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Angiotensin II AT₁ receptor blocker candesartan prevents the fast up-regulation of cerebrocortical benzodiazepine-1 receptors induced by acute inflammatory and restraint stress

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Abstract

Centrally acting Angiotensin II AT₁ receptor blockers (ARBs) protect from stress-induced disorders and decrease anxiety in a model of inflammatory stress, the systemic injection of bacterial endotoxin lipopolysaccharide (LPS). In order to better understand the anxiolytic effect of ARBs, we treated rats with LPS (50 µg/kg) with or without three days of pretreatment with the ARB candesartan (1 mg/kg/day), and studied cortical benzodiazepine (BZ) and corticotrophin-releasing factor (CRF) receptors. We compared the cortical BZ and CRF receptors expression pattern induced by LPS with that produced in restraint stress. Inflammation stress produced a generalized increase in cortical BZ₁ receptors and reduced mRNA expression of the GABA_A receptor γ₂ subunit in cingulate cortex; changes were prevented by candesartan pretreatment. Moreover, restraint stress produced similar increases in cortical BZ₁ receptor binding, and candesartan prevented these changes. Treatment with candesartan alone increased cortical BZ₁ binding, and decreased γ₂ subunit mRNA expression in the cingulate cortex. Conversely, we did not find changes in CRF₁ receptor expression in any of the cortical areas studied, either after inflammation or restraint stress. Cortical CRF₂ receptor binding was undetectable, but CRF₂ mRNA expression was decreased by inflammation stress, a change prevented by candesartan. We conclude that stress promotes rapid and widespread changes in cortical BZ₁ receptor expression; and that the stress-induced BZ₁ receptor expression is under the control of AT₁ receptor activity. The results suggest that the anti-anxiety effect of ARBs may be associated with their capacity to regulate stress-induced alterations in cortical BZ₁ receptors.

Keywords

Angiotensin II AT₁ receptor blockers; Cortical benzodiazepine receptors; GABA_A receptor subunits; CRF receptors; Anxiety; Stress

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1. Introduction

Peripheral and brain Angiotensin II (Ang II) play active roles in the response to stress [1–2]. Through stimulation of AT₁ receptors, brain Ang II participates in the regulation of hypothalamic-pituitary-adrenal (HPA) axis and the central and peripheral sympathetic activation during stress [1–3]. Brain and peripheral AT₁ receptors are antagonized by Ang II AT₁ receptor blockers (ARBs). Systemic ARB treatment inhibits the hormonal and central sympathetic stimulation during isolation [4–6], prevents the production of stress-induced gastric ulcers, decreases the central sympathetic response during cold-restraint [7–8], and limits the HPA axis stimulation during inflammation stress induced by systemic injection of the bacterial endotoxin lipopolysaccharide (LPS) [3,9].

ARBs effects have been proposed to be at least in part the result of AT₁ receptor inhibition in cerebral cortex and other subcortical structures regulating both behavior and the hypothalamic reaction to stress [10–11]. In addition, systemic ARB administration reduces stress-driven anxiety in rodents [5,12] and ameliorates LPS-induced sickness behavior, a measure of inflammation-induced anxiety and depression in rodents [13].

The anti-anxiety effect of ARBs is associated with inhibition of isolation stress-induced alterations in cortical gamma amino butyric acid A (GABA_A) and corticotrophin-releasing factor (CRF) systems [5], two major regulatory factors of the behavioral response to stress [14–17]. Sustained treatment with the ARB candesartan completely prevented the decrease in cortical CRF₁ receptor and BZ₁ binding produced by isolation stress [5]. These results suggested that a modulation of upstream neurotransmission processes regulating cortical CRF₁ receptors and the GABA_A complex were molecular mechanisms at least in part responsible for the anti-anxiety effect of centrally acting AT₁ receptor antagonists.

While this is valuable information, it does not clarify whether the effects of ARBs on CRF₁ receptors and the GABA_A complex are limited to the stress of isolation or are part of a more general anti-stress effect. The initial hypothesis of a unitary “stress syndrome” has been challenged [18–20]. Each stressor is associated with different brain neurochemical “signatures” depending on the particular challenge to homeostasis, the age, previous experience (which influences adaptation mechanisms), and the nature, intensity and duration of the stress [18–20]. For this reason it is essential to determine if the effect of ARBs extends to other stressors, indicating a more general anti-stress effect. Isolation is a social and psychological stressor [5]. Two very different stressors were selected for this study [20–22]: restraint, a physical stressor with a strong psychological component; and LPS-induced inflammation, a particular type of physical stressor that represents an immediate threat and is associated with anxiety and depression, behavioral changes that are prevented by candesartan treatment [13]. The aim of this study was to clarify whether the effects of candesartan on cortical CRF₁ receptors and the GABA_A complex were limited to isolation stress or part of a more general anti-stress effect extending to very different stressors.

2. Material and Methods

2.1 Animals

Eight weeks old male Wistar Hannover rats initially weighing about 250 g were used (Taconic Farms; Germantown, NY, USA). Upon its arrival, animals were acclimatized to our animal facility for one week. Rats 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 cycles of light and dark (lights on at 7:00 AM). A total of 48 rats were used for LPS treatment and 32 for restraint stress. The National Institute of Mental Health Animal Care and Use Committee (Bethesda, MD) approved all procedures. All efforts were made to

minimize the number of animals used and their suffering according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, NIH Publication No. 80-23, revised 1996.

2.2 Drugs

Candesartan (Astra-Zeneca, Mölndal, Sweden) was dissolved in 0.1 N Na₂CO₃ and then diluted in isotonic saline at a final pH of 7.5–8.0; this solution was protected from light and stored at 4°C. The ARB was administered s.c. at 1 mg/kg/day. At this dose, candesartan effectively blocks peripheral and central AT₁ receptors [3,9,13]. This AT₁ blockade is associated with a small decrease in the blood pressure, that nevertheless continue within the normal range [9]. Enough drug was prepared to provide the three injections required per experiment. Fresh drug was prepared for each experiment.

LPS (*Escherichia coli* serotype 055:B5; Sigma Chemical Co., St Louis, MO, USA) was dissolved in sterile saline and administered i.p. at 50 µg/kg; we have found that at this dose LPS produces brain and peripheral inflammation as well as sickness behavior response [3,13].

2.2 Experimental protocol

2.2.1 Inflammation stress—Animals were divided into four groups: vehicle-saline (n=5), candesartan-saline (n=5), vehicle-LPS (n=7) and candesartan-LPS (n=7). For 3 consecutive days at 9:00 AM, rats received s.c. injections of vehicle or 1 mg/kg/day of candesartan. On day 3 starting at 10:00 AM, rats received an i.p. injection of sterile saline or LPS 50 µg/kg. Three hours after saline/LPS injection, animals were removed from their cages and taken to a different room to be killed by fast decapitation between 1:00 PM to 2:30 PM). Trunk blood was collected in polyethylene tubes with EDTA (1 mg/ml blood). Plasma was obtained by centrifugation at 1800 × g, 15 min at 4°C and it was stored at –80°C until used. Brains were frozen in cold isopentane and stored at –80°C until used. This strategy was repeated in another set of animals; the first set of brains was used for BZ binding autoradiography and the second for real-time PCR after selective dissection of cingulate cortex.

2.2.2 Restraint stress—Rats were divided into four groups of eight animals each: vehicle-control (not stressed), vehicle-restraint, candesartan-control and candesartan-restraint. Candesartan was injected (s.c. 1 mg/kg/day) at 9:00 AM, daily for three days. After treatment, half of vehicle- and half of candesartan-treated rats were randomly selected and individually housed in restrainers (model 82, IITC.INC, Woodland Hills, CA) for 2 hours starting at 9:30 AM. Not stressed rats were left undisturbed in their cages. Stressed and control rats were killed by decapitation between 11:30 AM to 1:30 PM. Plasma and brains obtained were stored at –80°C until used.

2.3 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.

2.4 BZ receptors binding autoradiography

Fresh-frozen brains were sliced in a cryostat at –20°C. Sixteen µm thick coronal sections were thaw mounted on positively charged microslides and stored at –80°C until used (Daigger, Vernon Hills, IL). BZ binding sites were determined as previously described [23] with slight modifications. Brain sections were preincubated for 30 min at 4°C in freshly

prepared 170mM Tris-HCl, pH 7.4 buffer, followed by incubation for 60 min at 4°C in assay buffer containing 3 nM of the BZ agonist [³H]-flunitrazepam (71.0 Ci/mmol; Perkin Elmer, Boston, MA), a drug that binds to GABA_A α₁, α₂, α₃ and α₅ containing receptors. Non-specific binding was measured in consecutive sections co-incubated with the antagonist clonazepam 1 μM. A third set of slides was co-incubated with zolpidem 1 μM, an agonist that preferentially binds to GABA_A α₁-γ₂ containing receptors (resembling the pharmacologically described BZ₁ receptor) and with less affinity to GABA_A α₂ and α₃ containing receptors and no affinity for GABA_A α₅-γ₂ containing receptors (resembling the BZ₂ receptor; [24–26]. After incubation, sections were washed twice for 2 min in incubation buffer at 4°C and dipped once in ice-cold distilled water. Slides were dried and exposed to Kodak Biomax MR films for 2 weeks together with ³H-labeled standards. The films were developed in GBX developer (Eastman Kodak Company, Rochester, NY) for 4 min, fixed in Kodak GBX fixer for 4 min, and rinsed in water for 5 min.

Optical densities of autoradiograms were quantified by comparison with ³H-labeled standards (American Radiolabeled Chemicals, St Louis, MO) using the Scion Image Program 4.0.2 (Scion Corporation, Frederick, MD) based on the NIH Image Program of the National Institutes of Health. Optical densities were then transformed to the corresponding values of fmol/mg.

2.5 CRF receptors binding autoradiography

Sixteen μm thick coronal sections were cut and thaw mounted on positively charged microslides (Daigger). CRF receptors were label with [¹²⁵I]-sauvagine, a highly specific agonist for both CRF₁ and CRF₂ receptors. [¹²⁵I]-sauvagine binding was performed as earlier reported [27] with some modifications. Sections were pre-incubated twice for 10 min in 50 mM Tris-HCl pH 7.4 containing 10 mM MgCl₂ and 2 mM EGTA, followed by incubation for 120 min at room temperature in the same buffer plus 0.1 % BSA, 0.05 % bacitracin, 0.04 TIU/ml aprotinin and 0.15 nM [¹²⁵I]-sauvagine (Perkin-Elmer, Boston, MA, specific activity 2200 Ci/mmol). Two sets of adjacent section were used to quantify the receptor subtypes. Non-specific binding was defined by co-incubation with astressin 100 nM (Sigma). CRF₁ receptors were defined as the [¹²⁵I]-sauvagine binding displaced by 130 nM of the selective CRF₁ receptor antagonist antalarmin. CRF₂ receptors were defined as the [¹²⁵I]-sauvagine binding not displaced by antalarmin but displaced by astressin. Following the incubation period, slides were washed twice, 5 min each, in 50 mM Tris-HCl pH 7.4 containing 0.01% Triton X-100 at 4°C. Slides were deep once in distilled water and immediately dried under cold air. The slides were exposed to Kodak Biomax MR films (Eastman Kodak Company) for 3 days together with [¹⁴C] standards, developed and quantified.

Cerebral areas for both BZ and CRF receptor quantification were defined according to Paxinos and Watson [28].

2.6 Measurement of mRNA expression by real-time PCR

Cingulate cortex areas 1 and 2 were microdissected from 350 μm coronal brain sections. Punch microdissection was performed under stereomicroscope control using a 1.25 mm Harris Uni-Core microdissection needle. Up to four punches per brain were performed in sections between −0.2 mm to −1.5 mm from bregma [28]. Obtained tissues were store at −80°C until used.

Total RNA was isolated individually from homogenized cingulate cortex samples using 400 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 0.2 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), 3 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 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 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 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as arbitrary units relative to GAPDH. The expression of GAPDH was not affected by any of the treatments used (results not shown).

2.7 Statistical analysis

All results are expressed as means $\pm$ SEM, for groups of animals measured individually. One-way ANOVA and post-hoc Newman-Keuls' test were used to assess the significance of differences. $P < 0.05$ was considered as statistically significant. All statistics were performed with the use of Prism 5.02 software (GraphPad Software for Science, San Diego, CA, USA).

3. Results

3.1 Plasma hormones

Three days candesartan treatment (1 mg/kg/day) did not affect basal adenocorticotrophic hormone (ACTH) but significantly increased basal corticosterone plasma levels (Fig. 1). Plasma stress hormones were increased by three hours treatment with LPS (50 µg/kg i.p.): ACTH was more than 6-fold increased and corticosterone about 3-fold (Fig. 1A). Candesartan pre-treatment significantly reduced the LPS-induced ACTH plasma levels but it did not change that of corticosterone (Fig. 1A). Identical pattern of results was found for restraint stress (Fig. 1B).

3.2 Cortical BZ receptors

BZ receptor binding was quantified in cortical subdivisions and layers as described by Paxinos and Watson [28] (Fig. 2A). Four tiers were clearly visible on cortical BZ binding corresponding to layers 1–3, layer 4, layer 5 and layer 6 (Fig. 2B). BZ₁ receptor was the predominant subtype in the rat cortex with just a modest amount of BZ₂ subtype that was not statistically different to the non-specific binding (Fig 2B).

BZ₁ receptor binding was increased by LPS administration in almost all cortical areas and layers measured with only few exceptions where the increase in binding did not reach statistical significance. These exceptions were somatosensory cortex 2 layer 4, insular cortex layers 1–3, insular cortex layer 5, and agranular insular cortex (Fig. 3). Candesartan alone increased basal BZ₁ receptor binding in cingulate cortex area 2, and candesartan pre-treatment significantly prevented the generalized LPS-induced BZ₁ receptor binding increment (Fig. 3).

Restraint stress presented identical pattern of response. Restraint stress increased BZ₁ receptor binding; candesartan alone increased BZ₁ binding in cingulate cortex area 2, somatosensory cortex 1 layers 5 and 6, somatosensory cortex 2 layers 1–3, 4 and 6 and in all layers of insular cortex; and pre-treatment with candesartan prevented restraint-induced BZ₁ increase (Fig. 4).

3.3 GABA_A receptor subunits in cingulate cortex

Based in the binding results, cingulate cortex was selected as a representative area of the observed cortical changes and it was used to analyze gene expression in the LPS model.

Neither LPS nor candesartan nor their combination produced significant changes on mRNA expression of GABA_A α_1 and GABA_A β_2 subunits in cingulate cortex (Fig. 5). The GABA_A γ_2 subunit was down-regulated by both LPS and candesartan when administered alone (Fig. 5). Pretreatment with candesartan prevented the LPS-induced decrease in GABA_A γ_2 mRNA expression, and up-regulated GABA_A α_2 subunit expression (Fig. 5).

3.4 Cortical CRF receptors

Three cortical tiers were obvious on cortical CRF receptors binding corresponding to layers 1–3, layer 4 and layer 5–6 [28] (Fig 2A and 2C). CRF₁ receptors were the predominant subtype in the cerebral cortex with non-detectable binding for CRF₂ receptors (Fig 2C). CRF₁ binding was not significantly affected by any of the treatments either in immune (Table 2) or in restraint stress (not shown).

3.5 CRF receptor subtype mRNA in cingulate cortex

Expression of CRF₁ receptor mRNA was not changed by any of the treatments in the inflammatory stress model. On the other hand, CRF₂ mRNA expression was down-regulated by LPS administration (Fig. 6); and candesartan alone did not change CRF₂ receptor expression but it prevented LPS-induced CRF₂ down-regulation (Fig. 6).

4. Discussion

The main findings of the present study are that brain AT₁ receptor participates in the regulation of the cerebral cortical GABA_A system in control, non-stressed rats; and that AT₁ receptor blockade prevents the stress-induced alteration of cortical GABA_A complex.

Candesartan administration produced significant hormonal effects in inflammatory and restraint stress, further proof of AT₁ receptor regulation of the HPA axis response to stress [3,4,29]. While in isolation stress candesartan inhibits both the ACTH and corticosterone responses [4], in inflammation and restraint stress the ARB decreases ACTH release but does not affect corticosterone release (present results and [3,9]).

In addition, administration of candesartan alone enhanced basal corticosterone levels, an apparent paradoxical response, which is, however, in agreement with previous observations [3,6]. Candesartan decreases adrenal corticosterone content [6], and this may be interpreted as the result of increased corticosterone release. These observations indicate that enhanced corticosterone levels after candesartan administration may be mediated by direct effects on the adrenal gland, and that adrenal and/or circulating Ang II might inhibit basal corticosterone release. The report that Ang II decreases corticosterone production in adrenal cell cultures supports this hypothesis [30].

By contrast, Ang II infusion was reported to increase glucocorticoid release [31]. However, the amounts infused, 9 μ g/hour, may result in concentrations many times higher than normal circulating Ang II levels [32], and the effect occurs only in the obese Zucker, but not in lean control rats [31].

On the other hand, other studies do not support an interaction of the Ang II with corticosterone. In a maternal separation stress model, candesartan potentiates ACTH release but does not affect corticosterone [33]. In Spontaneously Hypertensive Rats, a model with over-stimulated Renin-Angiotensin System, ARBs do not affect basal corticosterone levels

[34]. In A-ZIP/F-1 transgenic mice, ARBs do not reduce the elevated corticosterone levels characteristic of this model [35]. The conclusion is that the effect of ARBs and Ang II on corticosterone levels depends on the model studied and the conditions of the experiments, and the precise mechanism of physiological control has yet to be elucidated.,

It is well established that the GABA_A complex is rapidly affected by acute stressors, indication of fast system plasticity. BZ receptors in the rat cortex are acutely modulated by cold swim, handling and isolation stress [5,36,37]. Their expression and stimulation is a major factor determining the GABA_A complex activation and its participation in the behavioral response to stress and anxiety [14–16]. Acute stressors like restraint, infection, hypoxia or combined mild stressors influence the GABA_A complex at two different levels: altering BZ₁ binding sites and modulating the expression of selective GABA_A receptor subunits [38–41]. Changes in GABA_A receptor subunits expression induced by stress will then promote differential clustering of selective subunits conditioning the receptor activity and its participation in stress and anxiety [25,42–46]. While the GABA_A γ_2 subunit is essential for the synaptic clustering of the GABA_A complex [39,41,47], anti-anxiety effects are commonly linked to receptors highly expressing the GABA_A α_2 subunits [42,43,48].

This study reveals fast, significant and widespread increases in cortical BZ₁ binding after acute restraint or inflammatory stress. In addition, inflammatory stress significantly decreases the mRNA expression of the GABA_A γ_2 subunit in the cingulate cortex, without affecting the α_1 , α_2 and β_2 subunits. These results support the hypothesis that the cortical GABA_A system regulates the response to stress [16,17,49]. While acute reduction of GABA_A γ_2 subunit may indicate decreased clustering of the GABA_A receptor complex and decreased inhibitory activity leading to anxiety [50], increases in BZ₁ binding should be interpreted as possible compensatory mechanisms to maintain the GABA_A complex function during stress.

Reducing the normal level of brain AT₁ receptor activation in non-stressed rats by administration of candesartan alone significantly enhanced cortical BZ₁ binding, increased cortical GABA_A α_2 subunit mRNA expression and reduced the expression of γ_2 subunit mRNA. Increased BZ₁ binding and α_2 subunit mRNA expression, which is associated with the anti-anxiety properties of the GABA_A complex [48] may explain why candesartan administration reduces anxiety in control rats unchallenged by stress [5,13]. The apparently contradictory decrease of γ_2 subunit mRNA as a result of candesartan treatment may possibly be interpreted as a compensatory mechanism to maintain the functional stability of the GABA_A complex.

In this study, systemic candesartan administration is very effective in preventing the consequences of inflammatory and restraint stress on the cortical GABA_A complex, abolishing the effects of both stress stimuli on cortical BZ₁ binding, and reversing the LPS-induced decrease in γ_2 subunit mRNA expression. Thus, candesartan eliminates the effect of inflammatory and restraint stress in the cortical GABA_A complex, facilitating the return of normal BZ₁ expression and restoring proper γ_2 subunit mRNA expression to control levels during the response to stress. Overall, we interpret the effects of candesartan as stimulation of adaptive mechanisms aiming to restore the resting inhibitory GABA transmission [43,47,51].

It must be noted that both inflammatory and restraint stress produced significant increases in BZ₁ binding, alterations in opposite direction from the significant decrease in BZ₁ binding we earlier observed after isolation stress [5]. Our experience is in agreement with the literature, since it has been reported that alterations in both BZ receptor and GABA_A subunit

expression after stress are not consistent and are strongly influenced by gender, intensity and length of stress, kind of stressor and are dependent on the area studied [36,37,40,41,52].

Nevertheless, the inhibitory effect of ARB treatment on the stress-induced cortical alterations in the GABA_A complex is shared across a variety of stress types: a) unpredictable restraint, a psychological stress perceived as a life threatening event where the body responds in anticipation to possible homeostatic needs [20]; b) systemic LPS administration, an immediate threat strongly activating the innate immune system and producing a robust inflammatory reaction associated with emotional responses characterized by sickness behavior [20–22]; c) isolation, a negative emotional stimulus representing a significant loss of normal social interaction [4–6]. In all cases, ARBs prevent stress-induced changes in the cortical GABA_A system, and these effects occur whether BZ₁ binding is decreased (isolation, [5]) or increased (inflammation and restraint, [present results]).

Common biological and molecular pathways may explain the similar effect of AT₁ receptor blockade on cortical benzodiazepine receptors during acute inflammatory and restraint stress. There is a close and inverse interaction between AT₁ receptor and GABA_A activity in the brain. AT₁ receptor activation directly inhibits GABAergic transmission and GABA_A function controls the effects of AT₁ receptor stimulation [53–56]. A similar direct control of GABA_A activity may occur in the cerebral cortex, as a consequence of activation of local AT₁ receptors [57].

In addition, regulation of GABA_A function by AT₁ receptor blockade may be indirect, the result of regulation of upstream molecular pathway(s) common to all the stress types studied. The hypothalamic paraventricular nucleus (PVN) is a major regulator of the hormonal and sympathetic response to stress [58]. In PVN, both inflammatory and restraint stress up-regulate AT₁ receptors [3,59] and inducible pro-inflammatory enzymes such as COX-2 and iNOS [60,13]. Inflammation and enhanced AT₁ receptor stimulation directly decrease GABA_A transmission desinhibiting PVN sympathetic activity and outflow which ultimately promotes the stress-induced sympathetic response [53,56,60,61]. In turn, enhanced central sympathetic activation plays a major role in the regulation of cortical GABA_A function and leads to anxiety [62,63]. Thus, ARBs may prevent stress-induced alterations in cortical GABA_A function also as a consequence of decreased inflammation in the PVN, preserving GABA_A inhibitory effects and reducing central sympathetic activation.

Conversely, none of the experimental conditions altered cortical CRF₁ receptor binding, the principal CRF receptor expressed in rat cortex [20,64,65]. This contrasts with the clear decrease in cortical CRF₁ receptor expression reported after isolation stress and with the prevention of such alterations by treatment with candesartan [5]. It is well established that activation of CRF₁ receptors promotes anxiety-like behavior [20,66,67]. These results may be related to the intensity, length and type of stress studied, since alterations in CRF binding are dependent on the characteristics of the stress applied [68].

In agreement with previous observations, we have not detected significant levels of cortical CRF₂ receptor binding [64,65], but we found clear expression of CRF₂ mRNA in cingulate cortical samples [69]. Activation of CRF₂ receptors has been proposed as antagonistic to CRF₁ stimulation [20,66,67], and CRF₂ receptor knock-out mice show increased anxiety behavior and higher sensitivity to acute stress [70–72]. In addition, central CRF₂ receptors regulate the neuronal activation of limbic areas, including cingulate cortex, in an anxiety model [73,74]. We hypothesize that prevention of CRF₂ receptor down-regulation by candesartan might be an additional mechanism explaining the anxiolytic and anti-stress effects observed with ARBs [75].

4.1 Conclusions

We conclude that cortical BZ₁ receptor expression is under AT₁ receptor control, and that very different stressors promote rapid and widespread changes in cortical BZ₁ receptor expression. While the direction of the changes depends on the particular challenge to homeostasis, candesartan treatment prevents the stress-induced alterations in BZ₁ receptor expression.

Conversely, the effects of candesartan in cortical CRF₁ receptor expression appear to be stress-specific, and not essential for the anxiolytic effects of ARBs.

Overall, our results and earlier studies suggest that ARBs prevent the alterations in the cortical GABA_A complex in very different stress models. The precise molecular mechanism for ARBs effect remains to be clarified; however, we propose that ARBs may affect cortical GABA_A receptors by modulating AT₁-GABA_A interactions in cortex and/or by regulating hypothalamic PVN sympathetic system. Nevertheless, cortical GABA_A regulation might be a common mechanism of ARBs associated with their anxiolytic effect. Since humans are often submitted to combinations of stressors, the present results provide additional and necessary information in support of the translational value of the studies.

Our results have important clinical implications. Abnormal responses to stress with alterations in the GABA_A complex response are a vulnerability factor for mood disorders like anxiety and depression [20,43,49,50,76]. We demonstrated, in pre-clinical rodent models, that ARB administration prevents development of stress-induced gastric ulcers, decreases anxiety and significantly reduced inflammation-induced sickness behavior [5,7,13]. In diabetic patients, candesartan administration resets the HPA-axis and ameliorates their depressive symptoms [77]. Furthermore, the Renin-Angiotensin system is activated in subjects living alone with depressive symptomatology [78]. Based on our results and those of the literature, we suggest that treatment with ARBs may be effective on a wide range of brain conditions and mood disorders where abnormal responses to stress play a significant role.

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Highlights

Acute stress promptly increases benzodiazepine-1 receptors in cerebral cortex

Candesartan prevents GABA_A receptor plasticity during stress

Candesartan prevents cortical CRF₂ gene expression during stress

Angiotensin II AT₁ receptors regulate stress and anxiety

Candesartan decreases anxiety

Figure 1. Plasma stress hormones in the LPS and restraint stress models

Animals pretreated with vehicle or ARB candesartan (1 mg/kg/day) were injected with LPS (50 µg/kg) for three hours (A) or subjected to restraint for two hours (B); control groups were either injected with sterile saline (A) or kept undisturbed in their home cages (B). Trunk blood was collected after decapitation. Values represent means ± SEM from groups of 7–9 rats measured individually. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to vehicle-control; # $p < 0.05$ as compared to vehicle-stress; one-way ANOVA and Newman-Keuls post-hoc test.

Figure 2. Cortical BZ and CRF receptors binding summary

A) Scheme of the rat brain coronal section showing the cortical areas where BZ and CRF receptors were measured. B) Representative pictures for BZ receptors binding; total binding ($BZ_1 + BZ_2$), binding after zolpidem displacement (BZ_2) and binding after clonazepam displacement (non specific). C) Representative pictures for CRF receptors binding; total binding ($CRF_1 + CRF_2$), binding after antalarmin displacement (CRF_2) and binding after astressin displacement (non specific). Cg1 and Cg2, cingulate cortex area 1 and 2; M2/M1, primary and secondary motor cortex; Ss1 and Ss2, somatosensory cortex 1 and 2; Ins, insular cortex; AI, agranular insular cortex; 1–6, cortical layers 1 to 6; LV, lateral ventricle; CPu, striatum.

Figure 3. Inflammation stress and BZ₁ receptor binding in the rat brain cortex

Animals pretreated with vehicle or ARB candesartan (1 mg/kg/day) were injected with sterile saline or LPS (50 µg/kg) for three hours. Values represent means ± SEM from groups of 7–9 rats measured individually. * p<0.05, ** p<0.01, *** p<0.001 as compared to vehicle-saline; # p<0.05 as compared to vehicle-LPS; one-way ANOVA and Newman-Keuls post-hoc test.

Figure 4. Restraint stress and BZ₁ receptor binding in the rat brain cortex

Animals pretreated with vehicle or ARB candesartan (1 mg/kg/day) were restraint for two hours or kept undisturbed in their home cages (control). Values represent means $\pm$ SEM from groups of 8 rats measured individually. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to vehicle-control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ as compared to vehicle-restraint; one-way ANOVA and Newman-Keuls post-hoc test.

Figure 5. mRNA expression of GABA_A receptor subunits in cingulate cortex after inflammation stress

Animals pretreated with vehicle or ARB candesartan (1 mg/kg/day) were injected with sterile saline or LPS (50 µg/kg) for three hours. Values represent means ± SEM from groups of 5–7 rats measured individually. * p<0.05 as compared to vehicle-saline; # p<0.05 as compared to vehicle-LPS; one-way ANOVA and Newman-Keuls post-hoc test.

Figure 6. mRNA expression of CRF receptors in cingulate cortex after inflammation stress
 Animals pretreated with vehicle or ARB candesartan (1 mg/kg/day) were injected with sterile saline or LPS (50 µg/kg) for three hours. Values represent means ± SEM from groups of 5–7 rats measured individually. * $p < 0.05$ as compared to vehicle-saline; ## $p < 0.01$ as compared to vehicle-LPS; one-way ANOVA and Newman-Keuls post-hoc test.

Table 1**Primers used for real-time PCR**

Gene	Forward (5' – 3')
	Reverse (5' – 3')
CRF ₁ receptor	TGCATCCGTGGACCTCATTG
	GGTAGCCATTGTTTGTCTGTTGT
CRF ₂ receptor	TGCCGCTGCGTCACCACCATA
	AGCAGCCTTCCACAAACATCCAGA
GABA _A α ₁ subunit	ACCAGGCTTGGGAGAGCGTGT
	CGTGGTCTGACACGGGTCCG
GABA _A α ₂ subunit	GCCAGAGAACAAGCCAGCCGA
	ACCCCTAATACAGGCTCCCGGT
GABA _A β ₂ subunit	GACCCCAGGAGCACAATGCTTGC
	CCACATGTCGTTCCAGGGCGT
GABA _A γ ₂ subunit	GCGGATGCTCACTGGATCACGA
	TGCAACTGGCACTCGGCATCA
GAPDH	ATGACTCTACCCACGGCAAG
	TGGAAGATGGTGATGGGTTT

Table 2
CRF₁ receptor binding after inflammation stress

Animals pretreated with vehicle or ARB candesartan (1 mg/kg/day) were injected with LPS (50 µg/kg) for three hours. Values are expressed as fmol/mg protein and represent means ± SEM from groups of 5–7 rats measured individually.

Cortical area	Vehicle-Saline	Candesartan-Saline	Vehicle-LPS	Candesartan-LPS
Cingulate cortex Area 1 and 2	3.0 ± 0.3	3.4 ± 0.3	2.9 ± 0.3	3.0 ± 0.2
Primary & Secondary Motor cortex	1.6 ± 0.1	1.9 ± 0.2	1.7 ± 0.2	1.7 ± 0.2
Somatosensory cortex Layer 1–3	3.3 ± 0.5	3.4 ± 0.3	2.7 ± 0.3	2.7 ± 0.1
Somatosensory cortex Layer 4	4.2 ± 0.5	4.7 ± 0.6	4.2 ± 0.4	4.2 ± 0.2
Somatosensory cortex Layer 5–6	1.5 ± 0.1	1.6 ± 0.2	1.3 ± 0.1	1.3 ± 0.1
Insular cortex Layer 1–3	2.6 ± 0.2	2.8 ± 0.4	2.5 ± 0.3	2.6 ± 0.2
Insular cortex Layer 4	4.7 ± 0.2	5.3 ± 0.7	4.7 ± 0.5	4.8 ± 0.2
Insular cortex Layer 5–6	1.8 ± 0.1	1.9 ± 0.2	1.6 ± 0.2	1.8 ± 0.1