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Angiotensin II mediates catecholamine and neuropeptide Y secretion in human adrenal chromaffin cells through the AT_1 receptor

Claudia Cavadas[a], Daniela Grand[a], François Mosimann[b], Maria Dulce Cotrim[c], Carlos Alberto Fontes Ribeiro[d], Hans R. Brunner[a], Eric Grouzmann[a,*]

[a] *Division of Hypertension and Vascular Medicine, Centre Hospitalier Universitaire Vaudois, 1011 Lausanne, Switzerland*
[b] *Department of Surgery, Centre Hospitalier Universitaire Vaudois, 1011 Lausanne, Switzerland*
[c] *Laboratory of Pharmacology, Faculty of Pharmacy, University of Coimbra, 3000 Coimbra, Portugal*
[d] *Department of Pharmacology, IBILI, Faculty of Medicine, University of Coimbra, 3000 Coimbra, Portugal*

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Abstract

The aim of the present work was to study the effect of angiotensin II (Ang II) on catecholamines and neuropeptide Y (NPY) release in primary cultures of human adrenal chromaffin cells. Ang II stimulates norepinephrine (NE), epinephrine (EP) and NPY release from perifused chromaffin cells by 3-, 2- and 12-fold, respectively. The NPY release is more sustained than that of catecholamines. We found that the receptor-AT_2 agonist, T_2-(Ang II 4–8)$_2$ has no effect on NE, EP and NPY release from chromaffin cells. We further showed that Ang II increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The selective AT_1-receptor antagonist Candesartan blocked $[Ca^{2+}]_i$ increase by Ang II, while T_2-(Ang II 4–8)$_2$ was ineffective. These findings demonstrate that AT_1 stimulation induces catecholamine secretion from human adrenal chromaffin cells probably by raising cytosolic calcium.

Keywords: Human adrenal chromaffin cells; Catecholamines; Neuropeptide Y; Angiotensin II; AT_1 receptor; AT_2 receptor

1. Introduction

It is well established that angiotensin II (Ang II) is able to stimulate catecholamine release from bovine, rat and porcine adrenal chromaffin cells [1–7]. However, controversy exists about whether AT_1 or AT_2 receptors are involved in this effect in several species [6–12]. No study is available to clearly demonstrate, in vitro, the effect of Ang II on human adrenal medulla on catecholamine release since this subject has been debated almost 40 years ago [13]. Neuropeptide Y (NPY) is a 36-amino-acid peptide present in the adrenal medulla of many species, including humans [14–16] and is co-localized with catecholamines in the same granules [17]. Functionally, NPY potentiates the effect of various agonists such as Ang II and norepinephrine (NE) in addition to exerting a direct contractile effect on the vasculature [18]. We have previously found that NPY, like nicotine, increases catecholamine release from human chromaffin cells through the NPY y3 receptor [19]. The aim of this work was to study the effects of Ang II on NE, epinephrine (EP) and NPY secretions in human adrenal chromaffin cells in culture.

2. Materials and methods

2.1. Peptides and antagonists

Angiotensin II was purchased from Novabiochem (Laufelfingen, Switzerland). T_2-(Ang II 4–8)$_2$ is a template-assembled peptide agonist for AT_2 receptors made of two angiotensin II 4–8 pentapeptide fragments (Ang II 4–8)$_2$, attached to a carrier molecule (T_2) which alone did not bind to either AT_1 or AT_2 receptors. Binding assays showed that in the presence of an AT_1 antagonist, T_2-(Ang II 4–8)$_2$ completely inhibited the specific binding of ^{125}I-AII to the AT_2 receptors of a rat adrenal membrane preparation and that half-maximal inhibition (IC_{50}) occurred at the concentration of 2×10^{-7} M. In contrast, T_2-(Ang II 4–8)$_2$ at the concentration of 10^{-5} M did not bind to AT_1 receptors of rat aortic

* Corresponding author. Present address: Centre Hospitalier Universitaire Vaudois, Division of Clinical Pharmacology and Toxicology, 1011 Lausanne, Switzerland. Tel.: +41-21-314-0741; fax: +41-21-314-4266.
E-mail address: eric.grouzmann@chuv.hospvd.ch (E. Grouzmann).

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smooth muscle cells. T_2-(Ang II 4–8)$_2$ mediates an agonistic angiotensin II effect on neurones of the inferior olive that express only AT_2 receptors [20]; the AT_1 antagonist Candesartan was obtained from Astra-Zeneca (Molndal) [21].

2.2. Adrenal glands and cell culture

The study was approved by the Hospital Transplantation Review Board and by the Medical Direction. For these studies, adrenal glands were obtained from four kidney transplant donors. All the donors were brain dead patients whose relatives had accepted multiorgan procurement. Chromaffin cells were isolated by the procedure described previously [19]. We shown that such preparation with differential plating allowed to obtain at least 95% pure chromaffin cell in culture.

2.3. Assay of $[Ca^{2+}]_i$

Intracellular free calcium concentration ($[Ca^{2+}]_i$) was determined using the fluorescent probe fluo-3/AM (Molecular Probes) as previously described [22]. Cells were preincubated for 2 min with the AT_1 antagonist Candesartan (1 μM) prior the addition of Ang II.

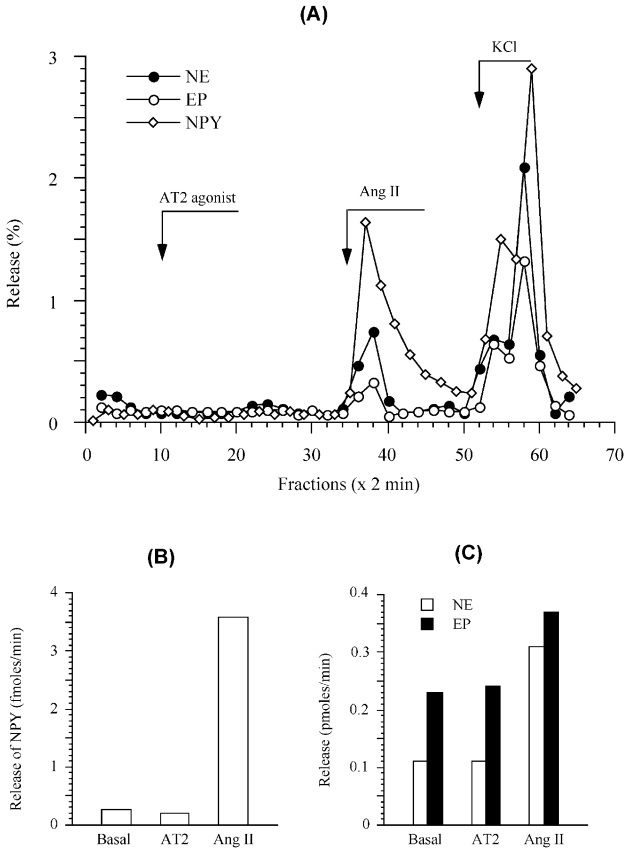

Fig. 1. Effect of AT_2 agonist, Ang II and KCl on basal release of NE, EP and NPY from human adrenal chromaffin cells. Cells were perifused for 20 min with KB followed by 20 min with 500 nM AT_2 agonist, 30 min with KB, 20 min with 100 nM Ang II, 14 min with KB and 16 min with 56 mM KCl. Every 2 min, perifusion samples were collected alternatively for NPY and for catecholamines assays. (A) Release of NE, EP and NPY is expressed as % of the intracellular content. Amount of NPY (B) and NE and EP (C) released per minute. One representative experiment of three is shown.

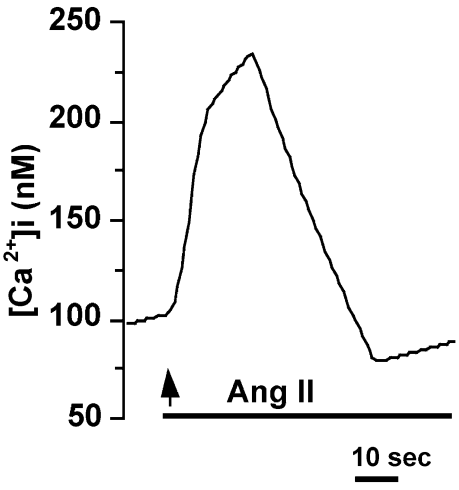

Fig. 2. Angiotensin II 100 nM increases $[Ca^{2+}]_i$ in human adrenal chromaffin cells. Typical trace taken from the recording of a single chromaffin cell loaded with fluo-3/AM and exposed with Ang II.

2.4. Release experiments

Cells were plated on glass coverslips placed in a perifusion chamber warmed at 37 °C and stabilized for 90 min at a flow rate of 0.35 ml/min with Krebs buffer (KB). The drugs were administered in KB as a prolonged infusion. Samples were then collected alternately every 2 min for NPY and catecholamine assays [19]. At the end of the perifusion, the coverslips were recovered and the cells lyzed with 0.5 ml of 0.4 M perchloric acid and sonicated. After centrifugation, an aliquot of supernatant was used to measure catecholamines and NPY. Catecholamines (NE and EP) were determined by HPLC with electrochemical detection (Waters, Milford, MA) [23]. NPY concentrations were measured by enzyme immunoassay [24].

2.5. Statistical methods

All values are expressed as mean ± S.E.M.. The data were compared by one-way analysis of variance with post hoc analysis using Student's unpaired *t*-test according to Bonferroni's method.

3. Results

3.1. Angiotensin II stimulates catecholamine and NPY secretions

In perifused chromaffin cells, catecholamines and NPY are constitutively released (Fig. 1A). NE, EP and NPY are

secreted at a rate of 0.08 ± 0.01%, 0.07 ± 0.007% and 0.07 ± 0.008% of intracellular content/2 min, respectively. The AT_2 agonist T_2-(Ang II 4–8)$_2$ (500 nM) infusion for 20 min had no effect on catecholamine, and NPY release by chromaffin cells with NE, EP and NPY are secreted at a rate of 0.06 ± 0.01%, 0.07 ± 0.008% and 0.06 ± 0.003% of intracellular content/2 min, respectively. Chromaffin cells constitutively released similar amounts of NE and EP representing 53% of total catecholamine secreted. In contrast, Ang II (100 nM) infusion for 20 min increased the release of both NPY and catecholamines by about 12- and 3-fold, respectively (Fig. 1B and C). Ang II produced a higher increase of NE secretion compared to the one observed for EP; therefore, AngII caused a preferential release of secretory granules that contain NE over those that are filled with EP or NPY, representing 80% of total catecholamine secreted.

The pattern of secretion is divided into two phases; a few seconds after exposure to Ang II, NE, EP and NPY were secreted apparently simultaneously with a peak at 4–6 min. However, NE and EP secretion returned to basal levels within 10 min after the addition of Ang II, whereas 10 min after removal of Ang II, NPY secretion rate was still higher than basal release (Fig. 1A). KCl was the most potent stimulus for catecholamine and NPY secretion (Fig. 1A).

3.2. Signal transduction induced by angiotensin II receptors in chromaffin cells

Next, we examined the pharmacology of functional Ang II receptors expressed on individual human chromaffin cells by measuring $[Ca^{2+}]_i$ increases evoked by Ang II, the AT_2 agonist T_2-(Ang II 4–8)$_2$ and Ang II after preincubation of the chromaffin cells with 1 μM Candesartan, an AT_1 antagonist. Ang II induced an increase in $[Ca^{2+}]_i$ peaking in 20 s followed by a rapid decrease within 30 s (Fig. 2). The basal $[Ca^{2+}]_i$ level was 127 ± 12 nM (n = 306). Ang II at 10 and 100 nM concentrations caused a similar mean increase in $[Ca^{2+}]_i$ of 68 ± 18 and 59 ± 16 nM, respectively (Table 1). Ang II stimulated 47% of the cells at 10 nM and 30% at 100 nM. Interestingly, when we take into account the responding cells, we found a stronger increase in $[Ca^{2+}]_i$ with the highest dose of Ang II. We did not observe any changes in calcium cytosolic concentrations when the cells were preexposed to Candesartan for 2 min, but the calcium response to 10 nM Ang II was strongly attenuated (only 10% of responding cells) increasing 7 ± 4 nM the $[Ca^{2+}]_i$ (Table 1). In addition, the AT_2 agonist T_2-(Ang II 4–8)$_2$ (100 nM) had no effect on $[Ca^{2+}]_i$ (Table 1).

Table 1
$[Ca^{2+}]_i$ in human adrenal chromaffin cells

	$\Delta[Ca^{2+}]_i$ (nM) in all cells	$\Delta[Ca^{2+}]_i$ (nM) responding cells	% of responding cells
Ang II (100 nM) (n = 156)	59 ± 16	218 ± 58	30
Ang II (10 nM) (n = 68)	68 ± 18	144 ± 34	47
Ang II (10 nM) + Candesartan (1 μM) (n = 72)	7 ± 4***	84 ± 41	10
AT_2 agonist (100 nM) (n = 33)	0	0	0

Data were obtained from four different glands. n is the total number of cells undergoing individual calcium measurement. Responding cells represent the proportion of cells that increase their free cytosolic calcium by at least 10 nM. Candesartan (1 μM) was added 2 min before Ang II 10 nM. AT_2 agonist used was T_2-(Ang II 4–8)$_2$. Data are expressed as the mean ± S.E.M. increase in $[Ca^{2+}]_i$.

***p < 0.001 compared to Ang II 10 nM.

4. Discussion

Numerous lines of evidence indicate that Ang II stimulates catecholamine secretion from the adrenal medulla of several species [1–7]. However, the involvement of AT_1 and AT_2 receptors in such effect is also dependent on the animal species studied [6–12]. It has been shown that cultured porcine chromaffin cells secreted catecholamines in response to an AT_2 agonist [9], whereas other studies have shown that Ang II increases catecholamine release from bovine chromaffin cells through functionally active AT_1 receptors [12]. Interestingly, some investigations reported mixed population of Ang II receptors in adrenal medulla depending on the species studied; for instance, AT_2 receptors accounted for 95% of Ang II binding sites in rat adrenal medulla, and 5% of the remaining binding sites were AT_1 [14]. Binding experiments are in agreement with functional studies since the AT_2 receptor agonist CGP42112 induced catecholamine release from the rat adrenal medulla and the effect of Ang II was markedly inhibited by the AT_2 antagonist PD123319, but was not affected by the AT_1 antagonist Losartan [7]. However, a slight inhibitory effect of Losartan on catecholamine release evoked by Ang II indicated that AT_1 receptors could be partially involved [7]. Indeed, another study reported that the catecholamine release from rat adrenal medulla was mainly mediated by AT_1 stimulation linked to an increase in cytosolic calcium concentrations [6]. The exact subtype of Ang II receptor(s) in human adrenal medulla is not clearly established since one study reports that mRNA encoding for the AT_1 receptor predominates (85%) over the AT_2 receptor (15%) [10], whereas contradictory autoradiographic studies reveal that AT2 receptors are found mainly in the medulla [11]. We have previously shown that AT_1 receptors are transcribed and translated in functional proteins in a human pheochromocytoma and that Ang II increases intracellular calcium and induces a dose-dependent secretion of NE and NPY in pheochromocytes [25]. Our present findings demonstrate that Ang II

increases catecholamine secretion on human chromaffin cells through the AT_1 receptor since our AT_2 agonist was ineffective. Therefore, we used a high concentration of T2-$(AngII\ 4-8)_2$ (500 nM) far above the IC_{50} (190 nM) on AT_2 receptor to ensure that the peptidomimetic occupied 90% of the binding site available in a preparation of cells expressing the AT_2 receptor [26]. Unfortunately, the cellular effects linked to AT_2 stimulation are generally difficult to be demonstrated and despite the lack of positive control for our AT_2 agonist with chromaffin cells, this molecule has proven to be active in various models where the AT_2 receptor was involved [20,21]. We observed a preferential secretion of NE rather than EP in response to Ang II, suggesting that stimulus-secretion coupling of the exocytic machinery may be different for EP- and NE-containing cells as previously found in bovine chromaffin cells [27]. The fact that the AT_1 antagonist Candesartan blocked the Ang II-induced $[Ca^{2+}]_i$ mobilization establishes that the link between transduction and secretion occurred via the AT_1 receptor. The finding that Ang II evokes a simultaneous release of NPY, EP and NE is in favor of a common storage of NPY within NE and/or EP granules; moreover, the long-lasting secretion of NPY even after the end of Ang II infusion might reflect the existence of a distinct pool of granules containing mainly NPY. We previously observed the same phenomenon when nicotine was used as a secretagogue [19]. Since NPY released from the adrenal medulla could locally enhances the secretion of catecholamine through a autocrine/paracrine mechanism [19], we postulated that the Ang II-induced NPY secretion also contributes to enhance a sustain secretion of catecholamine.

Acknowledgements

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