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 β-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 C₁₈ cartridge connected to the perfusion system. Indomethacin and meclofenamate (10⁻⁴ to 2 × 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 × 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 β-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 • β-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 β-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...
vassoactive hormone prostaglandins, mainly prosta
cyclin (PGI2). Ang II has an ability to stimulate
PGI2 synthesis in the vascular beds.14-16 On the
other hand, prostaglandins are known to participate
in the control of renin release from juxtaglomerular
cells of the kidney.17-19 These findings indicate that
a close relationship exists between the renin-
angiotensin system and prostaglandins. To examine
the hypothesis that prostaglandins may contribute
to the control of the vascular renin-angiotensin
system, we determined the effect of prostaglandin
synthesis inhibitors, indomethacin and meclofena-
mate, on the release of irAng I and irAng II from
isolated perfused rat hind legs. Further, we exam-
ined the effect of isoproterenol and propranolol on
the peptide release to ascertain if the β-adrenergic
mechanism is involved in the release of irAng II in
vascular beds of the hind leg.

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 pentobar-
bital (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 diam-
eter, 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 prep-
aration 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 to eliminate blood. After ligation of the
contralateral femoral artery, a hind leg was quickly
isolated by sectioning the animal. The whole prep-
aration 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 b(+)-
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 pres-
ture transducer (Model P23ID, Statham, Oxnard,
CA, USA) connected to a Grass polysgraph (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.

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 contam-
nation of plasma angiotensins, the tissue was thor-
oughly 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 \((10^{-6}-10^{-7} \text{ M})\) 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\% \((550.5 \pm 23.5 \text{ vs } 739.8 \pm 25.9 \text{ pg/30 min, basal; } p < 0.001)\) for irAng I and 32 \pm 4\% \((378.8 \pm 32.0 \text{ vs } 554.6 \pm 23.1 \text{ pg/30 min, basal; } p < 0.001)\) 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 for at least 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 I converting enzyme (ACE), and Ang II, all have been found in vascular tissues,\(^{1-5}\) 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\(^{21-23}\) 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

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

<table>
<thead>
<tr>
<th>Isoproterenol (M)</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>
</table>

Values are given as means \(\pm SE (n = 6)\). ir = immunoreactive.
TABLE 2. Effect of Propranolol 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^-8</th>
<th>10^-7</th>
<th>10^-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>irAng I (pg/30 min)</td>
<td>739.8 ± 25.9</td>
<td>657.9 ± 22.5*</td>
<td>619.4 ± 27.8t</td>
<td>550.5 ± 23.5t</td>
</tr>
<tr>
<td>irAng II (pg/30 min)</td>
<td>554.6 ± 23.1</td>
<td>457.9 ± 23.7*</td>
<td>400.6 ± 23.8t</td>
<td>378.8 ± 32.0t</td>
</tr>
</tbody>
</table>

Values are given as means ± SE (n = 6). ir = immunoreactive.
*p < 0.05, t p < 0.01, %p < 0.001, compared with basal values.

prostaglandin synthesis decreased renin release by the kidney.25 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 extrarenal 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.26 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,27 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.28-30 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. PG12 seems to be the best candidate for such a role, since PG12 is the major product of arachidonic acid metabolism in blood vessels; however, this possibility was not tested by examining the effect of PG12 on angiotensin release.

In our previous study using rat mesenteric arteries,12 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,12 leading to the hypothesis that, in mesenteric arteries, a β-adrenergic receptor mechanism participates in the regulation of Ang II release.

![FIGURE 5. Correlation between the amount of released immunoreactive Ang I (irAng I) and that of irAng II in isolated perfused rat hind legs before and after administration of propranolol (Prop).](image-url)
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 propranolol on the irAng II release. Surprisingly, and unexpectedly, isoproterenol had no effect on the release of irAng II or irAng I, even at the highest concentration 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 angiotensin 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 correlation between the released amount of irAng I and that of irAng II altered by indomethacin, meclofenamate, 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 extracellularly 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 inhibitors directly suppress the release of both peptides synthesized in the tissue. Alternatively, these substances may inhibit generation of Ang I in the tissue first and subsequently suppress Ang II formation. However, since there is no direct evidence suggesting that these inhibitors affect vascular renin or renin-like 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 regulation 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 mechanism(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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