Angiotensin II Type 2 Receptor Counter-Regulates Type 1 Receptor in Catecholamine Synthesis in Cultured Porcine Adrenal Medullary Chromaffin Cells

Kazuhiro Takekoshi, Kiyoaki Ishii, Shunsuke Shibuya, Yasushi Kawakami, Kazumasa Isobe, Toshiaki Nakai

Abstract—We previously showed that CGP 42112 (an angiotensin type 2 [AT₂] agonist) markedly reduces catecholamine biosynthesis by decreasing cGMP production mediated by AT₂, a subtype of Ang II receptor that is dominantly expressed in cultured porcine chromaffin cells. To elucidate the relationship of the 2 types of Ang II receptors, angiotensin type 1 (AT₁) and AT₂, in the synthesis of catecholamine in adrenal medullary cells, we have examined the effect of Ang II plus CV-11974 (an AT₁ antagonist that selectively simulates AT₂ stimulation) and the effect of Ang II plus PD 123319 (an AT₂ antagonist that selectively simulates AT₁ stimulation) on catecholamine synthesis. We found that Ang II reduced cGMP production via AT₂, in a similar manner to that found with CGP 42112. Stimulation of AT₁ significantly upregulated protein kinase C activity. Tyrosine hydroxylase (TH) is a rate-limiting enzyme involved in the biosynthesis of catecholamine, and this catecholamine synthesis depends both on TH enzyme activity and on the levels of TH protein after TH gene transcription. We found that AT₂ stimulation significantly inhibited TH enzyme activity, whereas AT₁ stimulation significantly upregulated TH enzyme activity. The stimulatory effect of AT₁ was completely inhibited by Ro-32-0432 (a protein kinase C inhibitor) and PD 98059 (a MAP kinase kinase-1 [MEK-1] inhibitor). Pretreatment of cells with either 8-Br-cGMP (a membrane-permeable cGMP analog) or Zaprinast (a phosphodiesterase inhibitor) abolished the inhibitory effect of AT₂ on TH enzyme activity, indicating that the stimulatory effect of AT₂ may be mediated through a reduction in cGMP concentration. Similar to the effect on TH enzyme activity, AT₂ stimulation significantly reduced TH mRNA and protein levels and net catecholamine content below basal levels, whereas AT₁ stimulation increased them. We confirmed these findings by gel mobility shift assay. Our results show that stimulation of AT₂ reduces catecholamine biosynthesis via a decrease in cGMP levels. In contrast, stimulation of AT₁ stimulates catecholamine biosynthesis through activation of PKC. Thus, we conclude that AT₁ and AT₂ have counter-regulatory roles in the synthesis of catecholamine in adrenal medullary chromaffin cells. (Hypertension. 2002;39:142-148.)

Key Words: receptors, angiotensin II ▶ tyrosine hydroxylase ▶ cyclic GMP ▶ angiotensin antagonist ▶ catecholamines

There are 2 major subtypes of Ang II receptor, type 1 (AT₁) and type 2 (AT₂).1 Most of the stimulatory effects of Ang II, such as those on the cardiovascular system and on fluid volume homeostasis, are mediated by AT₁. AT₁ is linked with activation of phospholipase C and with a subsequent increase in [Ca²⁺], and protein kinase C (PKC) activity.1 In contrast, the physiological role of and signal transduction pathways connected with AT₂ are not established. In adults, AT₂ expression is restricted to only a few organs, such as adrenal medulla.2 It has been shown that stimulation of AT₂ induces a decrease in intracellular cGMP levels in neuronal cells.3,4 However, the physiological roles of AT₂ in adrenal chromaffin cells remain to be clarified.

In adrenal medullary cells, tyrosine hydroxylase (TH) is a rate-limiting enzyme involved in the biosynthesis of catecholamines. TH activity can be regulated by both short-term and long-term mechanisms. Short-term regulation of enzyme activity occurs at the posttranscriptional level, where phosphorylation of TH leads to its activation.5 Long-term regulation occurs after TH gene transcription at the level of protein synthesis.5 Recently, it has been shown that the cGMP/cGMP-dependent protein kinase (PKG) pathway plays an important role in catecholamine biosynthesis.6-10 Indeed, agents that increase cGMP production, such as NO and natriuretic peptides, stimulate TH enzyme activity and gene transcription via activation of PKG.7-10 It is also of note that natriuretic peptides markedly increase the net content of intracellular catecholamine.10

Stachowiak et al11 previously showed that Ang II stimulates both TH enzyme activity and TH mRNA levels in rat

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adrenal medulla. However, the precise role of AT1 and AT2 in regulating catecholamine synthesis in adrenal medullary cells remains unclear.

Previously, we showed that activation of AT2 negatively regulates catecholamine synthesis in cultured porcine chromaffin cells.12 We found that AT2 is predominantly expressed in these cells; CGP 42112 (an AT2 agonist, 1 nmol/L) significantly inhibited cGMP production to below basal levels; CGP 42112 (1 nmol/L) significantly inhibited both the enzyme activity of TH and mRNA and protein levels of TH; and pretreatment of cells with 8-Br-cGMP (a membrane-permeable cGMP analogue) prevented the inhibitory effect of CGP 42112 on TH enzyme activity.4

To elucidate further the roles of AT1 and AT2 in catecholamine synthesis, we investigated the effect of Ang II alone (representing physiological conditions), Ang II plus CV-11974 (an AT1 antagonist, selectively simulating AT2 stimulation), and Ang II plus PD 123319 (an AT2 antagonist, selectively simulating AT1 stimulation) on the synthesis of catecholamine in cultured porcine adrenal medullary cells. In addition, we have examined the mechanisms underlying these effects on catecholamine synthesis.

Methods

Cell Culture

Cell culture, measurement of cGMP production, tyrosine hydroxylase enzyme activity, Northern blot analysis, Western blot analysis, and determination of intracellular levels were performed as described previously.12–15

PKC Activity

PKC activity was measured in cultured confluent cells as described previously.16

Results

Effect of Ang II Alone or Ang II Plus Either CV-11974 or PD 123319 on cGMP Production in Cultured Porcine Adrenal Medullary Cells

We have shown previously that CGP 42112 significantly inhibits cGMP production, as measured against basal levels, in cultured porcine adrenal medullary cells.12 To further investigate these findings, we examined the effect of Ang II alone on cGMP production. As shown in Figure 1, Ang II (1 nmol/L) inhibited cGMP production to levels significantly lower than the basal level. This inhibitory effect of Ang II on cGMP production was completely abolished by the PKC inhibitor Ro-32-0432 (100 nmol/L).

Gel Mobility Shift Assay

Nuclear extracts from cells were isolated as described previously.17 An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

Effect of Ang II Alone, or Ang II Plus Either CV-11974 or PD 123319 on cGMP Production in Cultured Porcine Adrenal Medullary Cells

As shown in Figure 2, Ang II alone (1 nmol/L) significantly increased PKC activity by ~2-fold. The co-addition of Ang II and 1 or 100 nmol/L PD 123319 significantly stimulated PKC activity by 1.8- and 2.1-fold, respectively. This stimulatory effect of Ang II and PD 123319 on PKC was completely abolished by the PKC inhibitor Ro-32-0432 (100 nmol/L). The co-addition of Ang II and 1 or 100 nmol/L CV-11974 did.
not affect PKC activity. PD 123319, CV-11974, and Ro-32-0432 alone did not affect basal PKC activity.

**Effects of Ang II Alone or Ang II Plus Either CV-11974 or PD 123319 on the Levels of TH Enzyme Activity in Cultured Porcine Adrenal Medullary Cells**

As shown in Figure 3a, Ang II alone (1 nmol/L) moderately but significantly increased TH enzyme activity to levels 1.3-fold higher than basal levels. Ang II plus 1 or 100 nmol/L CV-11974 significantly inhibited TH enzyme activity by 12% and 31%, respectively. In contrast, Ang II plus 1 or 100 nmol/L PD 123319 stimulated TH enzyme activity to levels 1.4- and 1.6-fold higher than basal levels, respectively. This stimulatory effect of AT1 was completely inhibited by both the PKC inhibitor Ro-32-0432 (Figure 3b) and the MEK-1 inhibitor PD 98059 (Figure 3b). PD 123319, CV-11974, Ro-32-0432, and PD 98059 alone had no significant effect on basal TH enzyme activity (Figure 3a and 3b).

**8-Br-cGMP and Phosphodiesterase Inhibitor Prevented the AT2-mediated Inhibitory Effect of TH Enzyme Activity**

We investigated whether a decrease in cGMP production is involved in the inhibition of TH enzyme activity caused by stimulation of AT2. As shown in Table 1, pretreatment with either 8-Br-cGMP (a membrane-permeable cGMP analog, 1 mmol/L) or Zaprinast (a phosphodiesterase inhibitor, 10 μmol/L) prevented the inhibitory effect of AT2 on TH enzyme activity.18

**Effect of Ang II Alone or Ang II Plus Either CV-11974 or PD 123319 on the Levels of TH mRNA in Cultured Porcine Adrenal Medullary Cells**

We examined the effect of Ang II alone or Ang II plus CV-11974 or PD 123319 on the levels of TH mRNA. As shown in Figure 4a and 4b, Ang II alone significantly stimulated TH mRNA production to levels 48% above the basal level. Ang II plus CV-11974 significantly inhibited production of TH mRNA to levels 20% below the basal level, whereas Ang II plus PD 123319 stimulated TH mRNA production to levels 71% above the basal level. Addition of either Ro-32-0432 or PD 98059 abolished the increase in TH mRNA levels induced by Ang II plus PD 123319. PD 123319, CV-11974, Ro-32-0432, and PD 98059 alone had no effect on basal TH mRNA levels (data not shown).

**Effects of Ang II Alone or Ang II Plus Either CV-11974 or PD 123319 on Levels of TH Protein and Intracellular Catecholamine**

To confirm that there was a change in catecholamine synthesis following induction of TH mRNA, we examined the effects of Ang II plus CV-11974 or PD 123319 on TH protein production. Cells were treated with Ang II alone or with Ang II plus CV-11974 or PD 123319 for 24 hours. The levels of TH protein were then examined by Western blot analysis (Figure 5). Ang II alone moderately but significantly increased levels of TH protein by 28%. Ang II plus CV-11974 significantly decreased levels of TH protein by 47%. Neither PD 123319 nor CV-11974 alone affected basal TH protein level. We also examined the intracellular levels of catecholamine. Cells were treated with Ang II plus either CV-11974 or PD 123319 for 24 hours, and the intracellular catecholamine levels were examined by high-performance liquid chromatography. As shown in Table 2, Ang II plus CV-11974 significantly decreased intracellular catecholamine levels by 9.7%, whereas Ang II plus PD 123319 increased intracellular catecholamine levels by 10.0%.

**Effect of Ang II Alone or Ang II Plus Either CV-11974 or PD 123319 on the DNA-Binding Activity of AP-1**

To determine whether AT1 or AT2 could affect the DNA-binding activity of the transcription factor AP-1, we incubated the nuclear extract from treated chromaffin cells with the [32P]-AP-1 consensus sequence. Ang II alone moderately increased AP-1 DNA-binding activity, Ang II plus CV-11974 markedly decreased the activity, and Ang II plus PD 123319 increased the activity. The specificity of AP-1 binding was verified by its marked reduction in the presence of an excess of unlabeled AP-1 probe. We also examined the effect of Ro-32-0432 and PD 98059 on the
AP-1 activation induced by Ang II plus PD 123319. Pretreatment of cells with either Ro-32-0432 or PD 98059 inhibited stimulatory effect of Ang II plus PD 123319 on AP-1 activity (data not shown).

Discussion
Here we have shown that the stimulation of AT₂ both inhibits production of cGMP (Figure 1) and decreases TH enzyme activity below basal levels (Figure 3a) in chromaffin cells. Also, pretreatment of the cells with either 8-Br-cGMP or Zaprinast (a phosphodiesterase inhibitor) prevents this inhibitory effect of AT₂ (Table 1). These results suggest that the inhibition of TH enzyme activity by AT₂ may be dependent on a decrease in cGMP production. As cGMP/PKG plays an important role in regulating TH enzyme activity,⁶–⁹ it is likely that AT₂ stimulation decreases the levels of cGMP, which in turn may reduce PKG activity, resulting in the inhibition of TH enzyme activity in chromaffin cells.

Figure 3. Effect of Ang II plus PD 123319 or CV-11974 on TH enzyme activity in cultured porcine adrenal medullary cells. Cells were incubated for 10 minutes with CV-11974 (100 nmol/L) alone, PD 123319 (100 nmol/L) alone, Ang II (1 nmol/L) alone, Ang II plus CV-11974 (1 or 100 nmol/L), or Ang II plus PD 123319 (1 or 100 nmol/L) (a) or Ro-32 to 0432 (100 nmol/L) alone, PD 98059 (100 nmol/L) alone, Ang II plus PD 123319 (100 nmol/L), Ang II plus PD 123319 (100 nmol/L) and Ro-32 to 0432 (100 nmol/L), or Ang II plus PD 123319 (100 nmol/L) and PD 98059 (100 nmol/L) (b), as indicated. TH enzyme activity was then measured as described in Materials and Methods. Values represent the mean±SEM (n=4 to 6). *P<0.05 vs basal level. #P<0.05 vs value obtained with Ang II (1 nmol/L) alone (a) or Ang II plus PD 123319 (b).
AT2 stimulation significantly reduced TH mRNA production to below the basal level (Figure 4). The precise mechanisms responsible for this AT2-mediated reduction in levels of TH mRNA are unclear. However, cGMP-inducing agents such as NO and natriuretic peptides also induce formation of an AP-1 complex composed of Fos/Jun dimmers, via activation of PKG.19,20 Jun and Fos proteins bind to the tetradecanoylphorbol 13-acetate–responsive elements (TRE) as homodimers or heterodimers.21 Binding of the AP-1 (Fos/Jun) heterodimer to the TRE site of the TH promoter is a prerequisite for TH gene activation in PC12 cells.22 Thus, we speculate that the decrease in TH gene expression induced by AT2 stimulation is mediated through a reduction in transcription factor binding to the specific TRE site of the 5′-flanking region of the TH gene. In support of this hypothesis, we found that AT2 stimulation induced a marked decrease in the binding activity of AP-1 (data not shown).

AT2 stimulation also suppressed TH protein levels and intracellular catecholamine content (Figure 5, Table 2), which indicates that AT2 stimulation induces a decrease in TH gene expression, which in turn results in the decrease of the TH protein levels and intracellular net catecholamine content. Belloni et al23 and we24 recently demonstrated that AT2 stimulation induces catecholamine release from the adrenal medulla. Thus, one explanation for the marked decrease observed in net catecholamine content is that AT2 stimulation depletes catecholamine stores in 2 ways: (1) by stimulating catecholamine secretion and (2) by inhibiting catecholamine synthesis (Figure 6).

In contrast to the inhibitory effects of AT2, AT1 stimulated both TH enzyme activity, TH mRNA levels, and AP-1 binding activity in a PKC- as well as a MAPK-dependent manner (Figure 3, Figure 4). Indeed, stimulation of AT1 significantly upregulated PKC activity (Figure 2). Consistent with these findings, Raizada et al25–27 recently demonstrated that AT1 activation induces TH mRNA in both a PKC- and a MAPK-dependent manner through activation of AP-1–binding activity in rat brain neuronal cells.

It is believed that the effects of Ang II binding to the AT1 are opposed by those of Ang II binding to the AT2 in several systems, including cell proliferation and differentiation, angiogenesis, and chronotropic effects in the heart.28 Along this line, we have shown here that AT2 stimulation induces activation of catecholamine synthesis, whereas AT1 stimulation inhibits catecholamine synthesis. These findings indicate that AT2 and AT1 appear to have counter-regulatory roles in the regulation of catecholamine synthesis. Also of note is that Ang II alone stimulates catecholamine synthesis, which suggests that under physiological conditions, AT1, as opposed to AT2, has a dominant regulatory role in catecholamine biosynthesis.

Recently, several AT1 antagonists (AT1 blockade: ARB) have been used to treat hypertension. Treatment with AT1 antagonists increases plasma levels of Ang II, which subsequently stimulate AT2. It is of interest, therefore, that stimulating AT2 significantly reduces the net intracellular content of catecholamine, probably through both inhibition of cate-
cholamine synthesis and stimulation of catecholamine secretion, which may subsequently lead to catecholamine depletion and sympathoadrenergic inhibition. Thus, these findings raise the possibility that AT₁ antagonists may inhibit sympathoadrenergic activation following not only the suppression of AT₁ blocking properties but also the activation of AT₂—a previously unknown, beneficial effect of its action (Figure 6).

Acknowledgments
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References

**TABLE 2. Effects of Ang II Alone, or Ang II Plus PD 123319 or CV-11974 on Intracellular Catecholamine Content**

<table>
<thead>
<tr>
<th></th>
<th>Nor epinephrine (nmol/mg protein)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>37.2±0.99</td>
<td>100.0</td>
</tr>
<tr>
<td>Ang II alone</td>
<td>39.8±0.79</td>
<td>107.8</td>
</tr>
<tr>
<td>Ang II plus CV-11974</td>
<td>33.6±1.01</td>
<td>90.3</td>
</tr>
<tr>
<td>Ang II plus PD 123319</td>
<td>40.9±0.56†</td>
<td>110.0</td>
</tr>
</tbody>
</table>

Cells were incubated for 24 hr with DMEM (as a control). Ang II (1 nmol/L) alone, Ang II plus CV-11974 (100 nmol/L) or Ang II plus PD 123319 (100 nmol/L), and then the intracellular catecholamine (noradrenaline + adrenaline) levels were measured as described in the Methods. Values represent the mean SEM (n=4–6).

†Significantly different (P<0.05) from the basal level.


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