Stress sensitivity is increased in transgenic rats with low brain angiotensinogen

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Abstract

AT₁ blockers attenuate hypothalamo–pituitary–adrenal (HPA) axis reactivity in hypertension independently of their potency to lower blood pressure. A reduced pituitary sensitivity to CRH and a downregulation of hypothalamic CRH expression have been suggested to influence HPA axis activity during chronic AT₁ blockade. This study was aimed at confirming the role of central angiotensin II in regulating HPA reactivity by using the transgenic rat TGR(ASrAOGEN), a model featuring low levels of brain angiotensinogen. Different stress tests were performed to determine HPA reactivity in TGR(ASrAOGEN) and appropriate controls. In TGR(ASrAOGEN), blood pressure was diminished compared to controls. The corticosterone response to a CRH or ACTH challenge and a forced swim test was more distinct in TGR(ASrAOGEN) than it was in controls and occurred independently of a concurrent enhancement in ACTH. Using quantitative real-time PCR, we found

increased mRNA levels of melanocortin 2 (*Mc2r*) and AT₂ receptors (*Agtr2*) in the adrenals of TGR(ASrAOGEN), whereas mRNA levels of *Crh*, *Pomc*, and AT₁ receptors (*Agtr1*) remained unchanged in hypothalami and pituitary glands. Since stress responses were increased rather than attenuated in TGR(ASrAOGEN), we conclude that the reduced HPA reactivity during AT₁ blockade could not be mimicked in a specific transgenic rat model featuring a centrally inactivated renin–angiotensin–aldosterone system. The ACTH independency of the enhanced corticosterone release during CRH test and the enhanced corticosterone response to ACTH rather indicates an adrenal mechanism. The upregulation of adrenal MC2 and AT₂ receptors seems to be involved in the stimulated facilitation of adrenal corticosterone release for effectuating the stimulated stress responses.

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Introduction

Over the last few years, it has been established that angiotensin II (Ang II) AT receptors (AGTR as listed in the MGI Database) are present in the organs of the hypothalamo–pituitary–adrenal (HPA) axis (Burson *et al.* 1994, Gasc *et al.* 1994, Llorens-Cortes *et al.* 1994, Jöhren *et al.* 1995, Jöhren & Saavedra 1996), that they are regulated during stress (Castren & Saavedra 1988, Aguilera *et al.* 1995, Leong *et al.* 2002), and that Ang II influences HPA axis reactivity by enhancing the synthesis and secretion of CRH, ACTH, and glucocorticoids (Rivier & Vale 1983, Abou-Samra *et al.* 1986, Schoenberg *et al.* 1987, Sumitomo *et al.* 1991, Naville *et al.* 1993, Jezova *et al.* 1998). This crosstalk between the renin–angiotensin–aldosterone system (RAAS) and the HPA axis is functionally relevant for hypertension. In the transgenic rat TGR(mREN2)27 (which develops a fulminant hypertension as a result of the integrated mouse Ren-2d renin gene into its genome), the urinary excretion of corticosterone is increased

during the developmental phase of hypertension, the adrenal response to ACTH is stimulated (Sander *et al.* 1992), and hypertension can be prevented when HPA reactivity is suppressed by dexamethasone (Djavidani *et al.* 1995). In spontaneously hypertensive rats (SHR), the Ang II-induced release of ACTH and corticosterone is stimulated compared to normotensive WKY rats, an observation that has been linked to a higher expression of AT_{1A} receptors (*Agtr1a*) in the pituitary gland (Jöhren *et al.* 2003). From a therapeutic viewpoint, chronic AT₁ receptor blockade diminishes HPA reactivity in rats in a blood pressure-independent manner (Seltzer *et al.* 2004, Raasch *et al.* 2006). Reductions in HPA reactivity induced by AT₁ blockade have been suggested to mediate metabolic benefits, since on the one hand the HPA axis is hyperreactive in diabetes (Chan *et al.* 2002, 2003, Jöhren *et al.* 2007) and on the other hand glucose utilization is enhanced after AT₁ blockade through a concurrent alteration in HPA reactivity (Uresin *et al.* 2004, Raasch *et al.* 2006, Müller *et al.* 2007).

Even though a functional impact of the interaction between the RAAS and the HPA axis has been demonstrated for hypertension, whether the reactivity of the HPA axis in response to peripherally applied AT₁ blockers or Ang II reflects a central (hypothalamic or pituitary) and/or a peripheral (adrenal) mechanism has not been sufficiently addressed. Both a reduced pituitary sensitivity to CRH and a downregulation of hypothalamic CRH expression have been discussed as reducing HPA axis activity during chronic AT₁ blockade or angiotensin-converting enzyme inhibition (Raasch *et al.* 2006). We aimed to answer this question in the present study by using the transgenic rats with low brain angiotensinogen (TGR(ASrAOGEN)) (Schinke *et al.* 1999). In TGR(ASrAOGEN), angiotensinogen protein concentration was reduced to 10% in medulla, pons, hypothalamus, thalamus, and cerebellum compared to controls, while angiotensinogen plasma concentrations and plasma renin activity were similar. This transgenic rat lacking in brain Ang II induced a 6–8 mmHg decrease in blood pressure (Schinke *et al.* 1999). The hypotension is comparable to the efficacy of AT₁ blockers at antagonizing an Ang II-induced increase in blood pressure when administered i.c.v. (Oliveira *et al.* 1996, Camara & Osborn 1998). Since the RAAS activity has been demonstrated to be impaired in the brain specifically, these transgenic rats may corroborate the functional significance of local Ang II production in the brain for centrally regulating the reactivity of the HPA axis.

Material and Methods

Animals

Eight-week-old transgenic rats deficient in brain angiotensinogen TGR(ASrAOGEN) were obtained from the Max-Delbrück-Center for Molecular Medicine (MDC), Berlin-Buch, Germany. Age-matched Hannover Sprague-Dawley rats (also from MDC) served as controls. The study was conducted according to the NIH guidelines for the care and use of laboratory animals, and was authorized by the local regulatory authority (Ministerium für Landwirtschaft, Umwelt und ländliche Räume des Bundeslandes Schleswig-Holstein). The animals were kept at room temperature with a 12 h/12 h darkness (1400–0200 h)/light (0200–1400 h) cycle. They received a standard diet and water *ad libitum*.

Study protocol

Protocol 1 At the age of 17 weeks, chronic polyethylene catheters were inserted during pentobarbitone anesthesia into the right femoral vein and artery. Catheters were tunneled under the back skin, exteriorized in the region of the cervical vertebra, and fixed at the skin. Thereafter, rats were housed individually in cages (height × width × length: 20 × 22 × 25 cm) until the end of the study. Blood pressure was monitored via arterial catheters 2 days after catheterization at between 0900 and 1000 h in conscious rats. Values were recorded for 5 min. Blood pressure and heart rate were

averaged over two time periods (30 s each) when conditions were stable. One day later, CRH tests were performed (Raasch *et al.* 2006, Müller *et al.* 2007). During the CRH tests, rats had free access to water and food. Three hours before starting the tests, arterial catheters were extended by ~4 cm to avoid stress reactions during blood withdrawal. CRH (10 µg/kg, i.v.) was injected 4 h before the light cycle. During the CRH tests, the lights at working benches were kept as dim as possible to maintain the darkness period. Before CRH injections and at regular intervals over a 4-h time period, 50 µl blood was withdrawn for analysis of corticosterone and ACTH. To avoid hemorrhage-induced alterations, platelets and erythrocytes were reconstituted and returned to each animal.

Protocol 2 A second set of age-matched rats undertook the forced swim test (FST). Before stress testing, blood samples were taken from a tail nick in order to distinguish the baseline conditions of the stress hormones. Thirty minutes after swimming (10 min) in a basin (diameter 35 cm, water depth 20 cm, and water temperature 15 °C), tail blood was taken again for the determination of corticosterone and ACTH.

Protocol 3 In a third set of animals (17 weeks), an ACTH test was performed 3 days after catheter insertions (see protocol 1). Before ACTH injections (2 µg/kg, i.v.) and at regular intervals within a 4-h time period, 30 µl blood was withdrawn. During ACTH tests, the lights at the working benches were kept as dim as possible.

Protocol 4 Organs and blood for the biochemical/molecular analysis were obtained from age-matched animals that were not included in any functional tests. Rats of this group were housed and handled similar to the rats of the other protocols. These rats had not been catheterized to avoid any surgery stress. Thus, surgery-induced alterations in baseline ACTH and corticosterone could be eliminated and MC2 receptor, CRH and AT receptors reflect native expression in organs of the HPA axis in these rats.

Biochemical analysis

Plasma concentrations of ACTH, corticosterone, and aldosterone were determined by RIAs using commercial kits (all from MP Biomedicals, Eschwege, Germany). Assays were performed as recommended by the manufacturers (Raasch *et al.* 2006, Müller *et al.* 2007). Norepinephrine and epinephrine were measured in plasma that was obtained one day after catheter insertion by HPLC and electrochemically as previously described (Raasch *et al.* 2004a, 2005).

RNA isolation and cDNA synthesis

Hypothalami were dissected according to Paxinos & Watson (1998). The brains were adapted to –10 °C, and coronal cuts were made 0.26 mm (at the optic chiasm) and 4.8 mm posterior to the bregma. To cut apart the hypothalamus, the

slice was turned on its posterior surface and cut sagittally 2.6 mm lateral to the midline directly before the amygdala and horizontally 7.4 mm under the cortical surface. The neurointermediate lobes were not removed before preparation of pituitary RNA. Hypothalami and pituitary and total adrenal glands were extracted on the ABI PRISM 6100 Nucleic Acid PrepStation (Applied Biosystems) according to the manufacture's instructions (Jöhren *et al.* 2003). Total RNA was synthesized using oligo-(dT)₁₅ primer and AMV Reverse Transcriptase (Invitrogen). Contamination with genomic DNA was avoided by thorough treatment with DNase I. cDNA was stored at -20°C until PCR.

qPCR

mRNA steady-state levels of AT_{1A} (*Agtr1a*), AT_{1B} (*Agtr1b*), AT_2 (*Agtr2*), and ACTH receptors (melanocortin 2 receptors=MC2 receptor; *Mc2r*), as well as of pro-opiomelanocortin (*Pomc*) and *Crh*, were quantified in organs of the HPA axis. Quantitative measurements of mRNA were performed by qPCR with the cycle threshold method using SYBR green I as a fluorescent dye on the GeneAmp 7000 sequence detection system (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany), and cDNA-specific primers. Primers for AT_{1A} , AT_{1B} , AT_2 receptors, MC2 receptor, POMC and CRH have been published elsewhere (Raasch *et al.* 2004b, 2006, Müller *et al.* 2007). All primers were obtained from Invitrogen. Product specificity was confirmed by dissociation curve analysis and agarose gel electrophoresis. No template controls served as negative controls (Jöhren *et al.* 2001). Expression values were normalized to total RNA content (Bustin 2002).

Statistical analysis

Data shown are expressed as means $\pm$ S.E.M. To quantify the total effect over the observation time in response to CRH regarding changes in plasma concentrations of corticosterone and ACTH, the area under the curves (AUC) was calculated for each individual animal on the basis of their delta values. Correlation analysis was carried out by using Pearson's product moment correlation. For pairwise comparisons, Student's *t*-test was employed, and differences in MC2 receptor mRNA were also analyzed by the median quantitative mutual information score (QMIS; Tsalenko *et al.* 2006). QMIS is powerful specifically when study groups exhibit different skewness or kurtosis. A two-way ANOVA followed by Bonferroni's *post-hoc* test for multiple comparisons was performed to examine the effects of two variables. Differences were considered to be statistically significant at $P < 0.05$.

Results

Blood pressure was slightly decreased in TGR(ASrAOGEN) compared to controls, whereas heart rate and left ventricular weight were not affected (Table 1). Weight of adrenals was

Table 1 Hemodynamic parameters of TGR(ASrAOGEN) and controls. Means $\pm$ S.E.M. ($n=13$)

	Control	TGR(ASrAOGEN)	P
Systolic blood pressure (mmHg)	124 $\pm$ 2	118 $\pm$ 2	0.028
Heart rate (beats/min)	370 $\pm$ 10	370 $\pm$ 6	0.969
Left ventricular weight (mg)	1067 $\pm$ 31	1034 $\pm$ 25	0.429

similar between the TGR(ASrAOGEN) and the controls (31.9 ± 2.1 vs 27.1 ± 1.1 mg; $P=0.1011$). Baseline levels of ACTH, corticosterone, and aldosterone did not differ between the two rat strains (Table 2). Moreover, controls and TGR(ASrAOGEN) featured similar plasma levels of norepinephrine and epinephrine (Table 2). Both norepinephrine and epinephrine exceeded the normal range, which might be due to the withdrawal modality.

In response to CRH stimulation, serum levels of ACTH and corticosterone sharply increased and returned to baseline levels within 4 h. The plasma profiles of ACTH after CRH stimulation did not differ between both rat strains (Fig. 1A). Despite similar ACTH profiles, the CRH-induced corticosterone response was enlarged in TGR(ASrAOGEN). The maximal concentration ($C_{\max}$; 362 ± 17 vs 437 ± 21 ng/ml; $P=0.0158$) was increased, but $t_{\max}$ (70 ± 8 vs 60 ± 7 min; $P=0.3589$) remained unchanged in TGR(ASrAOGEN) compared with the controls (Fig. 1B). The observation that the corticosterone response to CRH was enlarged in TGR(ASrAOGEN) independently of a concurrent ACTH increase was pointed out by plotting individual AUCs of concentration-time curves of ACTH (=AUC_{ACTH}) and corticosterone (=AUC_{corticosterone}) respectively. The regression analysis of corresponding AUCs verified a positive correlation between the ACTH and corticosterone response to CRH in TGR(ASrAOGEN) and controls (Fig. 1C). However, the regression line relating to TGR(ASrAOGEN) was shifted upwards (Fig. 1C) indicating an improved sensitivity towards ACTH. Accordingly, the mean AUC_{corticosterone} was significantly increased in TGR(ASrAOGEN) ($34\,535 \pm 3047$ vs $22\,209 \pm 3911$ pg/ml per min, $P=0.0202$), whereas the mean AUC_{ACTH} was similar between TGR(ASrAOGEN) and controls (Fig. 1C).

Table 2 Endocrine parameters of TGR(ASrAOGEN) rats and controls. Means $\pm$ S.E.M. ($n=8-13$)

	Control	TGR(ASrAOGEN)	P
Plasma ACTH (pg/ml)	110 $\pm$ 15	91 $\pm$ 9	0.148
Plasma corticosterone (ng/ml)	152 $\pm$ 34	135 $\pm$ 20	0.329
Plasma aldosterone (ng/ml)	191 $\pm$ 32	1091 $\pm$ 34	0.096
Plasma norepinephrine (pg/ml)	1039 $\pm$ 96	844 $\pm$ 96	0.164
Plasma epinephrine (pg/ml)	654 $\pm$ 95	512 $\pm$ 75	0.281

Figure 1 Plasma profiles of ACTH (A) and corticosterone (B) in response to CRH (10 µg/kg i.v.) in TGR(ASrAOGEN) (●) and controls (○). The increase in corticosterone response was statistically evaluated by two-way ANOVA. (C) Correlation between CRH-induced alterations of cumulative plasma concentrations of ACTH and corticosterone (depicted as AUC of the corresponding concentration–time curves; see Fig. 1) in TGR(ASrAOGEN) (●, solid line, Pearson $R=0.6447$, $P=0.0118$) and controls (○, dotted line, Pearson $R=0.5572$, $P=0.0471$). Means $\pm$ S.E.M. ($n=10$ –12).

After FST, the plasma levels of ACTH and corticosterone increased in TGR(ASrAOGEN) and controls to a similar extent compared to the CRH test (Fig. 2A and B). The corticosterone response was enlarged in TGR(ASrAOGEN), which was not again associated with a concurrent increase in ACTH. This was emphasized by the graphical analysis of the correlations between ACTH and corticosterone values. Consistently with the CRH test, the regression line representing the TGR(ASrAOGEN) was shifted upwards (Fig. 2C).

In order to determine the impact of the adrenals on the sensitization of the HPA axis in TGR(ASrAOGEN), an ACTH test was performed. Baseline corticosterone levels did not differ between both rat strains (129 ± 5 vs 100 ± 14 ng/ml; $P=0.088$). In response to ACTH, increase in corticosterone was slightly heightened in TGR(ASrAOGEN) compared to controls (Fig. 3).

Figure 2 Plasma concentrations of ACTH (A) and corticosterone (B) before and after TGR(ASrAOGEN) (closed bars) and controls (open bars) were stressed by a forced swim test (FST, 10 min 15 °C). The stress response and the increase in corticosterone after FST were statistically evaluated by two-way ANOVA. In (C), the correlation between ACTH and corticosterone after FST in TGR(ASrAOGEN) (●, solid line, Pearson $R=0.8771$, $P=0.0048$) and controls (○, dotted line, Pearson $R=0.7777$, $P=0.0115$) is depicted. Means $\pm$ S.E.M. ($n=7$ –8).

The steady-state mRNA levels of *Crh* and *Pomc* in the hypothalami and pituitary glands were similar between both strains (Fig. 4). The steady-state mRNA levels of MC2 receptors were increased by one-third in the adrenals of TGR(ASrAOGEN) (Fig. 5). Finally, while the mRNA levels of AT_{1A} and AT_{1B} receptors in the organs of the HPA axis did not differ between the two rat strains (Tables 3 and 4), the adrenal mRNA levels of AT₂ receptors were doubled in TGR(ASrAOGEN) (Fig. 5).

Discussion

Recently, we have shown that inhibition of AT₁ receptors attenuates HPA axis reactivity. A reduced pituitary sensitivity to CRH and a downregulation of hypothalamic CRH expression were speculated to underlie these effects (Raasch *et al.* 2006). Following on from this, in this study, we aimed to confirm the relevancy of Ang II-dependent central mechanisms for regulating HPA activity by using TGR(ASrAOGEN) that lacked brain Ang II (Schinke *et al.* 1999). Consistent with many other studies, a slight hypotension was observed in the TGR(ASrAOGEN), which served as the fundamental characteristic of this transgenic rat in most of the studies (Baltatu *et al.* 2000, 2001, Wang *et al.* 2004, Campos *et al.* 2006).

At first sight, TGR(ASrAOGEN) and controls do not seem to differ regarding stress parameters, since baseline levels of ACTH, corticosterone, aldosterone, norepinephrine, epinephrine, and adrenal weights were all similar. Even though morphometric examinations were not carried out, we speculate here that zonal differentiation was not altered in the adrenal cortex of TGR(ASrAOGEN), since no changes or hypertrophy in response to Ang II were observed in the zona fasciculata or the zona glomerulosa (Ogishima *et al.* 1992, McEwan *et al.* 1999, Aguilar *et al.* 2004). In accordance with these findings, baseline levels of stress hormones also remained unaffected in SHR after chronic AT₁ blockade

Figure 3 Maximal increase of corticosterone in response to ACTH (2 μg/kg i.v.) in TGR(ASrAOGEN) and controls; means ± S.E.M. (n=7–8). Significance of corticosterone response to ACTH was calculated by Student's *t*-test.

Figure 4 Steady-state mRNA levels of *Crh* (in the hypothalamus) and *Pomc* (in the pituitary gland) of TGR(ASrAOGEN) and controls. Means ± S.E.M. (n=7–10).

(Raasch *et al.* 2006). In contrast to our expectations and the observation that HPA reactivity is increased by Ang II and reduced by AT₁ blockade (Raasch *et al.* 2006, Müller *et al.* 2007), the CRH-induced corticosterone response in TGR(ASrAOGEN) was by no means diminished, but in fact increased. In order to confirm our results, a second stress test was performed. Accordant to the CRH challenge, the corticosterone plasma levels raised to the same extent after FST, and the stress-induced increase in corticosterone was enlarged in TGR(ASrAOGEN). The stimulated corticosterone response in TGR(ASrAOGEN) was not associated with a concurrent increase in ACTH in the CRH test or FST. As such, a combined hypothalamic and pituitary related mechanism seems rather unlikely. This conclusion is further supported by similar *Crh* and *Pomc* mRNA levels in the hypothalamus and pituitary glands of TGR(ASrAOGEN). However, i.c.v. or peripheral administrations of AT₁ blockers were found to alleviate the increases in hypothalamic *Crh* mRNA or pituitary ACTH in response to stress (Jezova *et al.* 1998, Raasch *et al.* 2006). While this inhibiting effect was observed in animals that were exposed to stress (Jezova *et al.* 1998, Raasch *et al.* 2006), the *Crh* and *Pomc* mRNA levels presented here reflect native expression in the organs of rats, thus accounting for the differences between our and the cited studies.

Figure 5 Steady-state mRNA levels of the MC2 receptor and the AT₂ receptor in the adrenal glands of TGR(ASrAOGEN) and controls. Means $\pm$ S.E.M. ($n=7-10$). Quantitative mutual information score (MC2 receptor) and Student's *t*-test (AT₂ receptor) were performed to prove statistical significance.

The ACTH independency of simulated corticosterone responses to stress indicates an adrenal mechanism. We asked whether the adrenal MC2 receptor expression is increased, since this subtype of MC receptor preferentially promotes the ACTH-induced corticosterone response (Chhajlani *et al.* 1993, Liakos *et al.* 1998). In human adrenal fasciculata cells, Ang II and ACTH stimulated the expression of the MC2 receptor (Lebrethon *et al.* 1994, Penhoat *et al.* 1994, 1995, Naville *et al.* 2001, Blondet *et al.* 2002). In bovine adrenal fasciculata cells, Ang II was hypothesized to induce a steroidogenic desensitization (Penhoat *et al.* 1995), justifying our speculation that the MC2 receptor is increased in TGR(ASrAOGEN). Indeed, steady-state levels of MC2 receptor mRNA were increased in TGR(ASrAOGEN). It therefore seems conceivable that an upregulation of the

Table 3 Steady-state levels of mRNA of AT_{1A} receptors in organs of the hypothalamo-pituitary-adrenal axis of TGR(ASrAOGEN) and controls. Means $\pm$ S.E.M. ($n=7-10$)

	Control	TGR(ASrAOGEN)	P
Hypothalami	$4.7 \pm 0.6 \times 10^5$	$5.3 \pm 0.7 \times 10^5$	0.547
Pituitary glands	$8.1 \pm 0.6 \times 10^5$	$7.3 \pm 0.6 \times 10^5$	0.431
Adrenal glands	$5.6 \pm 0.6 \times 10^5$	$7.3 \pm 0.9 \times 10^5$	0.066

Table 4 Steady-state levels of mRNA of AT_{1B} receptors in organs of the hypothalamo-pituitary-adrenal axis of TGR(ASrAOGEN) and controls. Means $\pm$ S.E.M. ($n=7-10$)

	Control	TGR(ASrAOGEN)	P
Hypothalami	$4.2 \pm 0.5 \times 10^4$	$6.2 \pm 0.9 \times 10^4$	0.068
Pituitary glands	$6.9 \pm 1.0 \times 10^4$	$6.1 \pm 0.7 \times 10^4$	0.532
Adrenal glands	$4.5 \pm 0.7 \times 10^4$	$5.5 \pm 0.8 \times 10^4$	0.366

MC2 receptor may account for the stimulated corticosterone response to stress in TGR(ASrAOGEN). In order to confirm this mechanism functionally, the adrenal responsiveness to ACTH was investigated. In accordance with our hypothesis, plasma corticosterone in response to ACTH was slightly enhanced in TGR(ASrAOGEN) compared to controls.

Whether other mechanisms besides the upregulation of MC2 receptor may be involved in the increased stress response in TGR(ASrAOGEN) is discussed in the following:

Ang II stimulates glucocorticoid release by enhancing the CRH-mediated ACTH release (Gaillard *et al.* 1981, 1985, Abou-Samra *et al.* 1986, Schoenberg *et al.* 1987) or by stimulating adrenal glucocorticoid production (Naville *et al.* 1993). We recently demonstrated that Ang II increased the corticosterone responsiveness to CRH, an effect that was associated with an upregulation of the AT_{1A} receptor (Müller *et al.* 2007). This poses the question whether AT₁ receptors are enhanced in the organs of the HPA axis of the TGR(ASrAOGEN), and whether this causes the observed stress sensitization. We found no alterations in the mRNA levels of the AT₁ receptor, indicating that the hypersensitization to stress is minor compared to alterations in the AT₁ receptor. A limitation of our study was that we determined mRNA levels rather than protein or AT₁ binding, even considering that AT₁ receptor binding was significantly higher inside various regions of the blood-brain barrier of TGR(ASrAOGEN) compared to controls (Monti *et al.* 2001). While hypothalamic AT₁ receptors are less stimulated in TGR(ASrAOGEN) by locally generated Ang II, it should be questioned whether peripherally generated Ang II causes the enhanced stress response. The following points tend to argue against an impact of peripherally arising Ang II: 1) if circulating Ang II is really involved in stress sensitization via hypothalamic AT₁ receptors, the ACTH response should also be enhanced; however, in our study, the potentiation of corticosterone response occurred ACTH independently; 2) even considering that glucocorticoids are potent activators of angiotensinogen gene expression (Brasier & Li 1996), Ang II does not penetrate the blood-brain barrier (Schelling *et al.* 1976) and 3) Ang II may indeed act upon AT₁ receptors in the circumventricular organs, but AT₁ receptor binding was clearly shown to be decreased in these areas (Monti *et al.* 2001). It is certainly true that the increase in AT₂ receptor mRNA in the adrenals of TGR(ASrAOGEN) may also have been related to the stimulated stress response of the transgenic rats, since another study on bovine fasciculata cells showed

that cortisol production was stimulated through AT₂ receptors, and thus independently of an AT₁-mediated mechanism (Defaye *et al.* 1995).

CRH is a key hormone in the integrated response to stress, which stimulates sympathetic outflow in addition to regulating pituitary ACTH release. Sympathoexcitatory responses to air-jet stress were found to be similar between TGR(ASrAOGEN) and controls, and between Wistar rats receiving i.c.v. infusions of losartan or vehicle (Zhang *et al.* 1999, Wang *et al.* 2004). This indicates that the brain RAAS appears to play only a small role in the control of sympathetic function during stress. Our findings that hypothalamic *Crh* expression as well as baseline epinephrine did not differ between controls and TGR(ASrAOGEN) may strengthen this idea, even though we are aware that we have not determined changes during stress reactions. Peripheral RAAS certainly does influence sympathoexcitatory responses to stress, since adrenal biosynthesis and secretion of catecholamines are reduced after AT₁ blockade (Armando *et al.* 2001).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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