Ang III induces the expression of inducible transcription factors of the AP-1 and Krox families in the rat brain

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Abstract

Besides rapid responses comprising increases in blood pressure, drinking and stimulation of natriuresis, angiotensin II (Ang II) induces the expression of transcription factors (TF) in the central nervous system. The Ang II metabolite Ang III (Ang 2-8) has been demonstrated to exert similar physiological effects as Ang II. We aimed to determine 1. whether Ang III induces TF expression in the brain; 2. which angiotensin (AT) receptor subtype is involved and 3. whether the two peptides, Ang II and Ang III, differ in their efficacy to stimulate TF expression. Ang II (100 pmol), Ang III (100 pmol) or vehicle were injected into the lateral brain ventricle of conscious rats alone or in combination with the AT₁ receptor antagonist losartan (10 nmol), the AT₂ receptor antagonist PD123319 (5 nmol) or the aminopeptidase inhibitor, amastatin (10 nmol). Similarly to Ang II, Ang III induced the expression of c-Fos, c-Jun and Krox 24 in four brain regions, the subfornical organ, median preoptic area, paraventricular nucleus and supraoptic nucleus of the hypothalamus with the same efficacy. This effect was AT₁ receptor-mediated. Pretreatment with amastatin reduced the expression of TF in response to Ang II, indicating that this expression is partly mediated by Ang III. Interestingly, the AT₂ receptor antagonist, PD 123319, alone slightly enhanced the expression of c-Fos, c-Jun and Krox 24 in different populations of neurons of the paraventricular nucleus. These data indicate that different populations of neurons in the paraventricular nucleus are tonically inhibited by AT₂ receptors under physiological conditions.

Keywords: c-Fos, c-Jun, Krox 24, hypothalamus, inducible transcription factor
Stimulation of brain AT\textsubscript{1} receptors by Ang II, the main effector peptide of the renin angiotensin system, induces a number of immediate effects comprising a rise in blood pressure, the release of arginine vasopressin (AVP) from the pituitary terminals of magnocellular neurons originating in the hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei, sympathetic modulation and drinking (5). However, Ang II, acting through its central AT\textsubscript{1} receptors, also induces a temporally and spatially highly differentiated expression of transcription factors of the AP-1 and Krox families which is restricted to the subfornical organ (SFO), median preoptic nucleus (MnPO) and the paraventricular (PVN) and supraoptic (SON) hypothalamic nuclei (10, 12, 16, 25). The distribution of c-Fos-positive cells in the SFO depends on the route of Ang II administration. While a stimulation of periventricular AT receptors increases c-Fos mainly in the cells adjacent to the ventricle, intravenous injection of Ang II, which reaches the brain via the circumventricular organs which lack the blood brain barrier, results in stimulation of neurons and c-Fos induction in the central parts of the SFO (15, 16, 24). Besides the SFO, regions like the PVN, SON or the MnPO display the same patterns of c-Fos expression to Ang II injected i.v. or icv (15, 16). When Ang II is injected icv, the expression of transcription factors in the SON and PVN can, at least partly, be ascribed to the activation of AT receptors in the SFO and MnPO. Lesions of the SFO or the anteroventricular region of the third ventricle were shown to inhibit Fos-like immunoreactivity in the SON and PVN (24, 31). The half life of Ang II in the brain tissue and cerebrospinal fluid is very short, only few seconds, because the enzyme aminopeptidase A (APA; glutamyl aminopeptidase, EC 3.4.11.7) catalyses the degradation of Ang II to angiotensin III (Ang III, (des-Asp\textsuperscript{1})-Ang II) (9). Both Ang II and Ang III bind with similar affinities to the two angiotensin receptor subtypes, AT\textsubscript{1} and AT\textsubscript{2}. When injected intracerebroventricularly (icv) or directly into
certain brain regions, Ang III produces an almost identical cardiovascular response and vasopressin release as Ang II (30, 33). However, Ang III injected centrally was less effective (about 50%) than Ang II in promoting dipsogenic responses (6). Both angiotensin peptides are equally released in the hypothalamic PVN following hyperosmotic stimulation in conscious rats (18).

A number of findings derived from experiments in which new, more specific aminopeptidase inhibitors or an anticatalytic aminopeptidase A antiserum were employed are in line with the previously formed hypothesis that Ang III is the main effector peptide of the RAS in the brain (26, 29, 33).

In the present study, we investigate whether Ang III is also involved in the Ang II-induced expression of inducible transcription factors (ITF) in the brain. We have compared the expression of c-Fos, c-Jun and Krox-24 following stimulation of periventricular angiotensin receptors with Ang II and Ang III in conscious rats and investigated the contribution of angiotensin receptor subtypes to these effects. For this purpose, rats were pretreated with the selective angiotensin AT₁- and AT₂ receptor antagonists, losartan and PD 123 319, respectively, prior to Ang II or Ang III injections. The aminopeptidase inhibitor, amastatin, was used to determine which of the two angiotensin peptides is more effective as inducer of transcription factor expression in the brain.

Methods

Animals

Male Wistar rats (280-300g BW) were obtained from Charles River, Sulzfeld, Germany. The animals were kept under controlled temperature, humidity and light/dark period and had free access to food and water.
Intracerebroventricular Injections

For intracerebroventricular (icv) injections, chronic cannulae were implanted into the lateral brain ventricle under chloralhydrate anaesthesia (400 mg/kg body weight, intraperitoneally). The animals were housed individually and were allowed a one week recovery period after surgery. During this time, rats were handled daily to avoid non-specific, stress-induced expression of transcription factors on the day of the experiment.

All icv injections were made between 8 and 11 a.m. in conscious, freely moving rats to avoid the interference of circadian rhythms with transcription factor expression. The injection volume was 5 µl (1 µl substance, followed by 4 µl isotonic saline, or 5 µl isotonic saline in controls.

Rats were allocated to 9 groups (n=5 animals per group). Group 1 received isotonic saline. Group 2 received Ang II (100 pmol). Group 3 received Ang III (100 pmol). Groups 4, 5, 6 and 7 received the AT₁ receptor antagonist losartan (10 nmol) or the AT₂ receptor antagonist PD 123 319 (5 nmol), respectively, alone or followed by Ang III (100 pmol). Group 8 and 9 received the aminopeptidase inhibitor amastatin (10 nmol) alone or in combination with Ang II (100 pmol), respectively. The inhibitors were injected 10 minutes before treatment with Ang III or Ang II. The doses of the antagonists were chosen according to the literature (3, 11, 28).

Immunohistochemistry

Ninety minutes after the last injection, rats were deeply anaesthetised with chloralhydrate (400 mg/kg body weight, intraperitoneally) and perfused intracardially with phosphate-buffered saline, followed by 4% parafomaldehyde solution for the
fixation of the brain tissue. Brains were removed, postfixed overnight in 4% paraformaldehyde and immersed in 30% sucrose for cryoprotection for three days. Immunohistochemistry was performed on coronal, cryostat-cut, free floating slices (50 μm). Incubation with the primary antiserum was followed by detection using the conventional avidin-biotin complex peroxidase reaction with diaminobenzidine as chromogen as described before (12). The dilutions of the antibodies were as follows: anti-c-Fos 1:20 000; anti-c-Jun 1:1000; anti-Krox-24 1:4000. The secondary antibody, goat-anti-rabbit IgG (H+L), was used in a dilution of 1:400 according to the manufacturer’s instructions (Vectastain Kit, Vector Laboratories, Burlingame, Ca, U.S.A). For double immunohistochemistry, slices were first incubated with the anti-peptide antiserum, followed by visualisation of the binding as described above with the vectastain SG substrate as chromogen. This was followed by incubation with the specific antisera against the transcription factors, visualized with DAB as chromogen. The dilutions of the anti-peptide antisera were 1:5000 for anti-oxytocin and anti-arginine vasopressin (AVP), 1:100 for anti-corticotropin releasing factor (CRF).

Drugs and antibodies used
Ang II and amastatin were purchased from Sigma-Aldrich (Taufkirchen, Germany), Ang III was from Bachem (Bubendorf, Switzerland). Losartan was a generous gift from Dr. R.D. Smith (Du Pont Merck Pharmaceutical Co., Wilmington DE, U.S.A.). PD 123 319 was a generous gift from Dr. H. Heitsch (Aventis, Frankfurt, Germany). Both peptides and the antagonists were dissolved in physiological saline. The anti-c-Fos and anti-c-Jun rabbit antisera as well as the anti-CRF antiserum were from Oncogene (Cambridge, MA, U.S.A.). The polyclonal anti-Krox-24 antibody was a
generous gift from Rodrigo Bravo (Bristol-Myers Squibb, Princeton, NJ, USA). The antibodies against arginine vasopressin and oxytocin were from Chemicon (Hofheim, Germany). The vectastain ABC kit and the blue vectastain SG substrate were purchased from Biologo (Kiel, Germany).

Statistics

Data represent means ± SD. Corresponding sections of different brain regions were photographed with a video camera. For the MnPO and SFO, 2 sections per animal, for the SON and PVN, 3 sections per animal were used. In case of the MnPO and SFO, the whole area of the respective nucleus was counted. In case of the bilateral SON and PVN, each side was counted separately. Stained neurons were counted with a computer program (Leica Qwin, Leica, Bensheim, Germany), which allows to directly select the area that should be counted and also calculates the size of the chosen area. Thus, the obtained counts were comparable. Average numbers and SDs were calculated per area and per group. Statistical analysis was carried out using ANOVA followed by the Bonferroni test. A value of P<0.05 was accepted as significant.

Results

c-Fos

A stimulation of periventricular angiotensin receptors with Ang III resulted in a strong expression of c-Fos in neurons of the SFO, MnPO, PVN and SON. The number of neurons in each of these four areas which expressed c-Fos was similar to the number of c-Fos-positive neurons after icv injection of Ang II (Table 1). The Ang III-
induced c-Fos expression was mediated by the AT1 receptor subtype, since
pretreatment with the selective AT1 receptor antagonist losartan at a dose which had
been shown previously to inhibit the expression of c-Fos to Ang II, almost completely
abolished the expression of c-Fos following icv Ang III (Table 1). Losartan, given
alone, was without effect (Table 1). Pretreatment with the selective AT2 receptor
antagonist, PD 123319, did not affect the Ang III-induced c-Fos expression,
indicating that the AT2 receptor was not involved. An interesting finding of the present
study is that the AT2 receptor antagonist PD 123 319 alone induced c-Fos
expression, which was almost exclusively restricted to the magnocellular part of the
PVN with single scattered neurons in the parvocellular part of the nucleus. The c-Fos
protein was co-localised with AVP, but not with oxytocin or CRF (Figure 1). Inhibition
of Ang II metabolism with the aminopeptidase inhibitor, amastatin, reduced the Ang
II-induced expression of c-Fos by about 50% in the SFO and SON and by about 30
% in the MnPO and PVN. Amastatin alone also slightly increased the expression of c-
Fos which was, however, much lower than c-Fos levels after a combined treatment
with amastatin/Ang II, but higher than the control levels (Table 1).

c-Jun
c-Jun levels tended to be lower than those of c-Fos except in the PVN. The Ang II
metabolite Ang III induced the expression of c-Jun in the MnPO, SFO and PVN to a
similar extent as Ang II (Table 1). In the SON, almost no expression of c-Jun was
detected 90 minutes after stimulation. This finding is in line with previous results
demonstrating that, in the SON, this transcription factor is induced at later time points
than in the other nuclei. Again, the expression of c-Jun following Ang III could be
inhibited with the selective AT1 receptor antagonist but not with the inhibitor of AT2
receptors (Table 1). Losartan alone had no effect on c-Jun expression as already
reported previously. Similarly to the c-Fos expression, PD 123 319 alone stimulated the expression of c-Jun in the magnocellular part of the PVN. When the aminopeptidase inhibitor, amastatin was given prior to Ang II to inhibit the generation of Ang III from Ang II, the number of cells positively stained for c-Jun was reduced to about 50% in the SFO, MnPO and PVN. Amastatin alone also increased the expression of c-Jun slightly above basal levels of control animals treated with vehicle (Table 1).

Krox 24
Ang II and Ang III were equipotent in stimulation of the Krox 24 expression (Table 1). Compared to c-Fos and c-Jun, the Krox 24 expression was especially high in the SON. Other nuclei showed no differences between the expression of Krox 24 and the AP-1 transcription factors (Table 1). The Ang III-induced Krox 24 expression was mediated by AT1 receptors, since pretreatment with losartan almost completely abolished the expression of this transcription factor in all four brain areas while pretreatment with PD123 319 had no effect (Table 1). Again, the AT2 receptor antagonist alone increased the number of Krox 24-positive neurons. In contrast to the expression patterns of c-Fos and c-Jun, Krox 24 protein was localised mainly in the parvocellular part of the PVN. The co-localisation experiments showed that neither vasopressinergic nor oxytocinergic neurons expressed this transcription factor (Figure 2). Pretreatment with amastatin diminished the Ang II-induced Krox 24 by about 60%, indicating that the increase in Krox 24 expression after Ang II was partly mediated by Ang III (Table 1). Animals treated with amastatin alone displayed a slightly enhanced Krox 24 protein when compared with vehicle-treated controls.
Discussion

The present study shows that Ang III is able to stimulate the expression of transcription factors of the AP-1 and Krox families. The Ang III-induced transcription factor expression was restricted to the same brain regions as the transcription factor expression induced by the main effector of the RAS, Ang II, namely the SFO, MnPO, PVN and SON. Our results clearly demonstrate that Ang III increased the transcription factor expression upon binding to the AT₁ receptor subtype. The aminopeptidase A inhibitor, amastatin, applied prior to Ang II reduced the expression of transcription factors in response to the peptide, indicating that at least part of the Ang II-induced transcription factor expression in vivo is mediated by the Ang II metabolite Ang III.

Ang III is generated from Ang II by an aminopeptidase A-catalysed removal of an N-terminal amino acid. Both peptides bind to the AT₁ and AT₂ receptor subtypes with similar affinities (8). As early as 1971, Blair-West and co-workers demonstrated that Ang III increased the secretion of aldosterone in a manner equipotent to Ang II (2). Since then, a number of studies have reported on effects of Ang III in peripheral tissues and organs. Most of these effects, which are similar to those produced by Ang II, are mediated by the AT₁ receptor subtype. Besides its effects in the periphery, Ang III is also generated in the brain and, similarly to Ang II, may act as a neurotransmitter/neuromodulator. Thus, Ang III has been demonstrated to be as potent as Ang II in the central regulation of blood pressure and renal functions, as evidenced by an increase in renal blood and urine flows, glomerular filtration rate or sodium and potassium excretion) (4). As mentioned above, stimulation of brain AT₁ receptors with Ang III increases the release of vasopressin from pituitary terminals of SON and PVN magnocellular neurons and stimulates drinking (19). Brain Ang III also
seems to exert a tonic stimulatory control over arterial blood pressure (20). Our results along with findings of others suggest that Ang III and not Ang II is the main effector peptide of the RAS in the brain.

In the present study, Ang II induced the expression of ITF also when its conversion to Ang III was inhibited. We employed a commercially available aminopeptidase A inhibitor, amastatin, which has been used in a number of previous studies (28). This inhibitor inhibits the aminopeptidase A and the aminopeptidase N with equal potencies. The latter enzyme catalyses the formation of angiotensin IV (Ang IV) from Ang III (22). The Ang IV binding site has been recently identified as the enzyme insulin-regulated membrane aminopeptidase (1). Ang IV can also stimulate the expression of ITF in the brain. The brain regions which respond to Ang IV with increased transcription factor expression also contain Ang IV binding sites and are not identical with the areas in which Ang II or Ang III induce ITF expression (23). Therefore, the observed reduction of ITF expression to Ang II after pretreatment with amastatin is rather caused by lack of Ang III resulting from its decreased generation from Ang II than by a perturbation of Ang IV levels.

We report in the present study that, compared to controls, treatment with the AT$_2$ receptor antagonist PD123 319 alone increased c-Fos, c-Jun and Krox 24 in the PVN. The AT$_2$ receptor is G protein-coupled like the AT$_1$ receptor, and is predominantly expressed in the brain during foetal development. In most regions of the adult rat brain this receptor subtype is found at very low levels. Although the AT$_2$ receptor appears to be suppressed in the adult brain, few areas, such as the cerebellum, superior and inferior olive and the locus coeruleus display remarkable levels of AT$_2$ receptors (13). The AT$_2$ receptor has been implicated in such diverse processes as wound healing (it is e.g. up-regulated in the cortex after brain ischemia (14, 32) ), the regeneration and differentiation of neuronal tissue as well as apoptosis.
The results of the present study demonstrate that AT2 receptors exert a tonic inhibitory control on neurons of the hypothalamic PVN. Once this tonic inhibition is removed by blockade of the AT2 receptor, the neurons respond with an expression of transcription factors. It appears that this reaction can only be observed within a very limited range of doses of the AT2 receptor antagonist. In previous experiments, when we used a higher dose of PD 123 319, we did not observe any effect of this compound on the expression of transcription factors in the PVN. This was possibly due to the fact that PD 123319 at higher concentrations can also interact with the AT1 receptor subtype. At lower doses, however, presumably only AT2 receptors are selectively blocked and angiotensin peptides can increasingly interact with AT1 receptors, as the AT2 receptor antagonist leaves the AT1 receptor unopposed and rather exposes it to elevated Ang II levels.

We have shown previously that the AT1 receptor-mediated vasopressin release and drinking response are potentiated after pretreatment with PD 123319 at a dose similar to that used in the present study (11). Interestingly, the transcription factors whose expression is up-regulated in both, the magnocellular and parvocellular parts of the PVN after stimulation of periventricular AT receptors, show distinct expression patterns after AT2 receptor blockade. The members of the AP-1 family of transcription factors were detected mainly in magnocellular vasopressinergic neurons, although single scattered c-Fos-positive neurons could also be found in the lower parvocellular part of the PVN close to the third ventricle. Co-localisation studies revealed no c-Fos expression in oxytocinergic neurons or CRF-positive neurons. On the other hand, Krox 24 was localised mainly in the upper parvocellular part of the PVN. Again, a few scattered neurons were detected close to the third ventricle in the lower PVN. Krox 24 was neither co-localised with AVP nor with oxytocin.
The number of AT$_2$ receptors in the PVN is very low. Nevertheless, the AT$_2$ receptor antagonist selectively increased transcription factors in the PVN. Other brain areas, the SFO, the MnPO and the SON did not respond to PD 123 319 with increased expression of transcription factor, although: i) they also contain AT$_2$ receptors; ii) the ratios of AT$_1$ and AT$_2$ receptors in the PVN, SFO, SON and the MnPO are almost identical and, iii) all brain areas, except for the SON, are localized in close vicinity to the third ventricle and can, therefore, be equally targeted with the antagonist. Obviously, angiotensin peptides acting on AT$_2$ receptors selectively inhibit neurosecretory neurons in the PVN but not other types of peptidergic neurons, e.g. in the MnPO.

Oxytocin- and vasopressin-synthesising neurons are also localised in the SON. However, the SON is localised far from the third ventricle and PD 123319 applied as a single icv injection, could hardly reach these neurons to produce an effective inhibition of AT$_2$ receptors. Therefore, our data does not allow any conclusion regarding the role of AT$_2$ receptors in this brain region. It appears that angiotensin peptides upon activation of AT$_1$- and AT$_2$ receptors can stimulate, but also inhibit neurosecretory neurons in the PVN to ensure a fine tuning of both, rapid responses, including hormonal regulations and the central control of blood pressure and volume homeostasis (11) and delayed effects comprising the activation of transcription factor and the regulation of the expression patterns of various inducible genes.

We conclude that both angiotensin peptides, Ang II and Ang III, upon binding to AT$_1$ receptors are able to stimulate the expression of transcription factors in the same brain regions. Our results also indicate that, under physiological conditions, Ang III considerably contributes to the increased activation of transcription factors in response to Ang II. Besides stimulatory effects, which are mediated by AT$_1$ receptors,
angiotensin peptides exert, by binding to AT₂ receptors, a tonic inhibitory control on the expression of transcription factors of the AP-1- and Krox families.

Thus, it seems that AT₂ receptors exert their tonic inhibition on different populations of neurons, and these neurons respond with distinct signals to the discontinuation of the inhibitory control. This finding makes the expression of transcription factors a powerful tool to study tonic inhibitory signalling systems.

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Table 1. Expression of c-Fos, c-Jun and Krox 24 by Ang II and Ang III and inhibition by pretreatment with losartan, PD 123 319 or amastatin.

Ang II (100 pmol), Ang III (100 pmol) or amastatin (10 nmol), losartan (10 nmol), PD 123 319 (5 nmol), isotonic saline or a combination of losartan (10 nmol) /Ang III (100 pmol), PD 123 319 (5 nmol) /Ang III (100 pmol) or amastatin (10nmol)/Ang III (100 pmol) were injected into the lateral brain ventricle of conscious rats. 90 minutes after treatment, the brains were fixed by intracardial perfusion with 4% paraformaldehyde. Expression of ITF was studied on coronal brain sections using polyclonal antisera followed by a peroxidase reaction with DAB as chromogen. The immunoreactive neurons were counted separately for each brain region with a computer program. Values are expressed as means ± SD (n=5 animals per group).
Figure 1. Effect of a blockade of AT$_2$ receptors with PD 123 319 on the expression of c-Fos in the PVN

PD 123 319 (5 nmol) was injected into the lateral brain ventricle of conscious rats. 90 minutes after treatment, the brains were fixed by intracardial perfusion with 4% paraformaldehyde. Expression of ITF was studied on coronal brain sections using polyclonal anti-c-Fos antiserum (1:20 000) followed by a peroxidase reaction with DAB as chromogen. Co-localisation of c-Fos with AVP (anti-AVP antiserum, 1:5000), oxytocin (anti-oxytocin-antiserum, 1:5000) or CRF (anti-CRF-antiserum, 1:100) was obtained by using a blue peroxidase substrate (SG, Vectastain) for the peptides and DAB as chromogen for c-Fos. (A), c-Fos in the PVN, (B) co-localisation of c-Fos and AVP, (C) co-localisation of c-Fos with oxytocin, (D) co-localisation with CRF

Figure 2. Effect of a blockade of AT$_2$ receptors with PD 123 319 on the expression of Krox-24 in the PVN

PD 123 319 (5 nmol) was injected into the lateral brain ventricle of conscious rats. 90 minutes after treatment, the brains were fixed by intracardial perfusion with 4% paraformaldehyde. Expression of ITF was studied on coronal brain sections using polyclonal anti-Krox 24 antiserum (1:4 000) followed by a peroxidase reaction with DAB as chromogen. Co-localisation of Krox 24 with AVP (anti-AVP antiserum, 1:5000) or oxytocin (anti-oxytocin-antiserum, 1:5000) was obtained by using a blue peroxidase substrate (SG, Vectastain) for the peptides and DAB as chromogen for Krox 24. (A), Krox 24 in the PVN, (B) co-localisation of Krox 24 and AVP, (C) co-localisation of Krox 24 with oxytocin.

mPVN: magnocellular part of the PVN; pPVN: parvocellular part of the PVN
derivatives. Effect of inhibitor structure on slow-binding processes.

intracerebroventricular injection of angiotensin II and IV in rats.

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<td>22±7+++</td>
</tr>
<tr>
<td>SON</td>
<td>2±2****</td>
<td>53±18</td>
<td>57±11</td>
<td>2±2***</td>
<td>2±1***</td>
<td>48±30</td>
<td>1±1***</td>
<td>12±6+++</td>
<td>2±1+++</td>
</tr>
</tbody>
</table>

*: p<0.05; **: p<0.01; ***: p<0.001 vs Ang III
+: p<0.05; ++: p<0.01; +++: p<0.001 vs Ang II