Angiotensinogen Is Cleaved to Angiotensin in Isolated Rat Blood Vessels

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The cleavage of synthetic tetradecapeptide renin substrate has been used to infer the presence of renin in the walls of isolated blood vessels; however, the conversion of natural angiotensinogen to angiotensin in isolated blood vessels has not been reported. We studied the release of angiotensinogen and the formation of angiotensins in a bloodless, perfused, isolated hind limb preparation of the rat. Perfusion with a modified Tyrode's solution resulted in spontaneous release of 4.7±1.5 pmol per 30 minutes of angiotensinogen as measured directly by radioimmunoassay. Western blot further identified the released material as angiotensinogen. Spontaneous release of angiotensins I and II was demonstrated by high performance liquid chromatography and radioimmunoassay. When highly purified rat angiotensinogen was added to the perfusate, release of angiotensin II was increased 14-fold compared with saline infusion. Captopril (10 μmol/L) inhibited angiotensinogen-induced angiotensin II release by 67% and led to an increase in angiotensin I release by 301%. Bilateral nephrectomy 24 hours before the experiments reduced basal angiotensin release below the detection limit and blunted angiotensinogen-induced angiotensin II formation by 95%. We conclude that active renin is present in the vessel wall and interacts with its natural substrate to form angiotensin peptides. Our data support the notion that the bulk of vascular renin is taken up from the circulation. (Hypertension 1993;21:1030–1034)

Key Words • renin • angiotensinogen • angiotensin • hind limb • blood vessels

During the last decade, evidence for a local renin-angiotensin system has accumulated. In the vasculature, there is local formation of angiotensin I (Ang I) and angiotensin II (Ang II), which may be derived from renin and angiotensinogen produced within the vessel wall itself or taken up from the blood. Angiotensinogen messenger RNA (mRNA) has been clearly demonstrated in the aortic wall, whereas the data on the presence of renin mRNA in the vessel wall are equivocal. The cleavage of the artificial substrate tetradecapeptide by isolated vessels to angiotensin peptides has been interpreted as evidence for the presence of renin. However, tetradecapeptide may be cleaved by nonrenin enzymes. Natural angiotensinogen, on the other hand, is effectively metabolized at physiological pH to Ang I solely by renin.

We investigated whether angiotensin peptides are formed in rat hind limbs perfused with highly purified rat angiotensinogen, whether such angiotensin formation is altered by angiotensin converting enzyme inhibitors and prior nephrectomy, and whether angiotensinogen is released from the vasculature into the perfusion medium.

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Methods

Animals

Male Sprague-Dawley rats (Ivanovas, Kisslegg, FRG) weighing 250–300 g were used. Four rats were nephrectomized 24 hours before hindquarter perfusion while they were under ether anesthesia. All procedures performed on animals were done in accordance with the guidelines of the American Physiological Society and were approved by the animal research ethics committee of the local government (Regierung von Mittelfranken, AZ 211-2531.3-1/92).

Hindquarter Perfusion

Preparation and perfusion of isolated rat hindquarters was performed with rats under thiobarbital anesthesia (60 mg/kg body wt i.p.) as previously described. After cannulation of the abdominal aorta and inferior vena cava, the perfusate was begun immediately. The hindquarters were perfused at a constant flow rate (10 ml/min) using a two-channel peristaltic pump (Harvard Instruments, South Natick, Mass.) in a nonrecirculating system with modified Tyrode's solution containing 2 g/L glucose and 20 g/L Ficoll 70 (Pharmacia, Freiburg, FRG). The perfusate was gassed with oxygen and carbon dioxide and maintained at pH 7.4 and 38°C.

Experimental Protocols

Perfusion for measurement of angiotensinogen was collected for three consecutive 30-minute periods (n=4) after an initial 30-minute washout period. The perfusate was cooled to 4°C by coils connected to the venous cannula. Immediately after collection, the per-
fusate was applied to phenylsepharose columns (5 mL volume) for extraction of proteins over 12 hours at 4°C.

To measure the release of Ang I and Ang II, we connected Sep-Pak C18 cartridges (Waters Chromatography Division, Milford, Mass.) "on-line" to the vena cava cannula for direct extraction of peptides as previously described.10 Per fusate was collected for three consecutive periods of 30 minutes each after an initial 30-minute washout period of perfusion. During the first 30 minutes, no substance was added (control period). During the second 30 minutes (experimental period), either angiotensinogen (5 pmol/mL, n=6 hindquarters) or 0.9% NaCl containing 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as a vehicle control (n=6) was infused. No substance was infused during the next 30-minute collection period (recovery period). Using the same protocol, we infused 12 more hindquarters with either angiotensinogen (n=6) or bovine serum albumin (n=6) in the presence of captopril (10 μmol/L added to the perfusate at the beginning of the perfusion). In addition, angiotensinogen (5 pmol/mL over 30 minutes) was given to four hindquarters from 24-hour bilaterally nephrectomized rats.

Rat angiotensinogen was prepared from 48-hour bilaterally nephrectomized rat plasma as previously described.14 The angiotensinogen charges used contained 35–56% angiotensinogen per weight, measured by direct and indirect radioimmunoassay.15 Angiotensinogen was also infused to perfusion channels without hindquarter, and samples were collected as described above (n=3).

**Demonstration of Angiotensinogen**

Proteins were eluted from phenylsepharose columns with 20 mL distilled water. Samples were concentrated to 1 mL, dialyzed against ammonium sulfate for 24 hours, frozen, and lyophilized. The recovery of iodinated angiotensinogen was 85%. The angiotensinogen content of the dry residues was quantified by direct radioimmunoassay using a polyclonal antisera against rat angiotensinogen.13 To confirm that the immunoreactive material was angiotensinogen, we performed sodium dodecyl sulfate disc electrophoresis followed by Western blot analysis. The protein probes were separated in a 10% polyacrylamide slab gel according to Laemmli.16 For molecular weight estimation, a standard kit from Pharmacia was used. Western blot was performed according to the method of Tsang et al.17 After the protein transfer and wash cycles, the blot sheet was incubated in monoclonal anti-rat angiotensinogen antibody solution (R1F6) for 1 hour and washed again. The sheet was incubated in peroxidase-labeled anti-mouse immunoglobulin M (Medac, Hamburg, FRG) for 2 hours, washed again, and incubated in a staining solution.

**Measurement of Peptides**

Eluates from Sep-Pak cartridges were lyophilized, and the dry residues were analyzed by high performance liquid chromatography (HPLC) and subsequent radioimmunoassay for Ang I and Ang II as described in detail elsewhere.10,18 After reversed-phase HPLC,10,18 peptides in HPLC fractions were quantified by radioimmunoassay for Ang I and Ang II (sensitivity, 1 pg per tube). The cross-reactivity of the Ang I antibody (K18) was 0.05% for Ang II. The cross-reactivity of the Ang II antibody (Celine) was 1% with Ang I and 100% with both the angiotensin (2–8) heptapeptide and the angiotensin (3–8) hexapeptide, respectively. Radioimmunoassays were performed using a modification of previously described methods; bound and unbound tracer were separated by a second antibody and polyethylene glycol instead of charcoal.10,19 Recovery from Sep-Pak cartridges and HPLC was 71% for Ang I and 92% for Ang II.

**Data are expressed as mean±SEM. Analysis of variance and subsequent Newman-Keuls test for post hoc analysis were used to assess significance of differences between groups. A value of p<0.05 was considered significant. CSS STATISTICA software (StatSoft Inc., Tulsa, Okla.) was used.**

**Results**

Isolated, perfused rat hindquarters released angiotensinogen spontaneously: 4.7±1.5 pmol was released during the first, 4.5±1.4 pmol during the second, and 4.1±1.2 pmol during the third 30-minute period. Western blot analysis of perfusate samples confirmed the presence of angiotensinogen (Figure 1). Hindquarter perfusate showed the same band pattern as did plasma (Figure 1).

Infusion of purified rat angiotensinogen into isolated, perfused rat hindquarters led to a 14-fold increase in vascular Ang II release (Figure 2). There was a tendency (p>0.05) toward higher Ang I release (Figure 2) during angiotensinogen infusion (1.4-fold compared with bovine serum albumin infusion). Ang II release from hind limbs perfused with angiotensinogen re-
Control Saline Recovery

**FIGURE 2.** Bar graphs show release of angiotensin (ANG) I and II from isolated, perfused rat hindquarters during three consecutive sampling periods of 30 minutes each. Top panel: Purified rat angiotensinogen (AOG) was infused during the second period. Bottom panel: Saline was infused as vehicle control. Data are mean±SEM, n=6 each.

*Significant differences (p<0.05 by analysis of variance and Newman-Keuls test) between AOG and saline infusion.

Control Captopril 24 h NX

**FIGURE 3.** Bar graph shows effects of converting enzyme inhibition (captopril, n=6) and bilateral nephrectomy 24 hours before perfusion (24 h NX, n=4) on angiotensinogen-induced angiotensin (ANG) formation. Data are mean±SEM.

*Significant differences (p<0.05 by analysis of variance and Newman-Keuls test) between control (angiotensinogen alone, n=6) and treatment.

Control Saline Recovery

Angiotensinogen after 24h NX

**FIGURE 4.** Representative examples of high performance liquid chromatographic elution profiles of perfusate samples collected during infusion of angiotensinogen into hindquarter of an untreated rat (top panel) and a bilaterally nephrectomized (NX) rat (bottom panel). Open bars represent angiotensinogen (ANG) I immunoreactivity; black bars, ANG II.

Angiotensin formation during angiotensinogen infusion was markedly diminished to approximately 5% of that in control rat hindquarters (Figure 3). Figure 4 shows a representative example of an HPLC elution profile. Very low amounts of ANG II metabolites (<5% of ANG II) were detected in most samples.

**Discussion**

This study demonstrates the formation of angiotensin peptides from angiotensinogen in a bloodless, perfused vasculature. Inhibition of the angiotensin converting enzyme with captopril blunted ANG II formation and increased ANG I. This finding is consistent with the presence of enzymatically active local renin in the vascular wall. The data with nephrectomized animals show that the bulk of such renin is taken up from the bloodstream. Clearly, angiotensinogen is also released from the vascular wall into the perfusate. Our data provide evidence not only for the presence of a local renin-angiotensin system but also for the physiological interaction of its main components within the vasculature to form substantial amounts of angiotensin peptides.

Our data are the first to show angiotensin formation from the natural substrate angiotensinogen in an isolated, perfused resistance vessel bed. Previous experiments with tetradecapeptide as a renin substrate provided only equivocal data: Hind limbs perfused with...
tetradecapeptide exhibited Ang II formation\(^{10,13}\) and increased vascular resistance,\(^{9,10,11}\) which was only partially inhibited by converting enzyme inhibitors. Furthermore, these inhibitors did not alter the ratio of Ang I to Ang II in the perfusate.\(^{10,13}\) Apparently, tetradecapeptide may be cleaved by enzymes other than renin.\(^{11}\)

In contrast, natural angiotensinogen is clearly preferable as a specific tool to study vascular renin.

Angiotensin formation from angiotensinogen was sensitive to captopril, because Ang II was suppressed and Ang I increased. The observation that mainly Ang II, rather than Ang I, was released was not unexpected, because Ang I is effectively converted to Ang II in the rat hindquarter.\(^{10,13}\) However, not all of the exogenous angiotensinogen-induced Ang II release could be suppressed by captopril. Contamination of angiotensinogen with angiotensin peptides or with renin was excluded by the use of a perfusion system without interconnected hind limb. Ang II could have been cleaved directly from angiotensinogen by serine proteases such as trypsin,\(^{20}\) or cathepsin G,\(^{21}\) or the conversion of Ang I to Ang II may have occurred at sites not accessible to captopril. Our data support the latter notion, because cleavage of angiotensinogen by nonrenin enzymes should be increased rather than decreased after bilateral nephrectomy.

Our data contrast with those of Campbell et al,\(^{12}\) who described a lack of vascular angiotensinogen formation in hindquarters perfused with a crude angiotensinogen preparation. These authors used nephrectomized rat plasma without further purification as a renin substrate, whereas we used purified angiotensinogen. Furthermore, the detection limit for angiotensinogen peptides was higher in the study of Campbell et al,\(^{12}\) which may explain why these authors failed to detect a spontaneous release of angiotensin peptides from perfused rat hind limbs, as described by Inagami's group\(^{22}\) and ourselves.

Angiotensinogen was released spontaneously from perfused hindquarters, along with Ang I and Ang II. The released angiotensinogen may be locally synthesized or taken up from plasma. Synthesis of angiotensinogen in the vascular wall has been demonstrated.\(^{4,5}\)

Our observation that the angiotensinogen-induced Ang II release persisted after the cessation of angiotensinogen infusion is consistent with the uptake of angiotensinogen by vascular tissue and its conversion into angiotensinogen by the vascular wall. We detected the two known plasma isoforms\(^{14}\) of angiotensinogen and another isoform of lower molecular weight, presumably a lower glycosylated isoform of des-Ang I angiotensinogen. The latter isoform was present in plasma and perfusate.

Angiotensinogen-induced angiotensinogen release was greatly reduced 24 hours after bilateral nephrectomy, supporting the notion that the bulk of vascular renin is derived from uptake of circulating renin of renal origin.\(^{19,20-23}\) The remaining vascular angiotensin formation in 24-hour nephrectomized rats could also be due to uptake of circulating renin, because the enzyme disappears more slowly from vascular tissue than from plasma.\(^{23}\)

The local synthesis of renin in vascular tissue is still a controversial issue.\(^{6-8}\) We studied release of angiotensinogen peptides from vascular tissue; therefore, we cannot exclude the possibility that locally synthesized renin might form angiotensin peptides intramurally that are not released. Local synthesis of renin may become predominant in specific pathophysiological situations.\(^{19,26}\) For example, we recently demonstrated that vascular angiotensogen formation in hypertensive rats transgenic for the mouse Ren-2 gene is independent of circulating renin.\(^{18}\)

In summary, we showed that the specific substrate for renin, namely angiotensinogen, is cleaved to Ang I and Ang II within the vascular wall. Our study provides further evidence that renin is present in the vascular wall and that it is able to interact rapidly with angiotensinogen perfused through the vessels. Our data are consistent with an uptake of renin from the blood into the vascular wall.

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References


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