary AT₁ receptor. Binding was present exclusively in anterior (arrow) but not posterior (arrowhead) lobe. Scale bar, 1 mm. All values are means ± SEM for groups of 7–9 animals measured individually. * $p < 0.05$, *** $p < 0.001$ as compared to vehicle-saline treated group. ### $p < 0.001$ as compared to vehicle-LPS treated group.

Figure 5. Pituitary expression of LPS receptors TLR4 and CD14

Data represent relative levels of mRNA normalized to 18S rRNA. Values are mean $\pm$ SEM for groups of 7–9 animals measured individually. *** $p < 0.001$ as compared to vehicle-saline treated group.

Figure 6. Pituitary cytokines gene expression

Data represent relative levels of mRNA normalized to 18S rRNA. Values are mean $\pm$ SEM for groups of 7–9 animals measured individually. * $p < 0.05$, *** $p < 0.001$ as compared to vehicle-saline treated group; ## $p < 0.01$, ### $p < 0.001$ as compared to vehicle-LPS treated group.

Figure 7. Pituitary cytokines content

Data represent the cytokine protein content. Values are mean $\pm$ SEM for groups of 7–9 animals measured individually. * $p < 0.05$ as compared to vehicle-saline treated group; ND, non detectable.

Figure 8. Pituitary nitric oxide synthase enzymes expression and activity

Data represent relative levels of mRNA normalized to 18S rRNA (A) and NOS activity measurement (B). Values are mean $\pm$ SEM for groups of 7–9 animals measured individually. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to vehicle-saline treated group; # $p < 0.05$, ## $p < 0.01$ as compared to vehicle-LPS treated group. ND, non detectable.

Figure 9. Pituitary COX enzymes expression and PGE₂ content

Data represent relative levels of mRNA normalized to 18S rRNA (**A**) and pituitary PGE₂ content (**B**). Values are mean $\pm$ SEM for groups of 7–9 animals measured individually. * $p < 0.05$, ** $p < 0.01$ as compared to vehicle-saline treated group; # $p < 0.05$ as compared to vehicle-LPS treated group.

Figure 10. Pituitary c-fos and IκB-α gene expression

Data represent relative levels of mRNA normalized to 18S rRNA. Values are mean $\pm$ SEM for groups of 7–9 animals measured individually. *** $p < 0.001$ as compared to vehicle-saline treated group. ## $p < 0.01$ as compared to vehicle-LPS treated group.

Figure 11. Circulating hormones and pituitary ACTH

A) Data represent levels of circulating ACTH and corticosterone and **B)** hormone content of ACTH in the anterior lobe. Values are mean $\pm$ SEM for groups of 7–9 animals measured individually. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to vehicle-saline treated group; # $p < 0.05$ as compared to vehicle-LPS treated group.

Table 1

List of the primers used for real time PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
18S	GACCATAAACGATGCCGACT	GTGAGGTTTCCCGTGTGAG
Angiotensinogen	CATGAGTTCTGGGTGGACAA	AAGTTGTTCTGGGCGTCACT
AT _{1A}	AGCCTGCGTCTTGTTTGTAG	GCTGCCCTGGCTTCTGTC
AT _{1B}	CACCTCGCCAAGGGAGAC	CAC TTGCAGGCTTTGAACC
CD14	ATGCCTCTCCCGCCACACCAG	GACCGCCGCGGTAGAACTCCA
c-fos	GGAGCCGGTCAAGAACATTA	GAAGGAACCAGACAGGTCCA
COX-1	ACTGGAACCCAGCACATTC	AAGGAGACATAGGGGCAGGT
COX-2	CGGAGGAGAAGTGGGGTTTAGGAT	TGGGAGGCACTTGCGTTGATGG
CRF ₁	TGCATCCGTGGACCTCATTG	GGTAGCCATTGTTTGTCTGTTGT
CRF ₂	TGCCGCTGCGTCACCACCATA	AGCAGCCTTCCACAAACATCCAGA
eNOS	GCCCCGGTACTACTCTGTCA	CCTTGAGTTGGCTCATCCAT
IL-1 β	CCTCTGCCAAGTCAGGTCTC	GAATGTGCCACGGTTTCTT
IL-6	CACAAGTCCGGAGAGGAGAC	CAGAATTGCCATTGCACAAC
iNOS	AGGCCACCTCGGATATCTCT	GCTTGTCTCTGGGTCTCTG
I κ B- α	GCAGTCTTGACTCAGACCTGTACG	GCCCTGGTAGGTTACTC
LIF	TCAACTGGCTCAACTCAACG	ACCATCCGATACAGCTCGAC
MIF	CAGAACC GCAACTACAGCAA	GAACAGCGGTGCAGGTAAGT
nNOS	CTGCCACGGAGTCCTTCATCAAAC	AGCCCCCTCTGCAGCGGTATT
Renin	CTGTGCATACTGGCTCTCCA	GAACCCGATGCGATTGTTAT
TLR4	AGCCGGAAAGTTATTGTGGTGGTG	CAACGGCTCTGGATAAAGTGTCT
TNF- α	GAGAGATTGGCTGCTGGAAC	TGGAGACCATGATGACCGTA

Table 2

Gene expression of other RAS components

	Vehicle-saline	Candesartan-saline	Vehicle-LPS	Candesartan-LPS
Renin	1.0 ± 0.27	1.3 ± 0.38	1.3 ± 0.38	1.0 ± 0.39
ACE	1.0 ± 0.22	0.8 ± 0.15	0.9 ± 0.14	0.8 ± 0.11
Angiotensinogen	1.0 ± 0.10	0.8 ± 0.09	0.8 ± 0.12	0.7 ± 0.11

Data represent relative levels of mRNA normalized to 18S rRNA. Values are mean ± SEM for groups of 7–9 animals measured individually.