Suppression of Angiotensin II Release by Prostaglandin Synthesis Inhibitors in Hind Legs

KENJI MIZUNO, KOICHI HIGASHIMORI, AND TADASHI INAGAMI

SUMMARY Previously we reported that immunoreactive angiotensin II (Ang II) release from isolated perfused rat mesenteric arteries was mediated by $\beta$-adrenergic receptor activation. However, the precise mechanism of regulation of vascular renin-angiotensin is not completely understood. In this study, we examined the effect of indomethacin and meclofenamate on immunoreactive angiotensin I (Ang I) and immunoreactive Ang II release from perfused rat hind leg vasculature to delineate the possible relevance of prostaglandins to the vascular renin-angiotensin system in vitro. We also examined the effects of isoproterenol and propranolol on the immunoreactive Ang I and II release. Isolated rat hind legs were perfused with Krebs-Ringer solution, and immunoreactive Ang I and II released into the perfusate were measured directly by using a Sep-Pak C$_18$ cartridge connected to the perfusion system. Indomethacin and meclofenamate (10$^{-6}$ to 2 x 10$^{-4}$ M) added to the perfusion medium suppressed immunoreactive Ang I and II release to similar extents in a dose-dependent manner ($p < 0.001$); the maximal percent inhibition of immunoreactive Ang II release evoked by these inhibitors (2 x 10$^{-4}$ M) was 60 ± 6% ($p < 0.001$) for indomethacin and 50 ± 4% ($p < 0.001$) for meclofenamate. Isoproterenol (10$^{-6}$ M) failed to cause a change in the release of both peptides, but propranolol (10$^{-6}$ M) slightly decreased the release of immunoreactive Ang I and II by 28 ± 4% ($p < 0.001$) and 32 ± 4% ($p < 0.001$), respectively. There was a highly significant positive correlation between the released amount of immunoreactive Ang I and that of immunoreactive Ang II altered by indomethacin ($r = 0.91$), meclofenamate ($r = 0.94$), or propranolol administration ($r = 0.90$). These results suggest that the renin-angiotensin in the hind legs is modulated by prostaglandins and that a difference exists in the $\beta$-adrenergic receptor-mediated release of Ang II among diverse vascular beds. (Hypertension 12: 67-73, 1988)

KEY WORDS renin-angiotensin system • rat hind leg • indomethacin • meclofenamate • prostaglandin • angiotensin II • $\beta$-adrenergic receptors

The presence of essential components of the renin-angiotensin system, such as renin enzyme, angiotensinogen, angiotensin converting enzyme, and angiotensin receptors, has been shown in vascular tissues. Although earlier reports of renin in homogenates of vascular tissues may be explained largely by adherence of plasma renin of renal origin to vascular beds, rigorous studies in recent years have revealed the presence of specific immunoreactive renin in the arterial tissue, cultured smooth muscle cells, and endothelial cells. In addition, angiotensinogen messenger RNA (mRNA) can be detected in arterial tissue, and angiotensin converting enzyme and angiotensin II (Ang II) receptors are present in the vasculature. Thus, Ang II probably is formed locally in the vascular tissues, is released from it, and binds to Ang II receptors in vascular smooth muscle.

Recently, we demonstrated that sizable amounts of immunoreactive angiotensin I (irAng I) and irAng II are released continuously at steady rates from isolated rat mesenteric arteries and hind legs perfused with angiotensinogen-free medium. Further, we reported that isoproterenol caused an apparent increase of irAng II release from the mesenteric arteries in vitro. This isoproterenol-induced release of irAng II was suppressed significantly by propranolol. Taken together, these results suggest that locally generated Ang II is released from peripheral vascular tissues and its release is mediated, at least in part, by $\beta$-adrenergic receptors. It is yet unknown if factor(s) other than the adrenergic mechanism can control the release of Ang II from vascular tissues. It is well known that vascular smooth muscle cells or endothelial cells, which are the target cells of Ang II activity, produce...
Angiotensins, the preparations were thoroughly washed with Krebs-Ringer solution for at least 60 minutes before the experiments were started. To avoid the contamination of plasma angiotensins, the preparations were thoroughly washed with Krebs-Ringer solution for at least 60 minutes before the experiments were started.

**Materials and Methods**

**Animals**

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN, USA) weighing 220 to 260 g were used in this study. The rats were maintained on a regular Purina chow diet (St. Louis, MO, USA) containing 0.39% sodium and 0.9% potassium and allowed free access to tap water.

**Hind Leg Preparations**

Rats were anesthetized with sodium pentobarbital (30 mg/kg i.p.) and given heparin (1300 IU/kg i.v.). The aorta was ligated at the sites distal to the renal arteries, and a cannula (PE-90; inside diameter, 0.86 mm) was inserted to the aorta. The hind leg was then flushed with 60 ml of Krebs-Ringer solution to eliminate blood. After ligation of the contralateral femoral artery, a hind leg was quickly isolated by sectioning the animal. The whole preparation was placed in a water-jacketed container maintained at 37 °C and perfused with Krebs-Ringer solution containing 0.4% (wt/vol) bovine serum albumin. The composition of the Krebs-Ringer solution was (in mM) NaCl, 112; KCl, 5.0; NaH₂PO₄, 1.0; CaCl₂, 2.5; NaHCO₃, 25; and d (+)-glucose, 11.2. This solution was maintained at 37 °C and aerated with a mixture of 5% CO₂ and 95% O₂ to obtain a pH of 7.4. The tissues were perfused at a constant flow rate of 4.0 ml/min with a roller pump (Polystaltic Pump, Buchler, Fort Lee, NJ, USA). The perfusion pressure was recorded with a pressure transducer (Model P23ID, Statham, Oxnard, CA, USA) connected to a Grass polygraph (Model 7D, Quincy, MA, USA). During perfusion of the hind legs the pressure was approximately 55 to 60 mm Hg. To avoid the contamination of plasma angiotensins, the preparations were thoroughly washed with Krebs-Ringer solution for at least 60 minutes before the experiments were started.

**Determination of Angiotensins**

Angiotensins that were released from isolated perfused hind legs were trapped by using Sep-Pak C₁₈ cartridges (Waters Association, Milford, MA, USA) as described previously. The cartridge was connected to the water-jacketed container to allow the perfusate to pass through the cartridge. The cartridges were moistened with 3 ml of methanol and prewashed with 10 ml of Krebs-Ringer solution just before use and were exchanged at a 30-minute interval. The cartridge was washed with 10 ml of 0.1% trifluoroacetic acid in distilled water, and trapped peptides were eluted with 3 ml of methanol/water/trifluoroacetic acid (80: 19.9: 0.1, vol/vol). The extracts were dried in a vacuum centrifuge (Speed Vac, Savant, Hicksville, NY, USA), and resultant residues were dissolved in 0.1 M Tris acetate buffer, pH 7.4, containing 2.5 mM Na₂EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.1% bovine serum albumin, and subjected to radioimmunoassay. The recovery of Ang I and Ang II (50 pg each) added to the perfusate and then sent to the Sep-Pak cartridge, was 96.5 ± 3.9% (mean ± SD; n = 5) for Ang I and 95.5 ± 6.2% (mean ± SD; n = 7) for Ang II. The coefficients of variations for the recovery were 5.9 ± 1.2% and 7.6 ± 2.2% for Ang I and Ang II, respectively. The Ang II antiserum showed less than 1% cross-reactivity with Ang I, but 100% cross-reactivity with angiotensin-(2–8) heptapeptide, angiotensin-(3–8) hexapeptide, and angiotensin-(4–8) pentapeptide. The detectability of the irAng II determination was 1 pg/tube. The blank values of Ang I and Ang II were less than 1 pg/tube.

**Drugs**

Indomethacin sodium salt (Merck Sharp & Dohme), meclofenamate (Warner-Lambert), l-isoproterenol bitartrate (Sigma Chemical), and dl-propranolol (Sigma) were dissolved in isotonic saline and diluted to the appropriate concentrations with Krebs-Ringer solution just before use. They were freshly prepared each day and were added to the Krebs-Ringer solution and infused continuously for a 30-minute period at each concentration.

**Statistical Analysis**

The values are expressed as means ± SE. Statistical analysis of data was performed by a one-way analysis of variance and Student’s t test when appropriate. A p value below 0.05 was accepted as the level of significance.

**Results**

The time course of the spontaneous release of irAng I and irAng II from isolated perfused hind legs was examined up to 3 hours. To equilibrate the preparations and to eliminate the possible contamination of plasma angiotensins, the tissue was thoroughly washed with Krebs-Ringer solution (approx-
imately 300 ml) before experiments were started. Under these conditions, the rate of release of angiotensins was as high as 636.5 ± 85.2 and 640.3 ± 51.9 pg (n = 6) for irAng I and irAng II, respectively, during the first infusion period of 30 minutes, and the release of irAng I and irAng II remained stable at least for 3 hours. The release of peptides at the end of the 3-hour perfusion period was 636.2 ± 59.6 and 630.2 ± 71.9 pg per 30 minutes for irAng I and irAng II, respectively.

Figure 1 shows the effect of various concentrations of indomethacin (10⁻⁸ to 2 × 10⁻⁶ M) in the perfusion medium on the release of irAng I and irAng II. Indomethacin suppressed irAng I and irAng II release in a dose-dependent manner (F = 27.67, p < 0.0001, for irAng I; F = 44.73, p < 0.0001, for irAng II). At the highest concentration of indomethacin (2 × 10⁻⁶ M), it caused an approximately 50 ± 6% (359.7 ± 90.9 vs 717.3 ± 50.2 pg/30 min, basal; p < 0.01) and 60 ± 6% (284.0 ± 84.8 vs 717.2 ± 39.5 pg/30 min, basal; p < 0.001) reduction in irAng I and irAng II release, respectively. There was a highly positive correlation between the amount of released irAng I and that of irAng II altered by indomethacin infusion (r = 0.91, p < 0.001), as shown in Figure 2.

To further ascertain that this effect of indomethacin was due to inhibition of cyclooxygenase rather than its cytotoxic action, we examined the effect of meclofenamate, a structurally unrelated inhibitor of the cyclooxygenase, on irAng I and irAng II release in a similar maneuver. As shown in Figure 3, meclofenamate (10⁻⁸ to 2 × 10⁻⁶ M) caused a significant reduction in irAng I and irAng II release in a dose-dependent fashion (F = 21.43, p < 0.0001, for irAng I; F = 28.34, p < 0.0001, for irAng II). At the highest concentration of meclofenamate (2 × 10⁻⁶ M), release of irAng I and irAng II was reduced by 49 ± 5% (351.5 ± 55.4 vs 702.2 ± 64.6 pg/30 min, basal; p < 0.01) and 50 ± 5% (337.8 ± 77.1 vs 676.2 ± 46.4 pg/30 min, basal; p < 0.001), respectively. As was the case with indomethacin, there was a highly significant positive correlation between the amount
of released irAng I and that of irAng II altered by meclofenamate infusion \((r = 0.94, p < 0.001)\), as shown in Figure 4.

The effect of isoproterenol on the release of irAng I and irAng II is shown in Table 1. Isoproterenol \((10^{-5}-10^{-6} \text{ M})\) did not cause any significant changes in the release of irAng I or irAng II, even at the highest concentration of \(10^{-6} \text{ M}\). On the contrary, propranolol \((10^{-7}-10^{-6} \text{ M})\) slightly but significantly reduced the release of irAng I \((F = 7.93, p < 0.002)\) and irAng II \((F = 8.46, p < 0.002; \text{ Table 2})\). The maximal percent inhibition of irAng I and irAng II release evoked by propranolol \((10^{-6} \text{ M})\) was 28 \(\pm\) 4% \((550.5 \pm 23.5 \text{ pg/30 min, basal; } p < 0.001)\) for irAng I and 32 \(\pm\) 4% \((378.8 \pm 32.0 \text{ pg/30 min, basal; } p < 0.001)\) for irAng II. There was a significant positive correlation between the released amount of irAng I and that of irAng II altered by propranolol \((r = 0.90, p < 0.001; \text{ Figure 5})\).

Discussion

The present observations confirm the previous findings that a large amount of irAng II was detected in the perfusate from isolated perfused rat hind legs.\(^{13}\) To avoid the contamination of plasma angiotensins, special caution was taken in these experiments; the hind leg preparations were thoroughly washed with approximately 300 ml of Krebs-Ringer solution for at least 60 minutes before experiments were started. Under these conditions, the basal release of irAng I and irAng II remained constant at least for 3 hours. Further, in a previous study we found that bilateral nephrectomy 24 hours before the experiments did not cause any significant change in irAng I and irAng II release from the hind legs, when plasma renin activity and plasma irAng II concentration were near undetectable levels. Thus, the possibility is eliminated that irAng I and irAng II determined in this study were due to contamination or sequestration of circulating plasma peptide.

Although essential components of the renin-angiotensin system, such as renin, angiotensinogen, Ang I, Ang II converting enzyme (ACE), and Ang II, all have been found in vascular tissues,\(^{1-3}\) the precise origin of each component of the vascular renin-angiotensin system is not completely known. Earlier reports of renin in homogenates\(^{1, 2, 6-7}\) of vascular tissues may be explained largely by adherence of plasma renin of renal origin to the vascular beds. However, recent findings that vascular smooth muscle cells\(^{10}\) and endothelial cells\(^{11}\) in culture synthesize renin enzyme and that angiotensinogen mRNA can be detected in arterial tissue\(^{3}\) provide strong support for the local production of renin and subsequent generation of angiotensins in vascular tissues. Evidence accumulates for such local synthesis of renin and angiotensins in other extrarenal tissues such as brain\(^{22-23}\) and testis.\(^{24}\)

However, little is known about the mechanism of generation and release of Ang II in vascular tissues. Available data obtained by our recent studies using isolated perfused rat mesenteric arteries demonstrated that release of locally generated irAng II was mediated by \(\beta\)-adrenergic receptor activation\(^{12}\) and was partially suppressed by ACE inhibition.\(^{12}\) These results indicate the importance of sympathetic tone and ACE activity in terms of generation and release of Ang II in vascular beds. Nevertheless, there seem to be other factors (or mechanisms) involved in the regulation of vascular Ang II release, inasmuch as the basal release of irAng II could be incompletely blocked even by a pharmacological dose of \(\beta\)-adrenergic antagonist\(^{12}\) and ACE inhibitor.\(^{12, 13}\) Prostaglandins participate in the local control of renin release from juxtaglomerular cells of the kidney in vivo and in vitro.\(^{17-19}\) Further, inhibition of

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**Table 1. Effect of Isoproterenol on Release of Immunoreactive Ang I and Ang II from Isolated Perfused Rat Hind Legs**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Basal</th>
<th>(10^{-9})</th>
<th>(10^{-8})</th>
<th>(10^{-7})</th>
<th>(10^{-6})</th>
</tr>
</thead>
<tbody>
<tr>
<td>irAng I (pg/30 min)</td>
<td>635.2 ± 33.9</td>
<td>678.0 ± 60.5</td>
<td>627.8 ± 42.3</td>
<td>635.2 ± 46.6</td>
<td>621.1 ± 46.2</td>
</tr>
<tr>
<td>irAng II (pg/30 min)</td>
<td>570.7 ± 22.9</td>
<td>517.3 ± 41.8</td>
<td>572.3 ± 34.0</td>
<td>567.5 ± 36.6</td>
<td>553.7 ± 35.3</td>
</tr>
</tbody>
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Values are given as means ± SE \((n = 6)\). ir = immunoreactive.
prostaglandin synthesis decreased renin release by the kidney. On the other hand, since prostaglandins may exert their numerous functions not only in the bloodstream but also in the target organ, it seems likely that prostaglandins can affect extra-renal tissue renin. In support of this concept, there is evidence that inhibition of prostaglandins by indomethacin significantly suppressed renin content in rat adrenal gland, concomitantly with a decrease of prostaglandin E2 production in the tissue. Therefore, we examined the effect of inhibitors of prostaglandin synthesis on irAng I and irAng II release from isolated perfused rat hind legs to gain insight into possible relevance of prostaglandins to the vascular renin-angiotensin system.

Our data clearly show that indomethacin, added to the perfusion medium, suppressed the release of irAng II from the hind legs. IrAng I release was also inhibited by indomethacin in a fashion similar to irAng II release. In view of the reported nonspecific action of indomethacin, we further examined the effect of a structurally different cyclooxygenase inhibitor, meclofenamate, on angiotensin release. It is apparent from our data that meclofenamate suppressed the release of irAng II and irAng I as well. The inhibitory effect on irAng I and irAng II release elicited by meclofenamate was practically comparable with indomethacin (see Figures 1 and 3), though the latter was more effective on irAng II release as compared with the former at the highest concentration (10%; p = NS).

These results indicate that inhibition of prostaglandin synthesis by cyclooxygenase inhibitors in isolated perfused rat hind legs leads to a decrease in irAng II release from the vascular beds and, hence, support the idea that prostaglandin is a candidate for a regulator of Ang II release in the tissue. One problem with this interpretation is that we cannot be certain that indomethacin and meclofenamate inhibited cyclooxygenase, because we did not measure prostaglandin production before and after the administration of these inhibitors. However, the dose of indomethacin and meclofenamate used has been shown by other investigators to inhibit cyclooxygenase. Thus, it is likely that inhibition of prostaglandin synthesis was achieved in this study.

The mechanism of regulation of angiotensin release by prostaglandins is not clear at present. One possibility is that prostaglandins regulate the rate of angiotensin formation that seems to take place in the vascular beds. An alternative explanation may be that prostaglandins directly regulate the release of angiotensin from the vasculature. Further studies are required to resolve this issue.

Although the present study has demonstrated the qualitatively similar effects of indomethacin and meclofenamate on irAng I and irAng II release, it is not readily apparent which prostaglandins are responsible for the regulation of vascular angiotensin release. PGF2 seems to be the best candidate for such a role, since PGF2 is the major product of arachidonic acid metabolism in blood vessels; however, this possibility was not tested by examining the effect of PGF2 on angiotensin release.

In our previous study using rat mesenteric arteries, we clearly demonstrated that isoproterenol (10^{-5}-10^{-4} M) added to perfusion medium caused a marked, dose-dependent increase in irAng II release. Further, this isoproterenol-induced increment of irAng II release was blocked by the addition of propranolol, leading to the hypothesis that, in mesenteric arteries, a beta-adrenergic receptor mechanism participates in the regulation of Ang II release.
from the tissue. Thus, to further ascertain if this mechanism is involved in the regulation of Ang II release in the vasculature of the hind legs, we examined the effect of isoproterenol and propranolo-
lon on the irAng II release. Surprisingly, and unex-
pectedly, isoproterenol had no effect on the release of irAng II or irAng I, even at the highest concentra-
tion of $10^{-6}$ M. Although this difference between the two types of vasculature suggests that different mechanisms are responsible for the regulation of angiotensin release in diverse vascular beds, further studies using other vascular beds will be needed to clarify this problem.

Interestingly, despite the lack of responsiveness of angiotensin release to isoproterenol, propranolol slightly suppressed the irAng I and irAng II release. We do not have a good explanation for this discrepancy. One possibility is that the adrenergic system is already activated fully due to the low perfusion pressure employed, and consequently, the antagonistic action of propranolol can be seen. However, this possibility does not seem likely, inasmuch as isoproterenol failed to cause any change in the angiotensin release, even at the highest concentration. Another possibility may be that the slight decrease in angiotensin release by propranolol was caused by its direct action (commonly called “membrane stabilizing” or “local anesthetic” action) on cell membranes of vascular smooth muscle, rather than on β-adrenergic receptors, to suppress angio-
tensin release. However, this possibility is also less likely, since such an action of propranolol can be observed only with high doses.31 Thus, further studies will be required to clarify this discrepancy by another approach.

In the current study, there was a positive corre-
lation between the released amount of irAng I and
that of irAng II altered by indomethacin, meclo-
fenamate, and propranolol. Previously, we reported that the ACE inhibitor captopril suppressed irAng II release from rat hind legs, with a reciprocal increase in irAng I release,13 suggesting that Ang I and Ang II are produced intracellularly or extracel-
larly in the vascular beds to be secreted into the extracellular space. The present observation not only supports such mechanisms of angiotensin generation but also implies that these synthesis inhibi-
tors directly suppress the release of both peptides synthesized in the tissue. Alternatively, these sub-
stances may inhibit generation of Ang I in the tissue first and subsequently suppress Ang II formation. However, since there is no direct evidence suggest-
ing that these inhibitors affect vascular renin or reninlike enzyme, the findings observed favor the idea of the direct effect on angiotensin release.

Finally, we have demonstrated that inhibition of prostaglandins by cyclooxygenase inhibitors can suppress the release of irAng II from isolated perfused rat hind legs. This decrease of irAng II correlated well with the decrease in irAng I release, suggesting that prostaglandins play a role in the regulation of release of angiotensins from vascular tissues. The physiological importance of such reg-
ulation remains to be clarified, but these data offer a new aspect for the local control of vascular tone through interactions between vasoconstrictor Ang II and vasodilatory prostaglandins.32, 33

Additionally, our data suggest that the mech-
nism(s) or stimuli responsible for the regulation of vascular Ang II release differ among diverse vascular beds. If such a hypothesis is correct, regulation of angiotensin release from vascular beds appears to be more complex than previously recognized, and hence, caution must be taken for better understanding of the physiological role of vascular angiotensin in blood pressure control.34, 35

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