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Angiotensin II AT_1 Receptor Blockade Ameliorates Brain Inflammation

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Brain inflammation has a critical role in the pathophysiology of brain diseases of high prevalence and economic impact, such as major depression, schizophrenia, post-traumatic stress disorder, Parkinson's and Alzheimer's disease, and traumatic brain injury. Our results demonstrate that systemic administration of the centrally acting angiotensin II AT_1 receptor blocker (ARB) candesartan to normotensive rats decreases the acute brain inflammatory response to administration of the bacterial endotoxin lipopolysaccharide (LPS), a model of brain inflammation. The broad anti-inflammatory effects of candesartan were seen across the entire inflammatory cascade, including decreased production and release to the circulation of centrally acting proinflammatory cytokines, repression of nuclear transcription factors activation in the brain, reduction of gene expression of brain proinflammatory cytokines, cytokine and prostanoid receptors, adhesion molecules, proinflammatory inducible enzymes, and reduced microglia activation. These effects are widespread, occurring not only in well-known brain target areas for circulating proinflammatory factors and LPS, that is, hypothalamic paraventricular nucleus and the subfornical organ, but also in the prefrontal cortex, hippocampus, and amygdala. Candesartan reduced the associated anorexic effects, and ameliorated associated body weight loss and anxiety. Direct anti-inflammatory effects of candesartan were also documented in cultured rat microglia, cerebellar granule cells, and cerebral microvascular endothelial cells. ARBs are widely used in the treatment of hypertension and stroke, and their anti-inflammatory effects contribute to reduce renal and cardiac failure. Our results indicate that these compounds may offer a novel and safe therapeutic approach for the treatment of brain disorders.
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INTRODUCTION

Brain inflammation is an essential mechanism to restore homeostasis in response to stress, infection, or injury (Yong and Rivest, 2009). Circulating proinflammatory cytokines and hormones, bacterial endotoxins and products of neuronal injury activate transcription factors, setting in motion inflammatory cascades with participation of brain parenchymal microglia and blood-derived infiltrating macrophages (Licinio and Wong, 1997). Although a well-regulated central inflammatory reaction contributes to reestablish homeostasis, exaggerated responses lead to chronic inflammation and neuronal damage (Dantzer *et al*, 2008). Such excessive activation of immune responses increases the risk of disease in vulnerable individuals, and is an important contributing factor underlying the pathophysiology of many psychiatric and neurological diseases. These include major depression, schizophrenia, autism, post-traumatic stress disorder, and neurodegenerative disorders, such as Alzheimer's and Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, HIV-associated dementia, and traumatic brain injury (Fassbender *et al*, 2004; Hope *et al*, 2009; Miller *et al*, 2009; Rivest, 2009).

Because drugs used in peripheral inflammatory conditions are not fully effective in the brain (Nimmo and Vink, 2009), the search for novel, safe, and effective central anti-inflammatory drugs is of major interest. Recent studies have focused on a neuropeptide, angiotensin II (Ang II) initially discovered as a circulating prohypertensive hormone. Ang II was later recognized as a pleiotropic factor locally formed in most tissues, including the brain (Saavedra, 1992). Most of the effects of Ang II are dependent on the stimulation of its AT_1 receptors (Saavedra *et al*, 2006), and AT_1 receptor overactivity promotes peripheral vascular and

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tissue inflammation (Savoia and Schiffrin, 2007). AT_1 receptor blockers (ARBs) not only antagonize the Ang II-mediated vasoconstriction but also reverse peripheral inflammation in hypertension, atherosclerosis, and diabetes (Savoia and Schiffrin, 2007). For this reason ARBs, compounds with excellent safety margins, are commonly used not only to regulate blood pressure, but also for their capacity to protect end organs, thus decreasing heart failure and renal damage (Savoia and Schiffrin, 2007).

We previously used the centrally acting ARB candesartan (Nishimura *et al*, 2000), a compound blocking central AT_1 receptor stimulation by Ang II when administered systemically (Seltzer *et al*, 2004). In spontaneously hypertensive rats (SHRs), a rodent model of genetic hypertension with overactive brain AT_1 receptors, behavioral and cognitive deficits, hypersensitive to stress and to brain inflammation (Kvetnansky *et al*, 1979; Saavedra, 1992; Sirén *et al*, 1992; Russell, 2003), candesartan reversed cerebrovascular inflammation and brain ischemia (Ando *et al*, 2004; Zhou *et al*, 2005). The effects of candesartan were not restricted to hypertensive models, because the ARB also reduced stroke-related inflammation in normotensive rats (Schmerbach *et al*, 2008).

We hypothesized that excessive AT_1 receptor activation may participate in other types of inflammatory responses in the brain, and we postulated a general anti-inflammatory effect of ARBs, unrelated to their blood pressure reducing effect. To test our hypothesis, we first selected a well-characterized model of inflammation, the activation of the innate immune response in the periphery and in the brain by a systemic administration of the bacterial endotoxin lipopolysaccharide (LPS) (Quan and Banks, 2007). AT_1 receptor blockade significantly decreased endotoxin-induced inflammation both *in vivo* in peripheral tissues of normotensive rats (Sánchez-Lemus *et al*, 2008, 2009a,b), and *in vitro* in circulating monocytes from normotensive human volunteers (Larrayoz *et al*, 2009). These studies demonstrated that ARBs could be considered potent anti-inflammatory agents by effects unrelated to their anti-hypertensive properties.

To establish whether ARBs can exert central anti-inflammatory activity, in this study we determined the effect of AT_1 receptor blockade on the LPS-induced brain inflammatory response. This study extends our preliminary results reporting anti-inflammatory effects of candesartan in the cingulate cortex (Benicky *et al*, 2009). To investigate whether anti-inflammatory effects observed *in vivo* were dependent on cross-system interactions, we included *in vitro* assays using LPS target cells. We studied cerebellar granule cells, a highly homogeneous population of neurons (Gao *et al*, 1995), cerebral microvascular endothelial cells (Nakagawa *et al*, 2009), and cortical microglial cells (Giulian and Baker, 1986). We show here that AT_1 receptor inhibition significantly and broadly decreases the brain immune response *in vivo* as well as the inflammatory responses in all LPS target cells studied. In addition, candesartan ameliorates LPS-induced sickness behavior, the acute anorexic effects and body weight loss (Konsman and Dantzer, 2001) and anxiety-like behavior in untreated and LPS-treated rats. These studies show that ARBs are potent central anti-inflammatory compounds, an important property of translational value.

MATERIALS AND METHODS

Detailed methods can be found in Supplementary Materials and Methods section.

Animals

Nine-week-old male Wistar Hannover (WH) rats and SHR (Taconic Farms, Germantown, NY) were used for *in vivo* experiments. Three-week-old male WH rats were used for isolation of brain microvascular endothelial cells. Eight-day-old and one-day-old Sprague Dawley male and female pups (Taconic Farms) were used for isolation of cerebellar granule cells and cortical microglia, respectively. The National Institute of Mental Health Animal Care and Use Committee (Bethesda, MD) approved all procedures.

In Vivo Studies

Short-term Ang II AT_1 receptor blockade. WH rats received daily subcutaneous injections of vehicle or candesartan (CV-11974, Astra-Zeneca, Mölndal, Sweden) at the dose of 1 mg/kg per day. This dose was similar to that used in the treatment of human cardiovascular disorders (Weinberg *et al*, 2004). The animals were injected intraperitoneally with 50 μg/kg LPS (*Escherichia coli* serotype 055:B5; Sigma-Aldrich, St Louis, MO) or saline on day 3 and were killed 3 h later by decapitation. Trunk blood was used for plasma preparation and brains were dissected, frozen in isopentane on dry ice, and stored at −80 °C until used.

Behavioral studies. For anorexia determination, separate groups of individually housed WH rats were subjected to short-term Ang II AT_1 receptor blockade followed by intraperitoneal injection of 50 μg/kg LPS as described above. Animals were euthanized 2 days after LPS injection. Body weight and food consumption were monitored daily for the duration of experiment. For anxiety determination, SHR were treated as above. At 3 h after LPS injection, the rats were tested on an elevated plus maze as described previously (Saavedra *et al*, 2006). The time spent and the number of entries into the open and closed arms were recorded during 5 min.

Sustained Ang II AT_1 receptor blockade. WH rats were implanted subcutaneously with osmotic minipumps (model 2002, Alza Scientific Products, Palo Alto, CA) containing candesartan (1 mg/kg per day) or vehicle. On day 14, the animals were injected intraperitoneally with 50 μg/kg LPS or saline and were killed 3 h later by decapitation. Trunk blood was used for plasma preparation and brains were dissected, frozen in isopentane on dry ice and stored at −80 °C until used. For immunohistochemical studies, separate groups of animals treated as above were anesthetized 3 h after LPS or saline injection and transcardially perfused with 4% buffered formaldehyde. The brains were dissected, post-fixed overnight, cryoprotected by 30% sucrose, frozen in isopentane on dry ice, and stored at −80 °C until used.

In Vitro Studies

Cerebellar granule cell culture. Cells were isolated from 8-day-old rat pups as described previously (Gao *et al*, 1995).

The isolated cells were plated at a density of 1.2×10^6 cells/ml and cultured for 6–14 days. Before the experiment, the cells were starved overnight in plain medium and subsequently treated for 4 h with 500 ng/ml LPS with or without 2 h pre-treatment with 5 μM candesartan. The dose of candesartan used was similar to those necessary to reduce Ang II effects in cell cultures (Seeger *et al*, 2001). Medium was saved and cells were lysed for RNA isolation as described below.

Brain microvascular endothelial cell culture. Cultures were prepared as described previously (Nakagawa *et al*, 2009). The cells were used after the first passage when they reached 80% confluence. Before the experiment, cells were starved overnight in a serum-free medium followed by treatment with 100 ng/ml LPS for 4 h with or without 2 h pre-incubation with 10 μM candesartan. Medium was saved and cells were lysed for RNA isolation as described below.

Cortical microglia cell culture. Cultures were prepared as described (Giulian and Baker, 1986). Initially prepared mixed glial cells were cultured for 10 days and subsequently used for microglia preparation. Purified microglial cells were allowed to grow for 24 h, starved overnight in a serum-free medium, and subsequently treated for 4 h with 100 ng/ml LPS with or without 2 h pre-incubation with 10 μM candesartan. Medium was saved and cells were lysed for RNA isolation as described below.

Determination of Cytokine Levels

Cytokines in plasma and medium were measured by commercial enzyme-linked immunosorbent assay kits from BioSource (Camarillo, CA) for interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α), and from R&D Systems (Minneapolis, MN) for IL-1β and IL-10.

Microdissection of Brain Structures

Coronal sections (300 μm) of fresh-frozen brains were cut on a cryostat, mounted on slides, and used for punch microdissection as follows (positions are relative to bregma): three sections between −1.3 and −2.2 mm for the paraventricular nucleus (PVN) and the central nucleus of the amygdala; two sections between −0.7 and −1.3 mm for the subfornical organ (SFO); three sections between −2.2 and −3.1 mm for the CA1 subdivision of the hippocampus; and three sections between 3.7 and 2.9 mm for the ventromedial prefrontal cortex (Paxinos and Watson, 1998). Punch microdissection was performed under stereomicroscope control using Harris Uni-Core microdissection needles of 0.75 mm diameter for the PVN and 1 mm for other brain structures. The individual punches dissected from the same nucleus were pooled and immediately homogenized for RNA isolation as described below. Tissues from each rat were studied individually.

Determination of mRNA Expression By Real-Time PCR

RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA) and purified using the RNeasy Mini kit (Qiagen, Valencia, CA). Total RNA (0.15 μg from brain tissue; 1 μg from cell lysates) was reversely transcribed into cDNA and quantified by real-time PCR as previously described (Sánchez-Lemus *et al*, 2009b). Primers used in the study are listed in Supplementary Table S1. Target cDNA from individual samples was normalized to GAPDH mRNA and expressed as a fold change relative to the vehicle-saline group.

Ang II Receptor Autoradiography

Binding of [^{125}I] Sarcosine^1-Ang II (ARC, St Louis, MO) to 16 μm coronal sections of fresh frozen brains was performed as described previously (Tsutsumi and Saavedra, 1991).

Localization and Quantification of mRNA Expression By *In Situ* Hybridization

Coronal sections (16 μm) of fresh frozen brains were cut on a cryostat, mounted on positively charged slides, dried for 5 min at 50 °C, and stored at −80 °C until used. *In situ* hybridization of AT_{1A} receptor mRNA was performed using [^{35}S]-labeled antisense and sense riboprobes corresponding to the 368 bp Pal1 fragment from the 3′-non-coding region of the rat AT_{1A} cDNA as described previously (Jőhren *et al*, 1995).

In situ hybridization of c-Fos and IL-1β mRNA was performed using [^{35}S]-labeled oligonucleotide probes. Antisense probes for the rat IL-1β, 5′-CCACGGGCAAGACA TAG GTAGCTGCCACAGCTTCTCCACAGCCAC-3′ (Kim *et al*, 2004), and c-Fos, 5′-GCAGCGG GAGGATGACGCCTC GTAGTCCGCGTTGAAACCCGAGAA-3′ (Vaisanen *et al*, 2004), and their corresponding sense probes were 3′ labeled with [α-^{35}S]-ATP (NEN/Perkin Elmer, Waltham, MA) to a specific activity of $3–4 \times 10^8$ c.p.m./μg. The sections were fixed in 4% formaldehyde, acetylated, delipidated, and exposed to hybridization buffer containing 2×10^7 c.p.m./ml sense or antisense probe. After hybridization for 18 h at 40 °C, the sections were washed, dehydrated, and exposed to Biomax MR film. The optical densities of autoradiograms were quantified by comparison with [^{14}C]-microscale standards (Tsutsumi and Saavedra, 1991) and expressed as nCi/g tissue.

Immunohistochemical Localization and Quantification of c-Fos and Microglia

Coronal sections (16 μm) from fixed brains were cut on a cryostat, mounted to positively charged slides, vacuum-dried, and stored at −80 °C until used. The sections were immersed into ice-cold acetone for 15 min, air dried, and pre-incubated 3×5 min in Tris-buffered saline (20 mM Tris, 136 mM NaCl, pH 7.4) containing 0.2% Triton X-100. After quenching of intracellular peroxidase and blocking of endogenous biotin, the sections were blocked with 10% of normal goat serum and exposed to primary antibody overnight at 4 °C. Rabbit polyclonal antibody against rat c-Fos (Ab-5, 1:10 000, Calbiochem, La Jolla, CA), or mouse monoclonal antibody against rat CD11b/c (Ox-42, 1:300, BD Pharmingen, San Jose, CA) were used as primary antibodies. After washing, the sections were exposed to goat biotin-conjugated antibody against rabbit or mouse IgG (1:1000, Jackson ImmunoResearch, West Grove, PA) followed by incubation with avidin and biotin-conjugated horseradish

peroxidase H. The sections were exposed to DAB reagent (Vector Laboratories, Burlingame, CA), counter-stained with hematoxylin, dehydrated in ethanol, cleared with xylene, and coverslipped with mounting medium.

Data acquisition and quantification. For data acquisition and quantification, sections were examined by an investigator blinded to the protocol used and the identity of the slides. Images were acquired by Axiocam digital camera (Zeiss, Jena, Germany) connected to a Zeiss Axioskop light microscope under constant illumination. Images were processed and analyzed using Image J software (Ruifrok and Johnston, 2001). Induction of c-Fos protein was expressed as the number of c-Fos-positive cells per PVN section. Microglia activation was expressed as a proportion of Ox-42-positive area to the total area of the PVN. The increase in the proportion of the area occupied by microglia was used as a measure of activation (Milligan *et al*, 1991; Popovich *et al*, 1997).

Statistical Analysis

Results are expressed as means ± SEM for groups of 5–10 animals measured individually. Results from cell culture experiments are expressed as means ± SEM of three independent cell preparations. To address the differences among all individual groups, we analyzed the data by one-way ANOVA followed by the *post hoc* Newman–Keuls' test for all determinations with significant omnibus ANOVA. Differences were considered significant at $P<0.05$.

RESULTS

Effects of LPS and an ARB on brain Ang II AT_{1A} mRNA expression and AT_1 receptor binding. When rats were studied at 3 h after LPS administration, the endotoxin upregulated AT_{1A} receptor mRNA expression in the hypothalamic PVN but not in the SFO, and did not alter AT_1 receptor binding in either structure (Supplementary Figure S1). Candesartan significantly blocked AT_1 receptor binding in the PVN and SFO in both vehicle- and in LPS-treated rats (Supplementary Figure S1).

Ang II AT_1 Receptor Blockade Decreases LPS-Induced Release of Proinflammatory Cytokines to the Circulation

The basal cytokine levels in plasma were below the limit of detection in vehicle-saline control rats and were not changed by AT_1 receptor blockade (Figure 1). LPS markedly elevated plasma levels of the proinflammatory cytokines TNF-α, IL-6 and IL-1β, and the anti-inflammatory cytokine IL-10 (Figure 1). Both short term and sustained AT_1 receptor blockade significantly reduced LPS-induced plasma levels of proinflammatory cytokines, but did not affect IL-10 concentrations (Figure 1).

Ang II AT_1 Receptor Blockade Reduces LPS-Induced Proinflammatory Cytokine mRNA in the Brain

LPS produced a widespread increase in TNF-α, IL-1β, and IL-6 mRNA expression in the PVN, SFO, ventromedial prefrontal cortex, central nucleus of the amygdala, and the CA1 subdivision of the hippocampus (Figure 2). The effects of LPS were significantly reduced by short-term AT_1 receptor blockade in all areas examined (Figure 2).

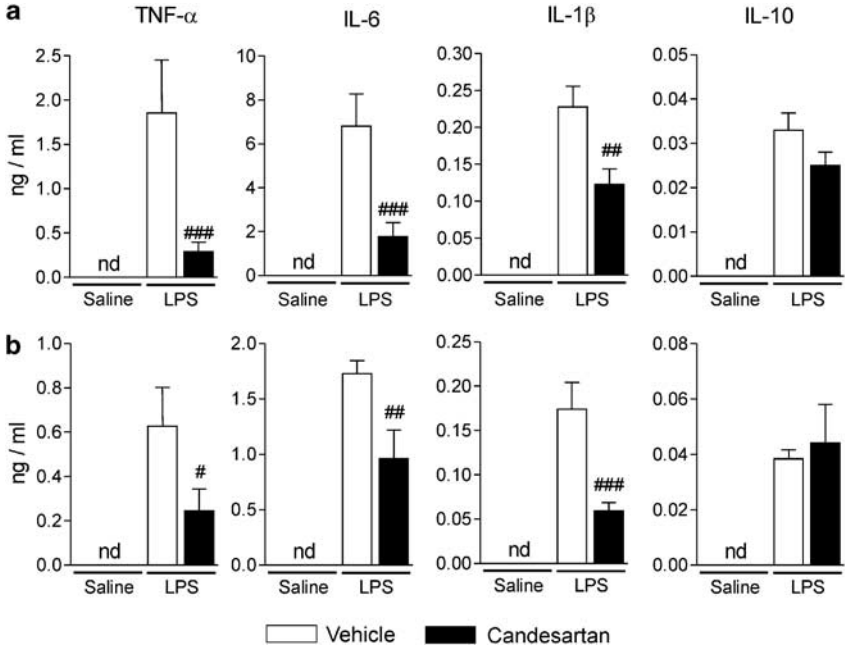

Figure 1 Selective decrease of LPS-induced proinflammatory cytokine release to the circulation by angiotensin II AT_1 receptor blockade. Short-term (a) and sustained (b) candesartan treatments were followed by LPS or saline injection as described in Materials and Methods. The levels of cytokines in plasma 3 h after LPS or saline injection were detected by specific ELISA. Data are expressed as means ± SEM ($n=5$–16). $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared with vehicle-LPS group; nd, non-detectable.

Ang II AT_1 Receptor Blockade Diminishes LPS-Induced mRNA Expression of Brain Proinflammatory Factors

Short-term candesartan pretreatment significantly reduced LPS-induced increase in mRNA expression of multiple proinflammatory factors in the PVN and the SFO (Figure 3 and Supplementary Figure S2). These include: (a) the LPS recognition molecule CD14; (b) the immediate early genes c-Fos and FosB, and the inhibitor of κB (IκB-α), representing the activation of the nuclear factor-κB (NF-κB); (c) inflammatory factor receptors, TNF-α R1, TNF-α R2, IL-1 R1, IL-6R, the IL-1R antagonist, and the prostaglandin E_2 (PGE_2)

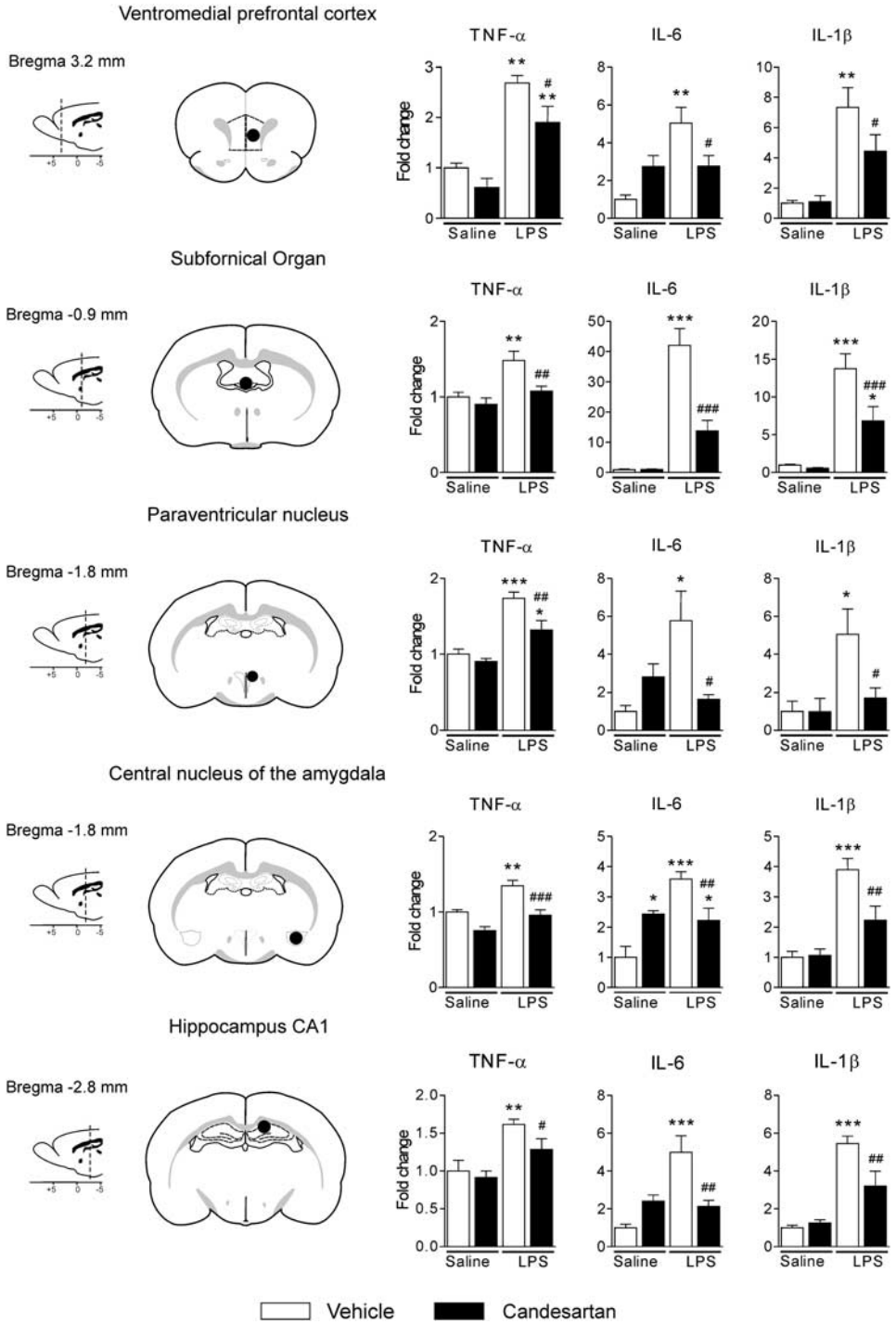

Figure 2 Widespread decrease of LPS-induced proinflammatory cytokine mRNA expression in brain by angiotensin II AT_1 receptor blockade. Short-term treatment with candesartan was followed by LPS injection as described in Materials and Methods. Expression of mRNAs of proinflammatory cytokines was detected by real-time PCR in brain structures isolated by punch microdissection from 300 μm coronal brain sections. The scheme on the left illustrates the distance from bregma and the punch area (black circle) in the corresponding coronal sections. The expression of all genes was normalized to the level of GAPDH mRNA. Data are expressed as a fold change relative to vehicle-saline group. Data represent mean ± SEM ($n = 5–8$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with vehicle-saline group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with vehicle-LPS group.

receptor EP4; (d) the nitric oxide (NO)-producing isoenzyme inducible NO synthase (iNOS) and the PGE_2-producing enzyme cyclooxygenase 2 (COX-2); (e) the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and the vascular cell adhesion molecule-1 (VCAM-1); and (f) the microglia activation marker CD40 (Figure 3 and Supplementary Figure S2). For the most part, the responses to LPS and AT_1 receptor blockade were similar in the PVN and SFO, with the ARB significantly decreasing expression of LPS-induced inflammatory factor mRNA expression. A few exceptions are noted in Supplementary Figure S2.

Sustained candesartan pretreatment yielded results similar to those obtained with short-term pretreatment for all factors examined in the PVN (Supplementary Figure S3). In addition, LPS upregulated, and short-term candesartan treatment prevented, c-Fos mRNA in the PVN, SFO, and choroid plexus, and IL-1β mRNA in the SFO and choroid plexus visualized by *in situ* hybridization. IL-1β mRNA was not detected in the PVN (Figure 4a–d).

Ang II AT_1 Receptor Blockade Lessens LPS-Induced c-Fos Protein Expression and Microglia Activation

Long-term ARB pretreatment suppressed, in the PVN, LPS-induced increase in the number of cells expressing c-Fos (Figure 5a and c) and microglial activation detected by OX-42 immunostaining (Figure 5b and d).

AT_1 Receptor Blockade Decreases LPS-Induced Stimulation of Proinflammatory Factors in Cultured Cerebellar Granule Cells

In primary cultures of cerebellar granule cells, candesartan suppressed LPS-induced upregulation of TNF-α, IL-1β, and IL-6, AT_{1A} receptor, CD14, and IκB-α mRNA (Figure 6a). LPS elicited an $\sim$100-fold increase in TNF-α release into the cultured medium, dose dependently inhibited by candesartan (Figure 6a).

LPS Increases AT_{1A} Receptor Transcription and AT_1 Receptor Blockade Diminishes LPS-Induced iNOS and Cytokine Expression in Cultured Cerebral Microvascular Endothelial Cells

In primary cultures of rat cerebrovascular endothelial cells, LPS induced expression of AT_{1A} receptor mRNA (Figure 6b). Incubation in the presence of candesartan decreased the LPS-induced expression of TNF-α, IL-1β, and iNOS mRNA (Figure 6b).

AT_1 Receptor Blockade Decreases LPS-Induced Stimulation of Proinflammatory Factors in Cultured Microglial Cells

In primary cultures of cortical microglia, candesartan reduced LPS-induced upregulation of IL-1β and CD40 mRNA (Figure 6c). LPS induced $\sim$20-fold increase in IL-1β release into the cultured medium; the effect that was significantly reduced by candesartan (Figure 6c).

AT_1 Receptor Blockade Reduces the Acute LPS-Induced Anorexic Effect and Body Weight Loss

Administration of LPS produced an acute anorexic effect and decrease in body weight (Figure 7a). These effects were significantly reduced by AT_1 receptor blockade (Figure 7a).

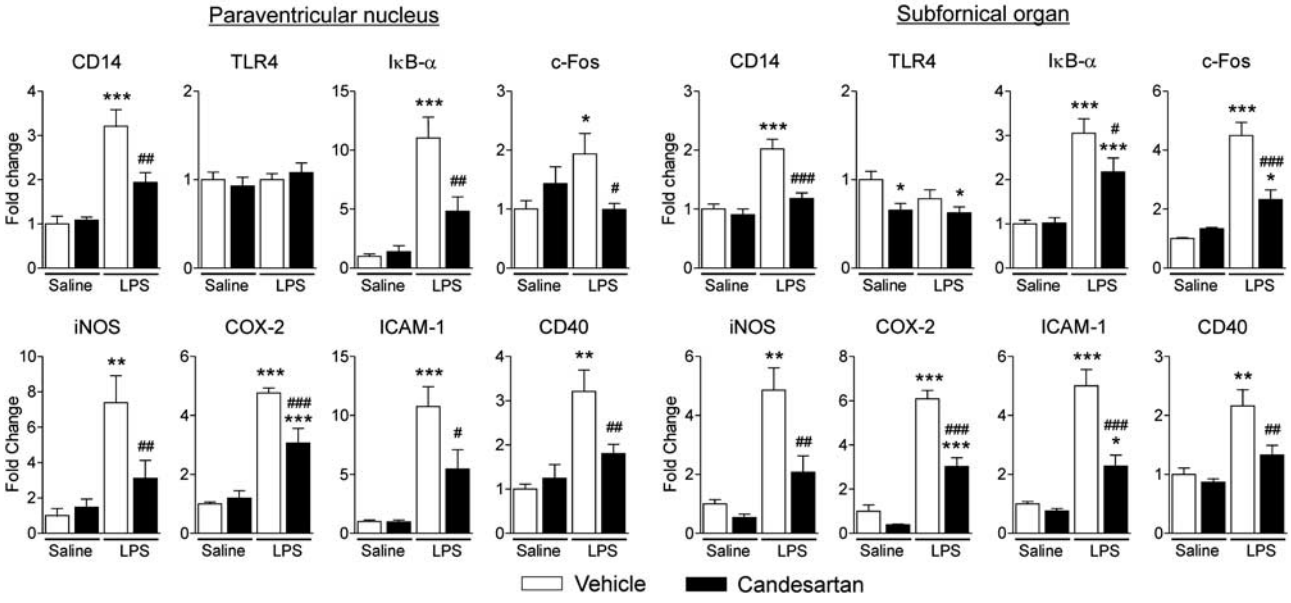

Figure 3 Decrease of LPS-induced inflammatory factor mRNA expression in PVN and SFO by angiotensin II AT_1 receptor blockade. Short-term candesartan treatment was followed by LPS injection as described in Materials and Methods. Expression of mRNAs of proinflammatory factors and mediators was detected in microdissected PVN and SFO by real-time PCR and was normalized to the level of GAPDH mRNA. Results are expressed as a fold change relative to vehicle-saline group. Data represent mean ± SEM ($n = 5$–8). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared with vehicle-saline group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#}P<0.001$, compared with vehicle-LPS group.

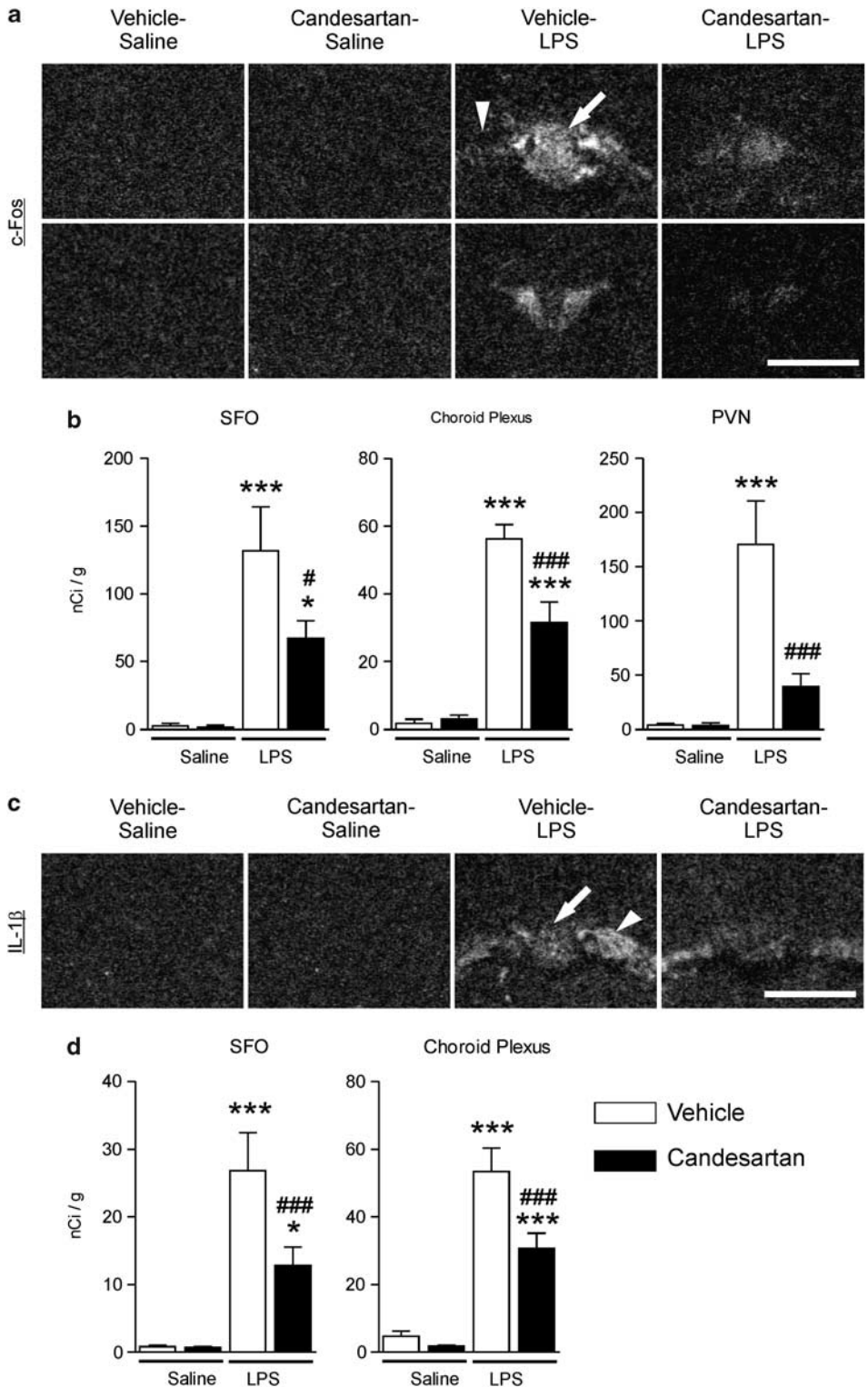

Figure 4 Angiotensin II AT_1 receptor blockade attenuates LPS-induced expression of c-Fos mRNA in PVN, SFO, and choroid plexus and IL-1β mRNA in SFO and choroid plexus. Short-term candesartan and LPS were administered as described in Materials and Methods. Expression of c-Fos and IL-1β mRNAs was analyzed by *in situ* hybridization (a) Representative dark field images of film autoradiograms illustrating the expression of c-Fos mRNA in SFO (top) and PVN (bottom). (b) Quantitative analysis of c-Fos mRNA in SFO and PVN. (c) Representative dark field images of film autoradiograms illustrating the expression of IL-1β mRNA in SFO and choroid plexus. (d) Quantitative analysis of IL-1β mRNA in SFO and choroid plexus. Data are expressed as means ± SEM ($n = 7$–9). $^{*}P < 0.05$, $^{***}P < 0.001$ compared with vehicle-saline group, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared with vehicle-LPS group. Scale bar represents 1 mm. Arrow points to SFO; arrowhead indicates adjacent choroid plexus.

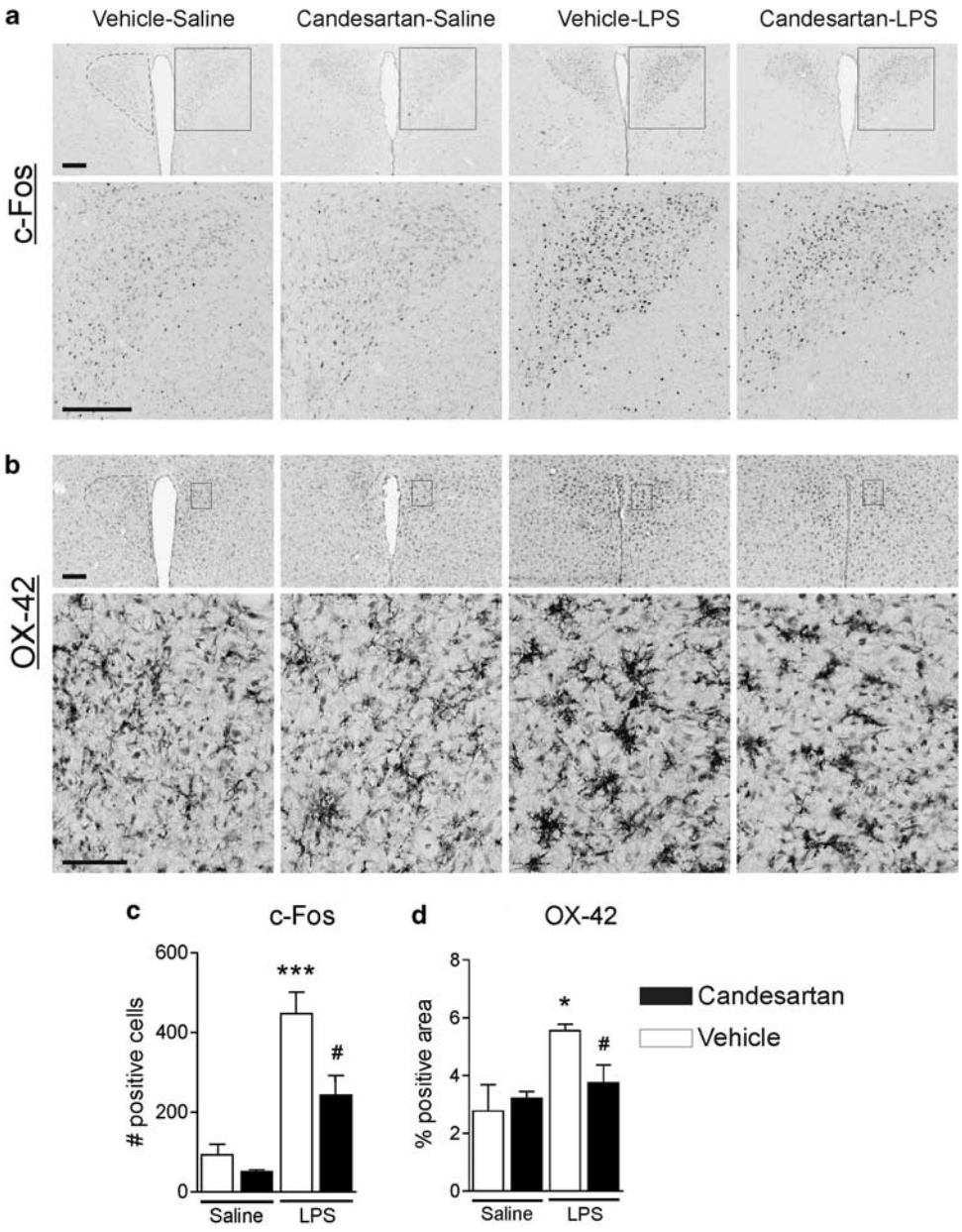

Figure 5 Angiotensin II AT_1 receptor blockade suppresses LPS-stimulated c-Fos induction and microglia activation in the PVN. Rats were treated with candesartan for 2 weeks followed by LPS administration as described in Materials and Methods. (a) Representative photomicrographs of c-Fos immunoreactivity in PVN. Rectangle area on the top image is magnified in the image below. Scale bar represents 200 μm. Dashed line outlines the area of PVN. (c) Quantitative analysis of c-Fos immunoreactivity in the PVN. Results are expressed as a number of c-Fos-positive cells per PVN section. (b) Representative photomicrographs of microglia in PVN stained with OX-42 antibody. Dashed line in the upper image outlines the area of PVN. Rectangle area in the upper image is magnified below. Scale bars represent 200 μm in the upper image and 50 μm in the lower image. (d) Quantitative analysis of microglia activation in PVN. An increase in the proportion of area positively stained with OX-42 antibody was used as a measure of microglia activation and is expressed as a percent of total area of PVN per section. Data are shown as means ± SEM ($n = 5$–8). $^{*}P < 0.05$, $^{***}P < 0.001$ compared with vehicle-saline group; $^{\#}P < 0.05$ compared with vehicle-LPS group.

AT_1 Receptor Blockade Ameliorates Anxiety in SHR

Anxiety-like behavior was determined by testing on an elevated plus maze. The time spent in the open arms and the number of entries into the open arms were not significantly affected by candesartan pre-treatment, although the consistent tendency toward the higher scores in the candesartan-treated groups was observed in both parameters (Figure 7b). The proportion of the open arm entries relative to the total number of entries was significantly increased by candesartan (Figure 7b) indicative of reduced anxiety.

DISCUSSION

Our results demonstrate widespread anti-inflammatory effects of Ang II AT_1 receptor blockade in the periphery and brain. Blockade of brain AT_1 receptors was achieved by

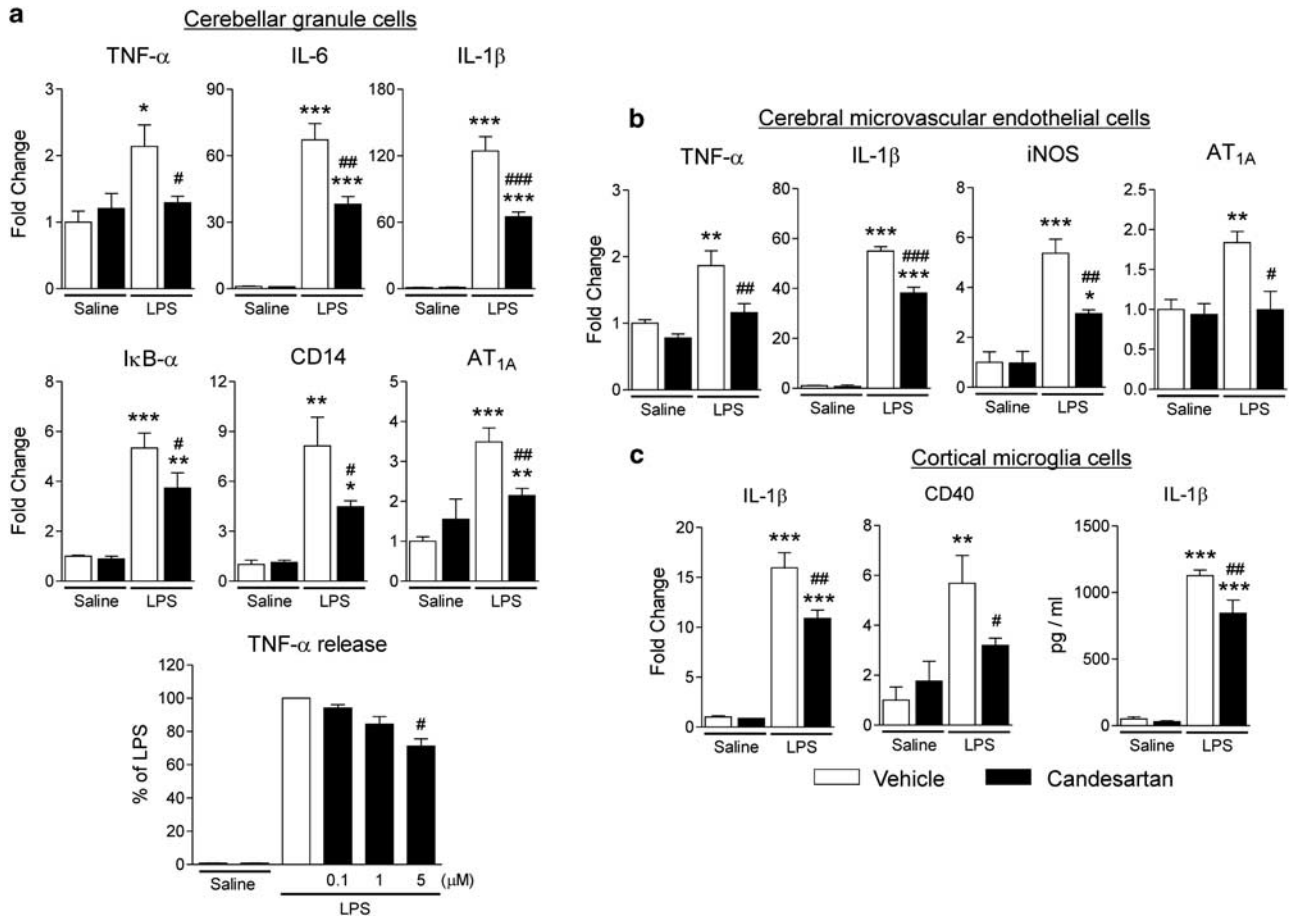

Figure 6 Angiotensin II AT_1 receptor blockade decreases LPS-induced inflammation markers in cerebellar granule cells (a), cerebral microvascular endothelial cells (b) and cortical microglia in culture (c). Cells were isolated and cultured as described in Materials and Methods. Expression of mRNAs was detected by real-time PCR and normalized to the level of GAPDH mRNA. Results are expressed as a fold change relative to vehicle-saline group. The release of TNF-α and IL-1β was determined by ELISA in the incubation media as described in Materials and Methods. Data are presented as means ± SEM of three independent experiments performed in duplicates. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with vehicle-saline group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared with vehicle-LPS group.

systemic administration of candesartan, an ARB with central AT_1 receptor blocking activity (present results; Nishimura *et al*, 2000; Sánchez-Lemus *et al*, 2009b) administered at a dose without major effects on systolic blood pressure in normotensive rats (Sánchez-Lemus *et al*, 2008). At this dose, candesartan prevented the consequences of not only of peripheral but also central Ang II-induced AT_1 receptor stimulation (Seltzer *et al*, 2004). The anti-inflammatory effects of AT_1 receptor blockade occurred both after short-term and sustained ARB pretreatment, indicating that the effects are not lost over time. At the early time point studied, LPS enhanced AT_{1A} receptor transcription in the PVN but not in the SFO (present results; Sánchez-Lemus *et al*, 2009b) and in cultured cerebral microvascular endothelial and cerebellar granule cells.

AT_1 receptor blockade reduced, as a consequence of effects on peripheral macrophages (Sánchez-Lemus *et al*, 2008, 2009a, b), the acute induction and release to the circulation of the major proinflammatory cytokines with direct actions in the brain without changing levels of the anti-inflammatory cytokine IL-10 (Maynard and Weaver, 2008). Candesartan decreased the LPS-induced formation and release of the proinflammatory hormone aldosterone and proinflammatory cytokines while preserving endotoxin-induced release of anti-inflammatory corticosterone (Sánchez-Lemus *et al*, 2008). This indicates that the overall peripheral effect of ARB pretreatment is to shift the balance in favor of anti-inflammatory factors in the circulation during the initial phase of the inflammatory response.

The ARB significantly reduced the inflammation-induced mRNA expression of proinflammatory cytokines and their receptors in all brain areas examined, a widespread effect not only restricted to previously identified inflammation target areas, such as the PVN and SFO, but also present in brain structures expressing AT_1 receptors (Jőhren *et al*, 1995; Lenkei *et al*, 1998) and involved in the regulation of emotion, cognition, and memory, such as the ventromedial prefrontal cortex, central nucleus of the amygdala, and CA1 subdivision of the hippocampus (McEwen, 2008).

AT_1 receptor blockade reduced inflammation-induced activation of transcription factors regulating expression of multiple inflammatory genes (Licinio and Wong, 1997; Quan and Banks, 2007), including c-Fos and FosB, members of the activator protein-1 (AP-1) transcription family, and NF-κB, visualized by IκB-α induction, which parallels NF-κB activation (Beg *et al*, 1993; Figure 8). This explains

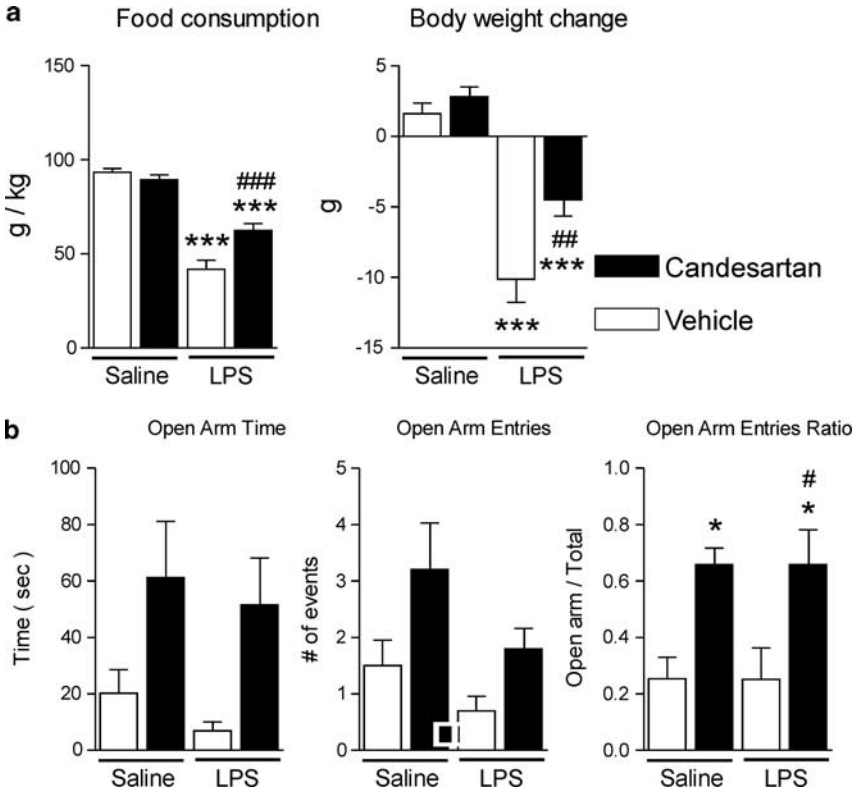

Figure 7 Candesartan ameliorates LPS-induced anorexia and weight loss in Wistar Hannover rats, and decreases anxiety-like behavior in SHRs. (a) Short-term candesartan and LPS were administered as described in Materials and Methods. Food consumption and body weight change were measured the day after LPS injection ($n = 8–10$). (b) Short-term candesartan and LPS were administered to SHRs as described in Materials and Methods. At 3 h after LPS injection, the rats were tested on an elevated plus maze for 5 min as described in Materials and Methods ($n = 9–10$). Data are expressed as means ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with vehicle-saline group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with vehicle-LPS group.

the inhibition not only of proinflammatory cytokines but also of enzymes, such as iNOS and COX-2, regulating production of inflammatory signals NO and prostanoids, such as PGE_2 (Figure 8). Our results are consistent with the established role of NF-κB as a major mediator of LPS effects in the brain (Pan *et al*, 2010), contradicting earlier observations reporting that intracerebral administration of LPS did not activate NF-κB or AP-1 in the brain (Watanabe *et al*, 2006).

Inhibition of gene expression of the PGE_2 receptor EP4 and many cytokine receptors reveals that the ARB limits not only the production of inflammatory mediators but also the capacity of the cellular targets to respond to inflammation. These results suggest the possibility that ARBs may also protect brain cells from other kinds of injury. For example, the EP4 receptor has been linked to production of amyloid β peptides characteristic of Alzheimer's disease (Hoshino *et al*, 2009), suggesting that its repression may be therapeutically beneficial.

ARBs exert brain anti-inflammatory effects by a number of parallel mechanisms (Figure 8). By decreasing circulating proinflammatory cytokines (present results; Sánchez-Lemus *et al*, 2008, 2009a, b), transcription of cytokine receptors, TLR4 (Dasu *et al*, 2009), and in particular CD14 (present results) in target areas, ARBs may reduce the capacity of the brain to recognize and respond to inflammatory factors evoked by the innate immune response. Our data suggest that the potent proinflammatory effects of Ang II in the periphery (Savoia and Schiffrin, 2007) and the brain (Zhou *et al*, 2005) involve sharing signal transduction pathways with inflammatory stimuli (Savoia and Schiffrin, 2007). Inflammation enhances brain AT_1 receptor transcription in the PVN and directly in cultured cerebellar granule cells, suggesting a clear link between inflammatory stimuli and Ang II. A reduction of Ang II-induced inflammatory cascades is an additional mechanism explaining the overall central anti-inflammatory effects of AT_1 receptor blockade.

Although the systemic administration of candesartan studied here effectively blocks both peripheral and brain AT_1 receptors and the effects of centrally administered Ang II (Nishimura *et al*, 2000; Seltzer *et al*, 2004), we cannot conclusively determine the relative role of central and peripheral AT_1 receptors in our model. To demonstrate that the changes observed in the brain may not exclusively depend on cross-system interactions, we studied the effects of LPS and AT_1 receptor blockade at the cellular level. We used primary cultures of isolated brain neurons, microglia, and endothelial cells, primary targets for circulating inflammatory cytokines and LPS (Quan and Banks, 2007). AT_1 receptor blockade exerted direct anti-inflammatory effects in a cerebellar cell culture highly enriched in granule cell neurons (Gao *et al*, 1995) and constitutively expressing AT_{1A} receptors, CD14, and TLR4. In addition, AT_1 receptor blockade reduced inflammation in cultured

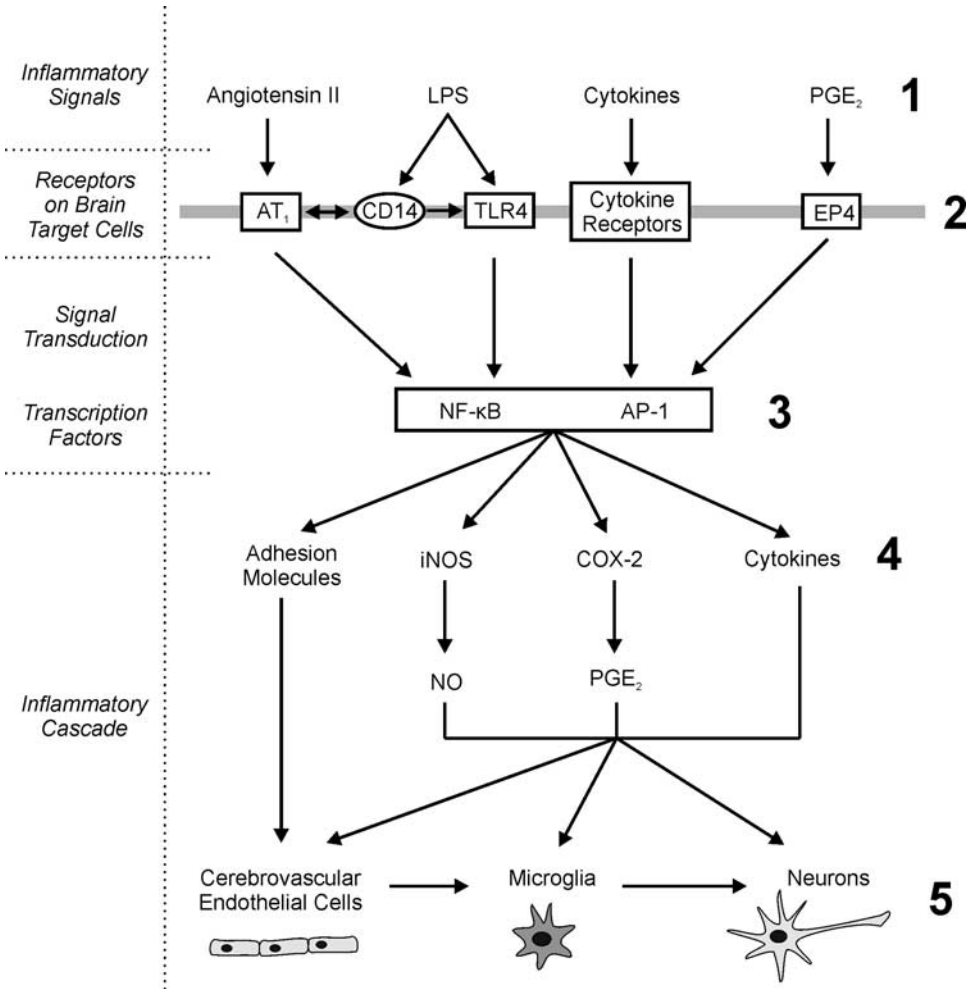

Figure 8 Angiotensin II and inflammatory signal pathways in the brain. AT_1 receptor activity is an important factor during brain inflammation. Angiotensin II and inflammatory stimuli interact, enhancing several common downstream signaling pathways. ARBs reduce inflammatory induction of peripheral cytokines and their release to the circulation (1). Inflammatory signals (1) induce transcription of angiotensin II AT_1, CD14, cytokine, and EP4 receptors in brain target cells (2). Angiotensin II AT_1 receptor blockade eliminates angiotensin II-stimulated downstream signaling and represses downstream transcription factors NF-κB and AP-1 (3). This limits inflammation-induced increase in brain inflammatory cascades: proinflammatory cytokines, cytokine and prostanoid receptors, inducible enzymes leading to PGE_2 and NO overproduction and adhesion molecules (4). In turn, this reduces microglia activation and neuronal injury in brain parenchyma (5) limiting the brain inflammatory reaction.

cerebral microvascular endothelial cells. These cells are involved in inflammatory leukocyte infiltration into the brain (Zhou *et al*, 2006; Man *et al*, 2007). Blockade of AT_1 receptors in cerebrovascular endothelial cells (Zhou *et al*, 2006) may reduce Ang II-mediated increase in blood brain barrier permeability and peripheral proinflammatory cell migration to the vasculature or brain parenchyma, explaining the beneficial effects of ARBs ameliorating brain inflammation in hypertension and diabetes (Fleegal-DeMotta *et al*, 2009). This anti-inflammatory activity is most likely sequentially replicated in adjacent neighboring microglia and neuronal cells. Stimulation of inflammatory cascades at endothelial target cells is followed by activation of microglia (Figure 8), the central resident immune cells (Henry *et al*, 2009), leading to induction of additional inflammatory cascades and ultimately producing, if unchecked, glial and neuronal damage (Henry *et al*, 2009). In particular, microglia activation increases LPS-induced neurotoxicity and consequently, the reduction of the proinflammatory microglia response enhances neuronal survival (Liu *et al*, 2010). We demonstrate here that the ARB candesartan inhibited the microglia activation marker CD40 and IL-1β mRNA expression and release in an enriched microglia cells culture. This is consistent with our finding of decreased microglia activation *in vivo* as a result of systemic candesartan administration, and it is in agreement with the previous observation of inhibition of inflammation-induced microglia activation, *in vitro*, by the ARB losartan (Miyoshi *et al*, 2008). Reduction of microglia activation might be another mechanism of ARBs promoting neuroprotection.

We report here direct anti-inflammatory effects of candesartan in LPS brain target cells. Our results do not exclude a major influence of peripheral anti-inflammatory effects in the candesartan-induced amelioration of brain inflammation. However, the *in vitro* experiments demonstrate that candesartan is able to directly and significantly reduce inflammation in LPS target cells in the brain. This suggests that direct central effects of candesartan may have a role in the amelioration of brain inflammation, which follows systemic administration of the ARB.

The central anti-inflammatory effects of the ARB have behavioral correlates as we demonstrate here. Candesartan reduced the acute sickness behavior produced by LPS, in particular its anorexic effect leading to acute weight loss (Konsman and Dantzer, 2001). In addition, candesartan ameliorated anxiety-like behavior in SHR, a strain with known hypersensitivity to stress and to LPS administration (Kvetnansky *et al*, 1979; Sirén *et al*, 1992). These effects were observed in both control and LPS-treated animals, indicating general anxiolytic properties that may be independent on the effect of LPS. Anxiolytic effects have been previously reported in normotensive rats exposed to prolonged treatment with candesartan (Saavedra *et al*, 2006), and both normotensive and hypertensive rats after acute treatment with another ARB, losartan (Srinivasan *et al*, 2003). Acute anxiolytic effects observed 5 min after intracerebroventricular injection of losartan (Kulakowska *et al*, 1996) further point to the direct involvement of brain Ang II AT_1 receptors in these behavioral changes.

In conclusion, our results demonstrate that direct brain inflammation, and peripheral inflammation affecting the brain are, to a significant degree, dependent on full AT_1 receptor function. ARBs limit, but do not eliminate the response of the brain to inflammatory signals. This is an important finding, as responding to inflammation is necessary to maintain and restore homeostasis, and pathological conditions may further develop as a consequence of unrestricted inflammation (Dantzer *et al*, 2008).

This report provides a basis to support recent findings in clinically relevant neuroinflammatory models. For example, brain RAS is upregulated in an animal model of multiple sclerosis, the experimental autoimmune encephalomyelitis, and ARBs ameliorate the characteristic paralysis and infiltration of inflammatory TH1 and TH17 cells in this model (Platten *et al*, 2009). Further evidence indicates that ARBs protect the brain from injuries promoting parenchymal inflammation and neuronal damage, such as irradiation-induced brain inflammation (Jenrow *et al*, 2010) and inflammation-induced dopaminergic neuronal death (Mertens *et al*, 2010).

Our results have major clinical significance. Excessive, uncontrolled inflammation is recognized as a major factor contributing to many psychiatric and neurological diseases, including but not limited to major depression and anxiety disorders, bipolar illness, schizophrenia, and neurodegenerative diseases such as Parkinson's and Alzheimer's disease (Dantzer *et al*, 2008; Doorduin *et al*, 2009; Glass *et al*, 2010; Fassbender *et al*, 2004; Hope *et al*, 2009; Miller *et al*, 2009). ARBs are compounds with excellent margin of safety, successfully used for years to treat not only increased systemic blood pressure but also to ameliorate the profound inflammatory component of peripheral cardiovascular disease and diabetes (Savoia and Schiffrin, 2007). We have previously found that ARBs reverse the cerebrovascular inflammation characteristic of genetic hypertension in an animal model (Ando *et al*, 2004; Zhou *et al*, 2005), and we demonstrate here that these compounds are very effective anti-inflammatory agents in the brain parenchyma of normotensive rats.

Our proposal to test ARBs for the therapy of inflammatory diseases of the brain is sustained by substantial clinical evidence indicating that ARBs are neuroprotective in humans (Anderson, 2010). ARBs prevent and ameliorate stroke in hypertensive patients to a greater degree than other similarly potent anti-hypertensive medications, in part as a consequence of effects beyond blood pressure control (Anderson, 2010). ARBs protect cognition both in hypertensive patients and in the elderly, significantly reducing the incidence and progression of Alzheimer's disease (Li *et al*, 2010). Other studies reported improvement in the quality of life, and amelioration of depression and stress (Phillips and de Oliveira, 2008). ARBs may represent a novel and safe approach for the treatment of acute and chronic neuropsychiatric disorders of increasing prevalence and socioeconomic impact where inflammation has a significant role.

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DISCLOSURE

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)