Mechanism of Renal Handling of Angiotensin II in the Dog

By Suzanne Oparil and Michael D. Bailie

ABSTRACT

The mechanism of renal handling of angiotensin II was studied in vivo in the renal circulation of the intact anesthetized dog and in vitro in whole blood using $^{14}C$-$5\text{-Ile}$-angiotensin II, $J$-$\text{Asp}$-$^{125I}$-angiotensin II, and $d$-$\text{Asp}$-$^{125I}$-angiotensin II. Seventy-five percent of a 600-pmole bolus of $^{14}C$-angiotensin II was degraded in a single passage through the kidney as measured by radioactive tracer and radioimmunoassay techniques. The metabolic products were $^{14}C$-$5\text{-Ile}$ (59%), $5\text{-Ile}$-$8\text{-Phe}$ (15%), and $3\text{-Val}$-$8\text{-Phe}$ (2%); recovery of the injected radioactive material was 99%. The degradation rate of $^{14}C$-angiotensin II in whole dog blood in vitro was only 17%/min. Similar metabolic patterns were seen in vivo and in vitro. Sixty-six percent of the injected $J$-$\text{Asp}$-$^{125I}$-angiotensin II was metabolized, but only 23% of the $d$-$\text{Asp}$-$^{125I}$-angiotensin II was metabolized in a single passage through the kidney. These observations indicate that, under the conditions of these experiments, (1) angiotensin II is rapidly metabolized in the dog kidney by multiple enzymes, including an aminopeptidase, (2) circulating plasma enzymes do not account for the renal handling of angiotensin II, and (3) angiotensin II is not sequestered in the kidney.

KEY WORDS renal circulation aminopeptidase endopeptidase angiotensinase chymotrypsin trypsin renin radioimmunoassay

Many proteolytic enzymes native to circulating blood (1, 2) and to several organs (3-5) are capable of attacking angiotensin II. Previous studies from our laboratories have demonstrated substantial losses of infused angiotensin II in a single passage through a number of vascular beds, including those of the hind limb (6) and the kidney (7). The rate of disappearance is too rapid to be due to plasma angiotensinases alone. Whether these losses are due to enzymatic degradation, sequestration in blood vessel walls, or both is unclear. The present study was undertaken to further characterize the handling of angiotensin II by the dog kidney. $d$-Amino acid-substituted analogues of angiotensin II and a system which prevented recirculation of peptides through the kidney were used.

Methods

The metabolism of synthetic $1$-$\text{Asp}$-$^{14}C$-$5\text{-Ile}$-angiotensin II ($^{14}C$-AII) (250 mc/mmole, New England Nuclear), $1$-$J$-$\text{Asp}$-$^{125I}$-$4$-$\text{Tyr}$-$5$-$\text{Val}$-angiotensin II ($^{125I}$-AII), and $1$-$d$-$\text{Asp}$-$^{125I}$-$4$-$\text{Tyr}$-$5$-$\text{Val}$-angiotensin II ($d$-$\text{Asp}$-$^{125I}$-AII) was evaluated following incubation in whole dog blood in vitro and after a single passage through the vascular bed of the dog kidney in situ. Radioactive tracer techniques and a radioimmunoassay for angiotensin II (AII) (7, 8) were used.

PEPTIDE CHARACTERIZATION

The purity of peptides was assessed by two-dimensional peptide mapping. Distilled water (50 uliters) containing either 0.2 fC (100 pmoles) of $^{14}C$-AII or 0.5 pmoles of unlabeled $1$-$J$-$\text{Asp}$-$5$-$\text{Ile}$-angiotensin II were spotted in three applications on Whatman 3MM paper (46x57 cm). The sample was then subjected to descending chromatography in a butanol-acetic acid-water (4:1:5) system for 17 hours at 21°C. The paper was dried in room air for 4 hours and then subjected to electrophoresis in a second dimension at 4,000 v for 60 minutes in pyridine acetate buffer, pH 3.6. To identify unlabeled peptides, the paper was cut into 1-inch squares and counted under a toluene-based scintillation fluid in a liquid scintillation counter (Packard). Each peptide produced a single ninhydrin-positive or radioactive spot.

To evaluate $d$-$\text{Asp}$-AII for racemization, $d$-$\text{Asp}$-AII and $l$-$\text{Asp}$-AII were incubated with leucine aminopeptidase (40 ng, 185 units/mg) in 0.11% ninhydrin collidine solution, and heated to 70-80°C in a chromatography oven. To identify labeled peptides, the paper was cut into 1-inch squares and counted under a toluene-based scintillation fluid in a liquid scintillation counter (Packard). Each peptide produced a single ninhydrin-positive or radioactive spot.

Received June 11, 1973. Accepted for publication September 8, 1973.

From the Department of Medicine, University of Chicago Medical School, Chicago, Illinois 60637, and the Departments of Human Development and Physiology, Michigan State University, College of Human Medicine, East Lansing, Michigan 48823.

This work was supported by U. S. Public Health Service Grants HL-14982 and HL-14075 from the National Heart and Lung Institute and by the Louis Block Fund of the University of Chicago.

Received June 11, 1973. Accepted for publication September 8, 1973.

500
buffer containing 2.5 mM MgCl₂, pH 8.5, for 60 minutes. Each reaction mixture was immediately lyophilized and subjected to amino acid analysis. The l-Asp-AII digestion released aspartic acid in quantitative yield. The d-Asp-AII digestion produced no detectable free amino acids, indicating that there was no measurable l-Asp at the amino terminus of the peptide.

IODINATION PROCEDURE

d-Asp-AII and L-Asp-AII were labeled with 126I by the chloramine T method of Greenwood et al. (9) as modified by Nielsen et al. (10). The procedures for iodination and purification of labeled peptides have been described in detail in a previous publication (11). Over 95% of the labeled material was monoiodinated and subjected to amino acid analysis. The Z-Asp-AII digestion released aspartic acid in quantitative yield.

PREPARATION OF PEPTIDE STANDARDS

To identify possible degradation products of 14C-AII, the peptide was digested with several enzymes and the products were subjected to peptide mapping as described previously. Trypsin, chymotrypsin, and carboxypeptidase A (DPP-treated) were used. Figure 1 summarizes the cleavages which should occur with these three enzymes. All enzyme incubations were carried out in 0.2 M sodium phosphate buffer, pH 7.0, for 30 minutes. Trypsin (20 µg, 205 units/mg) digestion was carried out in 0.2 M sodium phosphate buffer, pH 7.0, for 30 minutes. Chymotrypsin (20 µg, 50.9 units/mg) digestion was carried out in 0.1 M ammonium bicarbonate buffer, pH 8.0, for 30 minutes. Carboxypeptidase A (2 µg, 50 units/mg) digestion was carried out in 0.1 M ammonium acetate, pH 8.0, for 5 minutes. Each reaction mixture was lyophilized, spotted on Whatman 3MM paper, and subjected to two-dimensional peptide mapping as previously described. 14C-lysine (250 µc/m mole, New England Nuclear) was used as an additional standard.

IN VIVO AND IN VITRO METABOLISM

Ten mongrel dogs of both sexes weighing 15-20 kg were anesthetized with sodium pentobarbital (30 mg/kg, iv), intubated with a cuffed endotracheal tube, and placed on a Harvard positive-pressure respirator. A femoral vein was isolated and cannulated for the infusion of additional anesthetic. The left jugular vein was exposed, cannulated with Tygon tubing, and connected to the output side of a Sigma Motor pump. The left kidney was exposed via an extraperitoneal flank incision, and the renal artery and vein were isolated. The dogs were then given heparin sodium (1000 units/kg, iv). The renal artery was occluded with a bulldog clamp, and the renal vein was rapidly (3 minutes) cannulated with Tygon tubing. The bulldog clamp was removed, and blood was allowed to flow from the renal vein through the Tygon tubing into a reservoir from which it was pumped into the dog’s jugular vein at a rate adjusted to keep a constant level in the reservoir. A 23-gauge curved needle attached to a polyethylene catheter was placed directly in the renal artery for injection of labeled angiotensin II. Renal blood flow was determined by direct timed collection of the venous effluent in a graduated cylinder and was considered to be stable when three successive 1-minute collections varied by less than 10 ml. Blood was returned to the reservoir after each collection.

After renal blood flow had stabilized, control samples of renal venous blood were obtained; 15 ml of renal venous blood was collected in a flask containing 100 pmol of 14C-AII. The mixture was incubated at 37°C. Samples were removed at 30 seconds and 1, 2, 3, and 5 minutes and added to iced tubes containing 2.6 M ethylenediaminetetraacetic acid (EDTA), 1.6 mM dimer- capitol, and 3.4 mM 8-OH-quinoline (final concentration). The samples were spun immediately at 4°C in a refrigerated centrifuge, and the plasma fraction was removed and frozen. Control and experimental plasma samples were analyzed by the same techniques.

14C-AII (600 pmol) was injected as a single bolus into the renal artery of six dogs. 125I-AII (25 pmol) was injected into the renal artery of two dogs and d-Asp-125I-AII (25 pmol) was injected into the renal artery of two dogs. For 2.5 minutes following the
injection, the renal venous outflow was collected in 15-
mL samples in iced flasks containing EDTA, dimercap-
tol, and 8-OH-quinoline as previously described. Plasma samples obtained from the systemic circulation during this period contained no detectable radioactiv-
ity, ruling out recirculation of appreciable amounts of
peptides through the kidney.

The blood was immediately centrifuged at 4°C and
the plasma removed. Samples of plasma were taken for
determination of recoveries of radioactive material by
liquid scintillation counting and of immunoreactive
material by radioimmunoassay of AII (7, 8). In the 14C-
AII experiments, the remainder of the plasma was
passed over 2 × 50-mm columns of Dowex 50W × 2 for
concentration of peptides. The peptide concentration
technique has been described in detail previously (12).
Recoveries from the columns were 90–95%. The eluates
were lyophilized and subjected to peptide mapping as
previously described. In experiments utilizing 125I-
labeled peptides, unextracted plasma was subjected to
high-voltage paper electrophoresis and column chro-
matography on DEAE Sephadex A-25 as previously
described.

Results

PEPTIDE CHARACTERIZATION

Figure 2 is a map of 14C-AII, 14C-Ile, and
standard peptide fragments generated by enzymatic
digestion of 14C-AII. The resolution of the method
allowed identification of each peptide by position;
95% of all radioactivity applied to the map
appeared in identifiable spots. Sensitivity of detection
of a spot was 0.5% of the total radioactivity in the
sample.

PREPARATION OF PEPTIDE STANDARDS

Figure 3 summarizes the separation of the
peptide fragments of 125I-AII from one another and
from 125I-AII by electrophoresis and column
chromatography. d-Asp-AII and small peptide

![Image](https://example.com/fig2.png)

**FIGURE 2**

Peptide map of 14C-AII, 14C-Ile, and standard peptide frag-
ments generated by enzymatic digestion of 14C-AII or un-
labeled AII. The sequence of each peptide is indicated in
parentheses. Striped spots are radioactive; clear spots are
ninhydrin positive. T = product of trypsin digestion, CPA =
product of carboxypeptidase A digestion, ChT = product of
chymotrypsin digestion. HVPEP = high-voltage paper elec-
rophoresis and FC = paper chromatography.

![Image](https://example.com/fig3.png)

**FIGURE 3**

Separation of peptide fragments of 125I-AII from one an-
other and from 125I-AII by high-voltage paper electrophore-
sis and column chromatography on DEAE Sephadex. The
positions of 125I-AII (All), 125I-Tyr (Tyr), and the iodinated
products of hydrolysis by chymotrypsin (ChT) and trypsin
(Trp) are labeled.
Table 1

Angiotensin II Metabolism In Vitro: Percent of Total Counts

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>All</th>
<th>Trp (3-8)</th>
<th>ChT (5-8)</th>
<th>Ile (5)</th>
<th>Total metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>88</td>
<td>(84-93)</td>
<td>2 (0-4)</td>
<td>4 (2-5)</td>
<td>6 (4-9)</td>
</tr>
<tr>
<td>1</td>
<td>83</td>
<td>(79-87)</td>
<td>3 (2-5)</td>
<td>8 (6-9)</td>
<td>6 (3-9)</td>
</tr>
<tr>
<td>2</td>
<td>68</td>
<td>(62-75)</td>
<td>0</td>
<td>12 (6-15)</td>
<td>20 (17-25)</td>
</tr>
<tr>
<td>3</td>
<td>52</td>
<td>(44-67)</td>
<td>0</td>
<td>17 (9-19)</td>
<td>31 (26-34)</td>
</tr>
<tr>
<td>5</td>
<td>31</td>
<td>(28-36)</td>
<td>0</td>
<td>7 (3-9)</td>
<td>62 (56-70)</td>
</tr>
</tbody>
</table>

Mean values and ranges are given for three experiments.

IN VITRO METABOLISM

Table 1 summarizes the results of whole blood incubations of 14C-AII in vitro. The half-life of 14C-AII in this system was 3 minutes. All breakdown was only 17% in 60 seconds (the time required for most of the injected 14C-AII to traverse the renal circulation). The tryptic (3-Val-8-Phe) and chymotryptic (5-Ile-8-Phe) peptides were generated after short incubation periods; isoleucine was the major product of prolonged incubation. Results from three experiments were reproducible within 10%.

IN VIVO METABOLISM

Figure 4 shows the appearance of labeled and immunoreactive material in the renal venous effluent in a representative experiment; 95% of the immunoreactive material and 82% of the labeled material appeared within 60 seconds of injection. The prolonged transit time was due to decreased renal blood flow secondary to angiotensin-induced vasoconstriction. The peaks of labeled and immunoreactive angiotensin II occurred at 30 seconds; the peaks of peptide metabolites and isoleucine appeared somewhat later. The larger peptide fragments appeared before isoleucine, suggesting stepwise degradation of angiotensin II.

Figure 5 is a peptide map from a representative in vivo experiment with 14C-AII. The distribution of counts in the entire renal venous effluent is summarized. After a single circulation through the kidney, 22% of the radioactivity appeared as intact AII, and 78% appeared as metabolites, including Ile (59%), 5-Ile-8-Phe (17%), and 3-Val-8-Phe (2%). All of the labeled spots on the map corresponded to peptide standards.

Results from the six experimental dogs in which 14C-AII was used are summarized in Table 2.

Figure 4 shows the appearance of labeled and immunoreactive peptides in renal venous plasma plotted against time after injection into the renal artery. Peptides are quantified as percents of total radioactive or immunoreactive material in renal venous plasma. For abbreviations, see Figure 3.
FIGURE 5
Renal metabolism in vivo of $^{14}$C-AII. Peptide map of $^{14}$C-labeled peptides collected in renal venous effluent following bolus injection of $^{14}$C-AII into the renal artery. The quantity of radioactive material present in each spot is expressed as a percent of total radioactive material on the map. Abbreviations and standard peptides are the same as in Figure 2. Outlines of peptide spots are schematic.

$^{14}$C-AII was metabolized to the extent of 75% in a single passage through the kidney. The metabolic products were $^{14}$C-Ile (59%), 5-Ile-8-Phe (15%), and 3-Val-8-Phe (2%). The pattern of metabolic products was similar to that in the in vitro experiments and suggests the effects of multiple enzymes. Total recovery of immunoreactive material from experiments 5 and 6 was 26.3% and 21.9%, respectively. This procedure was in excellent agreement with the peptide mapping technique by which 19.7% and 21.0% of total counts recovered appeared as angiotensin II in the same experiments.

Figure 6 summarizes the results of a representative in vivo experiment with $^{125}$I-AII and the distribution of counts in the entire renal venous effluent. Sixty-six percent of injected $^{125}$I-AII was broken down in a single passage through the kidney. The metabolic products were $^{125}$I-Tyr (54%) and I-Asp-4-Tyr (12%). Results from two experiments were reproducible within 10%. Analyses by electrophoresis and chromatography were in close agreement. d-Asp-$^{125}$I-AII was more resistant to the metabolic effects of the renal circulation; 77% of injected d-Asp-$^{125}$I-AII survived the renal circulation intact (Fig. 7). Only 23% of the radioactive material recovered was in the form of metabolites: $^{125}$I-Tyr (21%) and 1-Asp-4-Tyr (2%). Results from two experiments were reproducible within 5%. Due to the small molar quantity injected, no decrease in renal blood flow was observed after injection of $^{125}$I-AII or d-Asp-$^{125}$I-AII.

**Discussion**

This study yielded chemical and immunologic evidence that angiotensin II is metabolized in the intact renal circulation of the dog at a rate that is too rapid to be due to plasma angiotensinases alone. The half-times for breakdown of angiotensin II were 3 minutes for whole dog blood in vitro and less than 1 minute (less than a single circulation time through the kidney in our preparation) for the canine renal circulation in vivo. The angiotensin II-destroying capacity of dog kidney was large: 75% of a dose of angiotensin II circulating at 200 times physiological levels was degraded in a single passage.

<table>
<thead>
<tr>
<th>TABLE 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Angiotensin II Metabolism In Vivo: Renal Circulation</strong></td>
</tr>
<tr>
<td>Expt.</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
</tbody>
</table>

*Subscript indicates the number of peptides in the set.*

*Circulation Research, Vol. XXXIII, November 1973*
RENAL HANDLING OF ANGIOTENSIN II

The finding of extensive and rapid metabolism of angiotensin II in the intact renal circulation in vivo confirms the results of others (13-16) using bioassay techniques. Destruction of natural angiotensin II and synthetic $\alpha$-(1-Asp-5-Val), $\alpha$-(1-Asp-5-Ile), and 1-Asn-5-Val-angiotensin II has been found in the kidneys of rat, rabbit, and dog. Isolated kidneys perfused with a plasma-free medium have been shown to inactivate angiotensin II (17-20), suggesting that the renal removal of angiotensin II does not depend on circulating angiotensinases. Studies in which bioassay techniques were employed did not differentiate between enzymatic breakdown and either receptor occupation or tissue sequestration as mechanisms for renal inactivation of angiotensin. The demonstration that renal removal of angiotensin can be suppressed by enzyme inhibitors favored an enzymatic mechanism (17, 19). Experiments in which 1-Asp-$^3$H-4-Tyr-5-Val-angiotensin II of high specific activity was infused into isolated rat kidneys perfused with Krebs-dextran solution showed a 67% inactivation of infused angiotensin II (20). In this study, peptide metabolites were not specifically identified and quantified, and 17% of the radioactive material infused was not recovered.

In the present study, peptide metabolites of angiotensin II were identified and quantified after a single passage through the renal vascular bed of the dog. Results of these experiments suggest that a complex enzymatic mechanism is involved in the renal handling of angiotensin II in vivo. Most of the previous attempts to characterize the mechanism of
action of renal angiotensinases have used cell-free extracts of renal tissue (5, 21-23). The physiological significance of data obtained from such studies is difficult to evaluate, since artifacts may be introduced by disruption of cells. In the present study, the pattern of metabolites appearing in the renal venous effluent was similar to that seen in whole blood in which an aminopeptidase and an endopeptidase contribute to angiotensinase activity. The behavior of 1-Asp-angiotensin II in the renal circulation further implicates the participation of multiple enzymes. Only one-third as much 1-Asp-angiotensin II as 1-L-angiotensin II was destroyed in a single passage through the kidney. This finding indicates that the kidney metabolizes angiotensin II largely by an aminopeptidase mechanism with lesser participation by an endopeptidase. Additional support for this mechanism comes from experiments in which the amino terminus of angiotensin II was protected by conjugation with phenyl isothiocyanate-35S (24). Following infusion of labeled phenyl isothiocyanate-35S-angiotensin II into the intact dog, peptides which appeared to result from endopeptidase activity were found in the circulation.

Recovery of administered radioactivity from the renal venous effluent was complete in the present study, ruling out tissue binding or sequestration as important mechanisms of renal handling of angiotensin II under our experimental conditions. The finding by Bailie et al. (7) that significant amounts of 14C-AII do not appear in renal hilar lymph or urine supports the view that metabolism takes place primarily in the vascular compartment. These experiments do not exclude circulating angiotensinases and tissue binding from contributory roles in the renal handling of angiotensin II. The dose of 14C-angiotensin II which we administered was biologically active and caused renal vasoconstriction, presumably by combination with receptor sites on the arteriolar wall. The finding of complete recovery of administered radioactivity then suggests that angiotensin II was removed from receptor sites as part of the inactivation mechanism. The delay in appearance time between angiotensin II and its metabolites in the renal venous effluent may have been due to transient receptor occupation and elution. There is evidence that plasma angiotensinases may participate in this process. Reversal of angiotensin II tachyphylaxis in isolated aortic strip preparations has been shown after perfusion with plasma or blood but not with Ringer's solution (25, 26). This reversal occurs with α- but not β-Asp-angiotensin II and has been attributed to removal of peptide from receptor sites by enzymatic destruction. The absence of plasma angiotensinases may explain the incomplete recovery of radioactivity in some experiments in which Ringer's solution was the perfusion medium (20).

The role of tissue sequestration in the peripheral handling of angiotensin II has recently been stressed (27). A decrease in pressor activity of angiotensin II in passage through the femoral arterial bed of the conscious dog has been related to delayed circulation due to profound arteriolar vasoconstriction. A vasodilating cofactor is effective in preventing these losses. The present study gave no evidence for sequestration of angiotensin II in the dog kidney, because (1) recovery of administered radioactivity was complete, (2) delayed release of intact angiotensin II was observed, and (3) 14C-angiotensin II and 125I-angiotensin II were metabolized in similar fashion despite large differences in the dose of peptide administered and the vasoconstrictor response induced.

References
Mechanism of Renal Handling of Angiotensin II in the Dog
SUZANNE OPARIL and MICHAEL D. BAILIE

Circ Res. 1973;33:500-507
doi: 10.1161/01.RES.33.5.500

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1973 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/33/5/500

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/