Regulation of angiotensin II type 2 receptor gene expression in the adrenal medulla by acute and repeated immobilization stress

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Abstract

While the renin–angiotensin system is important for adrenomedullary responses to stress, the involvement of specific angiotensin II (Ang II) receptor subtypes is unclear. We examined gene expression changes of angiotensin II type 1A (AT_{1A}) and type 2 (AT₂) receptors in rat adrenal medulla in response to immobilization stress (IMO). AT₂ receptor mRNA levels decreased immediately after a single 2-h IMO. Repeated IMO also decreased AT₂ receptor mRNA levels, but the decline was more transient. AT_{1A} receptor mRNA levels were unaltered with either single or repeated IMO, although binding was increased following repeated IMO. These effects of stress on Ang II receptor expression may alter catecholamine biosynthesis, as tyrosine hydroxylase and dopamine β-hydroxylase mRNA levels in PC12 cells are decreased with Ang II treatment in the presence of ZD7155 (AT₁ receptor antagonist) or with CGP42112 (AT₂ receptor agonist) treatment. Involvement

of stress-triggered activation of the hypothalamic–pituitary–adrenocortical or sympathoadrenal axis in AT₂ receptor downregulation was examined. Cultured cells treated with the synthetic glucocorticoid dexamethasone displayed a transcriptionally mediated decrease in AT₂ receptor mRNA levels. However, glucocorticoids are not required for the immediate stress-triggered decrease in AT₂ receptor gene expression, as demonstrated in corticotropin-releasing hormone knockout (*Crh* KO) mice and hypophysectomized rats, although they can regulate basal gene expression. cAMP and pituitary adenylate cyclase-activating polypeptide also reduced AT₂ receptor gene expression and may mediate this response. Overall, the effects of stress on adrenomedullary AT_{1A} and AT₂ receptor expression may contribute to allostatic changes, such as regulation of catecholamine biosynthesis.

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Introduction

The response of an organism to acute stress is necessary for survival. However, when stress is prolonged or repeated, the response is not only adaptive but also becomes maladaptive. With repeated exposure to stress, there is an increase in allostatic load (McEwen & Seeman 1999, McEwen 2007). As a result, stress is a major contributor to the development of cardiovascular disorders and neuropsychiatric illnesses and can also adversely influence the progression of chronic diseases, such as diabetes and cancer (reviewed in McEwen (1998) and Chrousos (2009)).

The physiological responses to stress involve multiple systems. The activation of the hypothalamic–pituitary–adrenocortical (HPA) axis and the catecholaminergic sympathoneural and sympathoadrenal systems is key neuroendocrine responses to stress (reviewed in Kvetnansky

et al. (2009)). The renin–angiotensin system (RAS), which is known for regulating blood pressure and fluid and electrolyte balance, also plays an important role in the stress response (reviewed in Saavedra *et al.* (2011) and Saavedra (2012)). Levels of plasma renin as well as circulating and adrenal angiotensin II (Ang II), the main active component of the RAS, are elevated in response to stress (Jindra & Kvetnanský 1982, Yang *et al.* 1993). The RAS likely contributes to the development of stress-related cardiovascular disorders (Saavedra *et al.* 2011); however, the underlying mechanisms are still unclear.

Ang II is a potent secretagogue that stimulates adrenomedullary norepinephrine and epinephrine release (Feldberg & Lewis 1964). Accordingly, nephrectomy or treatment with a nonselective Ang II receptor antagonist nearly abrogates catecholamine release from cat adrenal medulla in response to the stress of insulin-induced hypoglycemia

(Bumpus *et al.* 1980). Ang II also induces catecholamine biosynthesis by elevating gene expression of the catecholamine biosynthetic enzymes, tyrosine hydroxylase (TH), and phenylethanolamine *N*-methyltransferase (PNMT) (Stachowiak *et al.* 1990b). The results may depend on which Ang II receptor subtype is involved.

Ang II binds with equal affinity to two major sites, the angiotensin II type 1 (AT₁) and type 2 (AT₂) receptors (reviewed in de Gasparo *et al.* (2000)). In rodents, there are two subtypes of AT₁ receptor, the AT_{1A} and AT_{1B} receptor, which are pharmacologically indistinguishable but differentially regulated (reviewed in Inagami *et al.* (1994)). The AT₁ receptors mediate virtually all the well-known effects of Ang II, including vasoconstrictive, hypertrophic, proliferative, and pro-fibrotic effects. Although the expression of AT₁ receptors in rodent adrenal medulla is very low and limited to only the AT_{1A} receptor subtype (Leong *et al.* 2002), there is substantial evidence for a role of AT₁ receptors in maintaining basal adrenomedullary catecholamine synthesis and in mediating central and adrenomedullary responses to stress (Armando *et al.* 2001, 2007, Leong *et al.* 2002, Jezova *et al.* 2003).

AT₂ receptor activation has been proposed to oppose AT₁ receptor-mediated effects and is associated with vasodilation; nitric oxide release; and anti-hypertrophic, anti-proliferative, and anti-fibrotic effects (reviewed in de Gasparo *et al.* (2000)). Multiple *in vivo* studies using genetically altered animals or selective pharmacological agents suggest tissue protective properties of the AT₂ receptor subtype in a number of disease states (reviewed in Jones *et al.* (2008)). Unlike the AT₁ receptor, the AT₂ receptor is not very abundant in adult tissues, except for the adrenal gland where it is highly expressed in the zona glomerulosa and medulla (Israel *et al.* 1995).

While Ang II elevates *TH* gene expression in primary cultures of bovine adrenomedullary cells (Stachowiak *et al.* 1990b), more recently it was shown that activation of the AT₂ receptor inhibits catecholamine synthesis and TH expression and activity in porcine adrenomedullary chromaffin cells (Takekoshi *et al.* 2000, 2002). AT₂ receptor gene-deficient mice show elevated adrenomedullary *Th* and AT₁ receptor mRNA levels, norepinephrine and epinephrine levels, as well as enhanced HPA axis stimulation (Saavedra *et al.* 2001, Armando *et al.* 2002). This indicates that the AT₁ and AT₂ receptors may play opposite roles in the regulation of the adrenomedullary response to stress and that the expression of one receptor type may influence the expression of the other.

The relative expression of AT₁ and AT₂ receptors is an important modulator of adrenomedullary function. The current study is aimed to better understand the changes in Ang II receptor expression in the adrenal medulla in response to acute and repeated stress. Rats were exposed to immobilization stress (IMO), which is a stress model wherein the response of the adrenomedullary catecholaminergic system to various durations and repetitions of this stressor has been well characterized (Sabban & Kvetnansky 2001,

Liu *et al.* 2008). The findings reveal a biphasic response of AT₂ receptor gene expression to stress and provide insight into the mechanisms that trigger these dramatic changes.

Materials and methods

Animals

All animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996) and were approved by the NYMC Institutional Animal Care and Use Committee (rat experiments) or the Ethical Committee of the Institute of Experimental Endocrinology, Slovak Academy of Sciences (mouse experiments).

Male, Sprague Dawley rats (250–320 g) were obtained from Taconic Farms (Germantown, NY, USA). Male, corticotropin-releasing hormone knockout (*Crh* KO) mice (C57B1/129SV) and WT mice, age 150–180 days, were bred at the Institute of Experimental Endocrinology, as described previously (Kvetnansky *et al.* 2006). The *Crh* KO mouse line was originally a generous gift from Dr Joseph A Majzoub (Harvard Medical School, Department of Endocrinology, Boston, MA, USA). The animals were maintained under controlled conditions (23 ± 2 °C, 12 h light:12 h darkness cycle, lights on from 0600 h) with food and water *ad libitum*.

Hypophysectomized or sham-operated rats (Taconic Farms) were administered saline or 25 mg/kg per day (1 µl/h) hydrocortisone 21-hemisuccinate, sodium salt crystalline (cortisol; Sigma-Aldrich Corp.) for 7 days via osmotic minipump inserted s.c. in the interscapular area. All hypophysectomized rats received isotonic saline instead of drinking water. Complete removal of the pituitary in hypophysectomized rats was visually confirmed.

Stress

IMO, a strong noninvasive stress model, was performed as described previously (Nankova *et al.* 1994, Liu *et al.* 2008) on a metal board by taping the limbs with surgical tape and restricting the motion of the head exactly as originally described by Kvetnansky & Mikulaj (1970). Rats were subjected to single or repeated IMO. For single IMO (1 × IMO), rats were immobilized once for 2 h and subsequently killed either immediately (0 h) or 3 h after termination of the stress. For repeated IMO (6 × IMO), rats were immobilized for 2 h daily for 6 consecutive days and killed either immediately (0 h) or 3 h after termination of the stress. To elucidate the effect of the last IMO, one group of rats (the adapted control group) was immobilized for all but the last IMO and killed the next day (5 × IMO + 24 h). Absolute controls were not exposed to stress. For the hypophysectomy experiment, rats were killed immediately after 1 × IMO, performed on the seventh day of saline, or cortisol treatment.

Immobilization of WT and *Crh* KO mice was performed similarly, with the following modifications. For repeated stress, mice were immobilized for 2 h daily for 7 consecutive days (7×IMO). Consequently, the adapted controls were immobilized for 2 h daily for 6 consecutive days and killed 24 h later (6×IMO+24 h).

In all animal experiments, IMO was performed at the same time of the day (between 0800 h and noon). Following decapitation, the left and right adrenals were dissected. Subsequently, any cortex tissue adhering to the adrenal medulla was carefully removed. It has been estimated that the isolated medulla is >90% pure (Liu *et al.* 2005). The left and right adrenal medullae from each individual animal were frozen separately in liquid nitrogen and kept at −80 °C.

Primary cultures of rat adrenal medulla and PC12 cell cultures

Rat adrenal medullae were isolated as described earlier and collected in cold Hank's Balanced Salt Solution (HBSS; Invitrogen). Subsequently, the medullae were incubated with filtered dissociation solution containing 2.6 mg/ml collagenase type 1 (Worthington Biochemical Corp., Lakewood, NJ, USA), 0.15 mg/ml hyaluronidase type 1-S (Sigma-Aldrich Corp.), 3 mg/ml BSA (Fisher Scientific, Pittsburgh, PA, USA), and 20 U DNase (Ambion, Austin, TX, USA) in a 37 °C water bath for 20 min with periodic resuspension, followed by 20 min of continuous resuspension until the medullary tissue was dissociated. The reaction was stopped with cold HBSS and the digested tissue centrifuged at 2000 *g* for 3 min at 4 °C. The pellet was resuspended in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA, USA), 5% horse serum (Gemini Bio-Products), and 50 µg/ml streptomycin with 50 IU/ml penicillin (0.5%; Invitrogen). The cells were plated on collagen-coated 12-well tissue culture plates (BD Biosciences, San Jose, CA, USA) and incubated in a 37 °C humidified incubator with 5% CO₂ for 1 h before additional media were added. The media were replaced after 24 h. Cultures were used for treatment between 3 and 4 days after plating.

Rat adrenomedullary-derived PC12 cells were grown in the media described earlier and maintained at 37 °C in a humidified incubator with 5% CO₂, with media changed every other day, as described previously (Serova *et al.* 1997). Before treatment, cells were plated at about 50% density.

Cell culture treatment

For experiments with dexamethasone, 1 day before treatment, the media were replaced with stripped media containing DMEM supplemented with 10% charcoal-stripped FBS (Sigma-Aldrich), dialyzed horse serum (Gemini Bio-Products), and antibiotics. Cells were treated with 1 µM dexamethasone (Sigma-Aldrich), a dose that lies within the ranges typically used in PC12 cells and adrenomedullary primary cultures (Stachowiak *et al.* 1990a,

McMahon & Sabban 1992), in 0.01% ethanol for 3.5–24 h and subsequently harvested for total RNA isolation. To determine the role of transcription, 4 µM actinomycin D (Enzo Life Sciences, Farmingdale, NY, USA), a transcription inhibitor, dissolved in 0.04% DMSO was added to the cells immediately before dexamethasone treatment. In some experiments, cells were treated with dexamethasone alone or in combination with 200 µM 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate sodium salt (CPT-cAMP; Sigma-Aldrich), a membrane permeable cAMP analog, or with 0.01 pM to 1000 nM pituitary adenylate cyclase-activating polypeptide (PACAP) 1–38 (Tocris Bioscience, Minneapolis, MN, USA), dissolved in distilled water. Cells were alternatively treated for 8 h with the AT₂ receptor agonist, CGP42112 (1–1000 nM; Tocris Bioscience), or with 10 nM Ang II (Sigma-Aldrich) following 15 min pretreatment with the AT₁ receptor antagonist, ZD7155 (100 nM; Tocris Bioscience), all dissolved in distilled water. In all experiments, controls were treated with vehicle.

Isolation of RNA and quantification of changes in mRNA levels

Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen). Total RNA concentration was then quantitated using the NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and reverse transcribed using the RevertAid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific) using the oligo-dT primer. For quantitative real-time PCR, 2 µl cDNA product was mixed with 12.5 µl FastStart Universal SYBR Green Master Rox (Roche) and 1 µl of the following primer pairs: rat or mouse *Th*, dopamine β-hydroxylase (*Dbh*), AT_{1A} receptor, AT₂ receptor, or *Gapdh* (all purchased from Qiagen), and analyzed on an ABI7900HT Real Time PCR instrument (Applied Biosystems). Data are normalized to *Gapdh* mRNA levels and expressed as the relative fold change vs control, calculated using the $\Delta\Delta C_t$ method. *Gapdh* mRNA levels were not altered by any of the experimental conditions. In earlier experiments, real-time RT-PCR for *Th* and *Dbh* was performed on the LightCycler (Roche) as described previously (Sabban *et al.* 2010, Serova *et al.* 2011). A standard curve plotted using serial dilutions from 2 ng to 0.2 pg cDNA was used for quantification by the Fit Points method. Data are normalized to total RNA.

Autoradiography of Ang II receptor types

Binding experiments were performed as described previously (Nishimura *et al.* 2000) using consecutive sections (16 µm thick) from the adrenal gland. Sections were incubated with 0.5 nM [¹²⁵I]Sarcosine¹-Ang II (Sar¹-Ang II; ARC, St Louis, MO, USA) to determine total binding. Nonspecific binding was determined by incubation as earlier in the presence of 5×10^{−6} M unlabeled Ang II. The binding of [¹²⁵I]Sar¹-Ang II to AT₁ receptors was determined by incubation with 0.5 nM [¹²⁵I]Sar¹-Ang II in the presence of

10⁻⁵ M of the selective AT₁ receptor antagonist losartan (DuPont Merck). The binding of [¹²⁵I]Sar¹-Ang II to AT₂ receptors was determined by incubation with 0.5 nM [¹²⁵I]Sar¹-Ang II in the presence of 1 µM of the selective AT₂ receptor ligand PD 123319. AT₁ or AT₂ receptor binding was the binding selectively displaced by losartan or PD 123319 respectively.

Statistical analysis

All data are expressed as mean ± S.E.M., unless otherwise noted, with *n*=5–8 per group for animal experiments, *n*=4–6 per group for experiments in PC12 cells, and *n*=3 per group for experiments in primary cultures. Differences were analyzed by Student's *t*-test (if only two groups) or ANOVA followed by Bonferroni's *post hoc* analysis (if more than two groups) using GraphPad Prism 4 Software (GraphPad Software, Inc., La Jolla, CA, USA). A value of *P* ≤ 0.05 was considered significant.

Results

Effect of single and repeated immobilization stress on AT_{1A} and AT₂ receptor expression in the adrenal medulla

The stress-triggered changes in AT_{1A} and AT₂ receptor mRNA levels in rat adrenal medulla were first examined. Rats were exposed to the strong stress of immobilization once or repeatedly for up to 6 days as shown in Fig. 1A. The changes in mRNA levels were determined immediately and 3 h after the final immobilization, chosen based on optimal times for stress-triggered elevations of *Th* and *Pnmt* mRNA levels in the adrenal medulla (McMahon *et al.* 1992, Nankova *et al.* 1994). There were no significant changes in AT_{1A} receptor mRNA levels at any of these time points (Fig. 1B).

By contrast, gene expression of the AT₂ receptor was profoundly altered by the stress and displayed a biphasic effect. Levels of AT₂ receptor mRNA decreased by about 70% immediately and 90% 3 h after exposure to a single 2 h IMO (Fig. 1C). In the adapted controls, killed 24 h after the fifth consecutive daily IMO (i.e. on the sixth day), AT₂ receptor mRNA levels were elevated by 65%, compared with unstressed absolute controls. However, exposure of these animals to an additional immobilization (sixth daily IMO) also markedly lowered AT₂ receptor mRNA levels to about 50% of unstressed control levels. Moreover, the reduction was more transient than with the first IMO and did not continue to decline 3 h afterward.

Next, we examined the effects of IMO on AT₁ and AT₂ receptor expression using a radioligand binding assay. Three hours after exposure to 1×IMO, there was no change in AT₁ receptor binding and a tendency toward reduced AT₂ receptor binding (Fig. 2). However, 3 h after 6×IMO, there was an ~50% increase in both AT₁ and AT₂ receptor binding.

Figure 1 Time course of AT_{1A} and AT₂ receptor gene expression changes in the adrenal medulla in response to single and repeated IMO. (A) Rats were exposed to IMO for 2 h once (1×) or daily for 6 consecutive days (6×) and then killed either immediately (IMO + 0 h) or 3 h after the final IMO (IMO + 3 h). The adapted control group was exposed to 5×IMO, then killed on the sixth day (5×IMO + 24 h), representing both the starting point for the sixth IMO and the sustained effects of repeated IMO. Absolute controls were not exposed to stress. AT_{1A} (B) and AT₂ (C) receptor mRNA levels were determined in the adrenal medulla by real-time RT-PCR, normalized to *Gapdh* mRNA levels, and expressed relative to unstressed absolute controls, taken as 1. **P* < 0.05, ****P* < 0.001 vs absolute control; ##*P* < 0.01, ****P* < 0.001 vs adapted control; aa*P* < 0.01 vs indicated groups.

Figure 2 AT₁ and AT₂ receptor binding in the adrenal medulla in response to single and repeated IMO. Adrenal glands were isolated from rats exposed to 2-h IMO once (1×) or daily for 6 consecutive days (6×) and killed 3 h after the final IMO. Sections of the adrenal gland were incubated with 0.5 nM [¹²⁵I]Sar¹-Ang II and either 10⁻⁵ M of the selective AT₁ receptor antagonist losartan to determine AT₁ receptor binding or 1 μM of the selective AT₂ receptor antagonist PD 123319 to determine AT₂ receptor binding. ***P* < 0.01 vs absolute control.

Role of the AT₁ and AT₂ receptors on catecholamine biosynthetic enzyme gene expression in PC12 cells

As our results suggest that IMO is changing the expression or binding of the AT_{1A} and AT₂ receptors in the adrenal medulla under conditions that are also known to elevate expression of the catecholamine biosynthetic enzymes (reviewed in Sabban & Kvetnanský (2001)), we examined the relationship between them in adrenomedullary derived PC12 cells. Although the AT₁ receptor is expressed at very low levels in PC12 cells, pretreatment with the AT₁ receptor antagonist ZD7155 led to a significant reduction of *Th* and *Dbh* mRNA levels in response to Ang II, likely by way of AT₂ receptor activation (Fig. 3A). This was further studied with the selective AT₂ receptor agonist CGP42112. Treatment with CGP42112 for 8 h led to a dose-dependent decrease in *Th* (*F* = 4.01; *P* < 0.05) and *Dbh* (*F* = 14.28; *P* < 0.001) mRNA levels (Fig. 3B).

Mechanism of the stress-triggered modulation of AT₂ receptor gene expression

One of the major responses to stress is the activation of the HPA axis. We speculated that the stress-triggered rise in glucocorticoids may mediate some of the observed changes in AT₂ receptor gene expression. Primary rat adrenomedullary cultures were treated with the synthetic glucocorticoid, dexamethasone. Similar to the response with IMO, *Th* and *Dbh* mRNA levels were elevated, as expected (Stachowiak *et al.* 1990a, McMahon & Sabban 1992). AT_{1A} receptor mRNA levels were unchanged, while there was a precipitous decline in AT₂ receptor mRNA levels (Fig. 4A).

The time course of changes in *Th*, *Dbh*, and AT₂ receptor mRNA levels in response to dexamethasone was determined in PC12 cells (Fig. 4B). Cells were treated with dexamethasone for various times up to 24 h. *Th* and *Dbh* mRNA levels were elevated as previously reported (Stachowiak *et al.* 1990a, McMahon & Sabban 1992). There was a time-dependent decrease in AT₂ receptor mRNA levels (*F* = 96.29; *P* < 0.001) from ~80 to 10% of basal levels from 3.5 to 24 h with dexamethasone. This response is mediated largely at the transcriptional level, as 8-h treatment with dexamethasone in the presence of 4 μM of the transcription inhibitor, actinomycin D, abolished the decrease in AT₂ receptor mRNA levels (Fig. 4C).

To further understand the importance of glucocorticoids *in vivo*, we analyzed the changes in AT₂ receptor mRNA levels in the adrenal medulla of *Crh* KO mice following single (1×) or repeated (7×) IMO (Fig. 5A). These mice are

Figure 3 AT₁ and AT₂ receptor-mediated regulation of *Th* and *Dbh* gene expression in PC12 cells. (A) PC12 cells were pretreated with 100 nM ZD7155 (AT₁ receptor antagonist) for 15 min, then treated with 10 nM Ang II for 8 h. Subsequently, *Th* and *Dbh* mRNA levels were determined by real-time RT-PCR, normalized to total RNA levels, and expressed relative to control, taken as 1. ##*P* < 0.01, ###*P* < 0.001 vs Ang II-treated group. (B) PC12 cells were treated with 1–1000 nM CGP42112 (AT₂ receptor agonist) for 8 h. Subsequently, *Th* and *Dbh* mRNA levels were determined by real-time RT-PCR, normalized to total RNA levels, and expressed as the percentage of control levels (dotted line), taken as 100%. **P* < 0.05 vs control.

Figure 4 Effect of dexamethasone on AT_{1A} and AT₂ receptor gene expression in rat adrenal medulla primary cultures (A) and PC12 cells. Rat adrenal medulla primary cultures (A) or PC12 cells (B) were treated with 1 μM dexamethasone (Dex) or vehicle (control) for up to 24 h. Subsequently, total RNA was isolated and *Th*, *Dbh*, AT₂ receptor, or AT_{1A} receptor mRNA levels were determined by real-time RT-PCR. Data are normalized to *Gapdh* mRNA levels and expressed relative to their respective time-matched control, taken as 1, indicated as a dotted line in (B). (C) PC12 cells were treated for 8 h with 1 μM Dex in the presence or absence of 4 μM actinomycin D (transcription inhibitor). Total RNA was isolated and AT₂ receptor mRNA levels were determined by real-time RT-PCR. Data are normalized to *Gapdh* mRNA levels and expressed relative to vehicle-treated control, taken as 1. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs vehicle-treated control.

unable to synthesize CRH and do not display the large elevation in plasma ACTH and corticosterone in response to stress (Muglia *et al.* 2000, Kvetnansky *et al.* 2006). The reduction in AT₂ receptor mRNA levels in the WT mice in response to single or repeated IMO (Fig. 5B) was similar to the changes observed in rats (Fig. 1C). A comparison between the WT and *Crh* KO mice demonstrated no difference in basal AT₂ receptor mRNA levels in unstressed absolute controls.

Immediately following 1×IMO, AT₂ receptor mRNA levels were decreased by ~75% in both the WT and *Crh* KO mice, indicating that the rise in glucocorticoids is not essential for AT₂ receptor downregulation in the immediate response to acute stress. Similarly, there was a 45–65% decline in AT₂ receptor mRNA levels relative to respective adapted controls immediately after exposure to the seventh IMO in both the WT and the *Crh* KO mice.

However, in the adapted controls (studied 24 h after the sixth IMO and not exposed to the seventh IMO), AT₂ receptor mRNA levels were elevated by approximately twofold in the *Crh* KO mice but not significantly in the WT mice, relative to respective genotype-specific unstressed absolute controls. This suggests that the stress-triggered elevation in glucocorticoids may attenuate upregulation of AT₂ receptor gene expression in the prolonged response to repeated stress exposure.

We further analyzed the role of glucocorticoids in the regulation of AT₂ receptor gene expression in hypophysectomized rats under basal conditions and in response to 1×IMO. Compared with sham-operated controls, AT₂ receptor mRNA levels were increased by approximately threefold in hypophysectomized rats (Fig. 6). This induction is reversed by cortisol supplementation, indicating that glucocorticoids are potent negative regulators of AT₂ receptor gene expression under basal conditions. Immediately following 1×IMO, however, AT₂ receptor mRNA levels are significantly reduced in both sham-operated and hypophysectomized rats compared with respective unstressed controls. These data provide further evidence that glucocorticoids are not essential for the decrease in AT₂ receptor mRNA levels immediately following IMO.

As the stress-triggered reduction in adrenomedullary AT₂ receptor mRNA levels was observed in *Crh* KO mice and hypophysectomized rats, a non-HPA mechanism likely mediates this response. Stress-triggered activation of the splanchnic nerve markedly increases nicotinic cholinergic stimulation of the adrenal medulla as well as release of PACAP, which can elevate cAMP levels in chromaffin cells. Therefore, we looked at the response of AT₂ receptor gene expression to combined elevations in both cAMP and glucocorticoids. PC12 cells were treated for 24 h with 200 μM CPT-cAMP, a membrane permeable cAMP analog, alone or together with 1 μM dexamethasone. AT₂ receptor mRNA levels were decreased by approximately three orders of magnitude in response to CPT-cAMP (Fig. 7A). The combined treatment of CPT-cAMP and dexamethasone lowered AT₂ receptor mRNA levels to a similar extent. The response to PACAP was also examined. Treatment of PC12 cells for 7 h with concentrations of 0.01 pM to 1000 nM PACAP 1–38, the predominant endogenous form, elicited a dose-dependent reduction in AT₂ receptor mRNA levels ($F = 71.13$; $P < 0.0001$), while elevating *Th* ($F = 26.45$; $P < 0.0001$) and *Dbh* ($F = 130.40$; $P < 0.0001$) mRNA levels (Fig. 7B). A 90% reduction in AT₂ receptor mRNA levels was observed with 1000 nM PACAP 1–38, the highest concentration

Figure 5 Effects of single and repeated immobilization stress on AT₂ receptor gene expression in the adrenal medulla of WT and *Crh* KO mice. (A) WT and *Crh* KO mice were exposed to IMO for 2 h once (1×) or daily for 7 consecutive days (7×) and then killed immediately after the final IMO (IMO+0 h). The adapted control group was exposed to 6×IMO, then killed on the seventh day (6×IMO+24 h), representing both the starting point for the seventh IMO and the sustained effects of repeated IMO. Absolute controls were not exposed to stress. (B) AT₂ receptor mRNA levels in the adrenal medulla of WT or *Crh* KO mice were determined by real-time RT-PCR, normalized to *Gapdh* mRNA levels, and expressed relative to WT absolute control levels, taken as 1. **P*<0.05, ****P*<0.001 vs genotype-matched absolute control; #*P*<0.01, ###*P*<0.001 vs genotype-matched adapted control; ^a*P*<0.05 vs WT adapted control relative to absolute control for the respective genotype.

tested. These results indicate that PACAP-triggered elevation of cAMP could be mediating downregulation of AT₂ receptor gene expression immediately and shortly after stress.

Discussion

This study showed, for the first time, the kinetics of AT_{1A} and AT₂ receptor gene expression changes in the adrenal medulla in response to single and repeated exposure to IMO. AT_{1A} receptor mRNA levels are unaltered by any of the conditions examined, although binding is increased following repeated IMO. By contrast, AT₂ receptor gene expression is especially sensitive to IMO and demonstrated a biphasic response. Immediately following either single or repeated IMO, AT₂ receptor gene expression is rapidly downregulated. However, after 24 h recovery from repeated exposures to the stress, AT₂ receptor gene expression is higher than in unstressed absolute controls.

(as seen in adapted controls). AT₂ receptor binding is actually elevated 3 h after 6×IMO, similar to the AT_{1A} receptor. Together, these findings suggest that IMO alters the ratio of AT_{1A} and AT₂ receptor subtypes, which can affect catecholamine biosynthesis. Indeed, IMO changes the expression or binding of these receptors at a time when expression of the catecholamine biosynthetic enzymes is elevated (reviewed in Sabban & Kvetnanský (2001)). A role of AT₁ receptors is supported by the finding that *Th* and *Dbh* mRNA levels in PC12 cells are decreased by Ang II treatment in the presence of ZD7155 (an AT₁ receptor antagonist). Conversely, treatment with the AT₂ receptor agonist CGP42112 decreased *Th* and *Dbh* mRNA levels. Experiments to determine the mechanism by which stress alters AT₂ receptor gene expression show that glucocorticoids are able to lower AT₂ receptor mRNA levels in a transcriptionally mediated pathway. In this regard, glucocorticoids can act as negative regulators of basal adrenomedullary AT₂ receptor gene expression and appear to attenuate the upregulation of AT₂ receptor gene expression in the prolonged response to repeated stress. However, they are not essential for the rapid drop in AT₂ receptor gene expression in the immediate response to stress. Instead, this study suggests a possible role of PACAP and cAMP in the regulation of AT₂ receptor gene expression.

Stress-triggered changes in Ang II receptor expression in the adrenal medulla

AT₁ and AT₂ receptor gene and protein expression were studied using real-time RT-PCR and receptor binding,

Figure 6 Regulation of AT₂ receptor gene expression in the adrenal medulla of hypophysectomized rats under basal conditions and in response to single immobilization stress. Sham-operated or hypophysectomized (Hypox) rats, supplemented with or without cortisol, were exposed to a single 2-h immobilization stress and killed immediately (1 × IMO + 0 h). AT₂ receptor mRNA levels were determined in the adrenal medulla by real-time RT-PCR, normalized to *Gapdh* mRNA levels, and expressed relative to sham-operated unstressed controls, taken as 1. ***P* < 0.01 vs sham-operated unstressed control; ^a*P* < 0.05 vs Hypox unstressed control; [#]*P* < 0.05, ^{##}*P* < 0.01 vs indicated groups.

Figure 7 Effects of cAMP and PACAP on AT₂ receptor gene expression in PC12 cells. (A) PC12 cells were treated for 24 h with 1 μ M dexamethasone (Dex) in the presence or absence of 200 μ M CPT-cAMP (cAMP), a membrane-permeable cAMP analog. Subsequently, total RNA was isolated and AT₂ receptor mRNA levels were determined by real-time RT-PCR. Data are normalized to *Gapdh* mRNA levels and expressed relative to vehicle-treated control, taken as 1. *** P <0.001 vs control; # P <0.05, ## P <0.01 vs Dex-treated group. (B) PC12 cells were treated for 7 h with 0.01 pM to 1000 nM PACAP 1–38. Subsequently, total RNA was isolated and *Th*, *Dbh*, and AT₂ receptor mRNA levels were determined by real-time RT-PCR. Data are normalized to *Gapdh* mRNA levels and expressed relative to vehicle-treated control, taken as 1. *** P <0.001 vs control.

as the commercially available antibodies for the AT₁ (Benicky *et al.* 2012) and AT₂ receptors (R Nostramo, E L Sabban, J M Saavedra 2012, unpublished observations) are not specific. The downregulation of AT₂ receptor mRNA levels to as low as 1/10 of basal levels, observed 3 h after 1 $\times$ IMO, is also reflected by a tendency for reduced AT₂ receptor binding. The decline in AT₂ receptor binding with 1 $\times$ IMO is less pronounced than the changes in mRNA levels. This is probably due to the time point examined (3 h after the IMO), as the reduction in mRNA levels is likely to precede the changes in protein expression. Conversely, neither AT_{1A} receptor mRNA levels nor AT₁ receptor binding is altered by 1 $\times$ IMO. Therefore, acute exposure to IMO may trigger an increase in the relative expression of AT_{1A} to AT₂ receptors.

The changes in Ang II receptor expression following repeated stress exposure are different. With repeated IMO, there is a drop in AT₂ receptor mRNA levels immediately after the stress, yet at the time examined (3 h afterwards)

binding is elevated compared with unstressed absolute controls. This could reflect 1) the decrease in AT₂ receptor mRNA levels from a higher starting point before the sixth IMO (adapted controls) than before the first IMO (absolute controls) and 2) the more transient reduction in AT₂ receptor mRNA levels after repeated compared with single IMO. AT₁ receptor binding is also elevated 3 h after 6 $\times$ IMO, although there is no change in mRNA levels. This could reflect increased receptor stability, decreased recycling, or altered dimerization patterns without alterations in receptor gene transcription.

The changes in adrenomedullary AT_{1A} and AT₂ receptor mRNA levels were previously examined immediately following exposure of rats to restraint stress using *in situ* hybridization and ligand binding assays (Leong *et al.* 2002). Similar to our results with IMO using quantitative RT-PCR, AT₂ receptor mRNA levels decreased immediately following a single 2-h restraint. In contrast to our finding of a transient decline in AT₂ receptor mRNA levels with repeated (6 daily) IMO, AT₂ receptor mRNA did not differ from basal levels immediately following repeated (7 daily) exposures to restraint stress (Leong *et al.* 2002). Additionally, AT₁ receptor binding was unaltered immediately following repeated restraint stress, yet increased 3 h after repeated immobilization stress. These differences may reflect the decreased severity or more rapid habituation to the milder stress of restraint, compared with immobilization.

Effects of stress-triggered modulation of Ang II receptor expression on adrenomedullary function

The altered expression of AT₁ and AT₂ receptors likely has important consequences for the adrenomedullary response to stress, and in particular the degree of catecholamine production and release. Previous data support an inhibitory role of the AT₂ receptor and a stimulatory role of the AT₁ receptor on adrenomedullary catecholamine biosynthesis in response to selective Ang II receptor subtype activation in cultured porcine adrenomedullary chromaffin cells and in mouse adrenal medulla (Takekoshi *et al.* 2000, 2002, Armando *et al.* 2002). Through the pharmacological application of the compounds CGP42112 and ZD7155, we found that the AT₁ and AT₂ receptors mediate similar converse effects on *Th* mRNA levels, and for the first time, *Dbh* mRNA levels in PC12 cells. CGP42112, a partial AT₂ receptor agonist, can displace AT₂ receptors selectively and with high affinity (Heemskerk & Saavedra 1995). At the higher concentrations used here, however, it may exert additional effects unrelated to AT₂ receptors, as is the case for compound 21 (reviewed in Henrion (2012)). Similarly, ZD7155, a high-affinity AT₁ receptor antagonist (Remuzzi *et al.* 1996), has been used at high concentrations and the results obtained may be in part the consequence of nonselective effects. To completely elucidate the role of AT₂ receptors, it will be necessary to develop novel very specific, potent, and selective receptor agonists and antagonists.

As AT₁ receptor expression is very low in PC12 cells (data not shown), the altered response of *Th* and *Dbh* gene expression to Ang II in the presence of ZD7155 suggests that small changes in the ratio of AT_{1A}/AT₂ receptor expression may have significant effects on catecholamine biosynthesis following receptor activation. We hypothesize that the decrease in AT₂ receptor activation in response to acute stress may remove an inhibitory factor in catecholamine production, thus allowing AT₁ receptor stimulation by increased circulating and/or locally produced Ang II to increase catecholamine formation. In this regard, adrenal and urinary norepinephrine and epinephrine levels were decreased following isolation stress in rats treated with the AT₁ receptor antagonist candesartan (Armando *et al.* 2001). Conversely, the increase in AT₂ receptor gene expression following prolonged exposure to stress (observed in adapted controls) may be a protective mechanism, as AT₂ receptor upregulation mediates neuro-, vaso-, and reno-protective effects in numerous pathologies (reviewed in Jones *et al.* (2008)).

Role of the HPA and sympathoadrenal axes in the down-regulation of AT₂ receptor gene expression in response to stress

One of the major responses to stress is the activation of the HPA axis with elevations in plasma ACTH and corticosterone levels. Treatment of rat adrenal medulla primary cultures and PC12 cells with the synthetic glucocorticoid dexamethasone decreases AT₂ receptor mRNA levels. In this regard, dexamethasone decreased AT₂ receptor transcription and mRNA levels in serum-depleted PC12 cells (Kijima *et al.* 1995). This response in PC12 cells is progressive and is inhibited by actinomycin D, indicating that it is likely transcriptionally mediated. Accordingly, the promoter of the AT₂ receptor is reported to contain a glucocorticoid regulatory motif (Martin & Elton 1995, Ichiki *et al.* 1996). Our *in vivo* data demonstrate that glucocorticoids can act as negative regulators of basal adrenomedullary AT₂ receptor gene expression as cortisol supplementation reversed the hypophysectomy-induced increase in AT₂ receptor mRNA levels. However, this effect on basal AT₂ receptor gene expression was not observed in *Crh* KO mice. This difference may be due to activation of compensatory pathways or strengthening of redundant pathways, the consequences of the life-long *Crh* KO.

However, in response to stress, AT₂ receptor mRNA levels are decreased in WT and *Crh* KO mice. While this suggests that the stress-triggered rise in glucocorticoids is not essential for AT₂ receptor downregulation, it does not rule out a role for aldosterone. The absence of CRH has no effect on levels of plasma aldosterone (Muglia *et al.* 1995), which is released in response to stress (Stier *et al.* 2004) and can inhibit adrenomedullary AT₂ receptor mRNA levels (Wang *et al.* 1998). Conversely, hypophysectomy reduces basal plasma aldosterone levels (Rauschkolb *et al.* 1956, Balment *et al.* 1986) and probably aldosterone release during stress. As AT₂ receptor mRNA

levels are decreased following acute stress in sham-operated and hypophysectomized rats, the involvement of aldosterone is unlikely.

Even though glucocorticoids do not appear to be required for the downregulation of AT₂ receptor mRNA levels immediately following IMO, the findings suggest that they may attenuate AT₂ receptor gene expression in the prolonged response following repeated exposure to stress. Glucocorticoids may dampen the effects mediated by elevated AT₂ receptor expression in the long-term response to repeated stress exposure.

Much like the absence of an effect of IMO on adrenomedullary AT_{1A} receptor mRNA levels, dexamethasone also did not change AT_{1A} receptor gene expression. This suggests that there may be tissue-specific differences in glucocorticoid-mediated regulation of the AT_{1A} receptor gene as its promoter contains a glucocorticoid response element and dexamethasone treatment increased promoter activity in rat vascular smooth muscle cells (Guo *et al.* 1995, Murasawa *et al.* 1995).

In addition to glucocorticoids, IMO has been shown to activate multiple signaling pathways in the adrenal medulla (reviewed in Kvetnansky *et al.* (2009)) as well as lead to the induction of growth factors and cytokines (Liu *et al.* 2008), which are also implicated in the downregulation of AT₂ receptor gene expression in PC12 cells (Kijima *et al.* 1995). Elevated cAMP, by CPT-cAMP, was found to be very effective in lowering AT₂ receptor mRNA levels in PC12 cells, as previously observed in dibutyl cAMP-treated serum-depleted PC12 cells, which triggered destabilization of AT₂ receptor mRNA (Murasawa *et al.* 1996). After 24 h treatment with CPT-cAMP, there is almost a complete loss (three orders of magnitude decline) of AT₂ receptor mRNA levels, whether in the presence or absence of dexamethasone. Elevated cAMP could be involved in mediating the large reduction in AT₂ receptor gene expression with IMO. Stimulation of the adrenal medulla by release of acetylcholine and PACAP from the splanchnic nerve, with subsequent elevations in calcium and cAMP and their signaling pathways, are important in mediating the adrenomedullary response to stress (Hamelink *et al.* 2002, Kvetnansky *et al.* 2009, Stroth & Eiden 2010). Our results show that PACAP decreases AT₂ receptor mRNA levels in PC12 cells and thus may mediate the observed stress-induced changes in AT₂ receptor gene expression.

Overall, the changes in AT₁ and AT₂ receptor expression in the adrenal medulla likely contribute to the regulation of adrenomedullary function in response to stress. The rapid decrease in AT₂ receptor expression observed immediately following a single exposure to stress would result in increased AT₁ receptor activation, increasing catecholamine biosynthesis, and release to handle the immediate threat to homeostasis. Conversely, upregulation of the AT₂ receptor may serve a protective function after prolonged repeated stress exposure.

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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