Role of Endothelium in Conversion of Angiotensin I to Angiotensin II in Rabbit Aorta

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SUMMARY Rabbit aortic rings with either an intact endothelium or a disrupted endothelium were used to generate dose response curves to angiotensin I (AI) in the presence (ED90 = 3 × 10−7 M) and absence (ED90 = 1.7 × 10−8 M) of 10 µg/ml teprotide, a converting-enzyme inhibitor. Treatment with teprotide did not alter responses to angiotensin II (AII). Comparable dose-dependent responses were obtained with AII regardless of endothelial integrity. Contraction velocities in response to angiotensin I (10−7 M) and AII (10−7 M) were also measured. Angiotensin II produced a significantly greater contraction velocity (p <0.001) than that produced by AI. The amount of conversion to AII by both intact rabbit aortic rings and rings following removal of the endothelium was determined using 125I-AI and 125I-AII. Waters C18 SEP-PAK columns were used to separate AI and AII. During the first 3 to 4 minutes after the addition of AI, contraction velocity measurements and conversion were greater in intact rings than rings without endothelium. Conversion of AI to AII in endothelial-disrupted rings was the same as in intact rings by 5 minutes after the addition of AI. Conversion of AI to AII was inhibited by 30 µg/ml teprotide at all times measured, and there was no evidence of an alternate route of metabolism. Angiotensin I contraction velocity measurements after 10 µg/ml teprotide also demonstrated impaired efficiency of conversion of AI to AII. Thus, it was established that a lack of endothelium attenuated the rate of conversion of AI to AII initially, and formation of AII with or without endothelium was blocked by teprotide. This evidence strongly suggests that the rates of conversion of AI to AII are influenced by endothelial cells in the arterial wall, and AI has a weak direct action on vascular smooth muscle. (Hypertension 6: 216–221, 1984)

KEY WORDS • endothelium • angiotensin I • angiotensin II • teprotide • converting enzyme • aorta

IN 1956 Skeggs et al.1 identified two angiotensin peptides and showed that conversion of angiotensin I (AI) to angiotensin II (AII) was dependent on a plasma enzyme. Later investigators found angiotensin-converting enzyme (ACE) in the pulmonary vasculature,2,3 kidney,4 mesenteric arcade,2 and isolated tissue preparations such as rabbit aortic strips.5 Since the quantity of AI produced across several intact vascular beds was greater than that attributable to plasma ACE activity,2,2 it was hypothesized that ACE was located on the luminal surface of the vascular endothelium. This hypothesis was confirmed by Ryan et al.6 and Johnson and Erdos.8 In 1981, a study6 with immunofluorescent microscopy demonstrated the presence of ACE in both the intima and adventitia of guinea-pig artery. In 1982, Velletri and Bean10 identified ACE activity in homogenates of rat aortic tunica media and adventitia, but not of the intima. They also presented evidence that aortic enzyme activity was not blocked by large amounts of the ACE inhibitors teprotide and captopril.

Dose-dependent responses to AI in rabbit aortic strips have been documented by Ackerly et al.6 and Trachte et al.11 These contractile responses were attributed primarily to conversion of AI to AII. After pretreating the aortic strips with teprotide, however, dose-dependent contractions still occurred, but high concentrations of AI were required.6 Although teprotide has been shown to be a competitive inhibitor of ACE, it was demonstrated that 100 and 300 µg/ml of teprotide did not shift the AI dose-response curve beyond that achieved with 10 µg/ml of teprotide. This suggested the possibility for a weak direct action of AI on arterial smooth muscle.9 However, measurements of ACE activity were not made.

Several recent studies12–15 have addressed the ease with which endothelium can be removed or sloughed during preparation of vascular segments for bioassay. Since ACE is known to be located in the plasma membrane of vascular endothelium, variations in endothelial integrity should influence conversion of AI to AII by an arterial preparation. Previous studies have present-
ed evidence of reproducible conversion of AI to All by aorta, but they were done under conditions that should have resulted in the loss of most of the endothelium. Therefore, our present studies were performed to determine if: 1) the aortic conversion of AI to All was associated with the integrity of the endothelium; 2) a significant conversion occurred in the presence of teprotide, as suggested by Velletri and Bean; and 3) AI had a direct action on arterial smooth muscle.

Methods and Materials

Methods

Male New Zealand white rabbits (2-3 kg) were sacrificed by cervical dislocation, and the thoracic aortas removed. After carefully stripping the excess connective tissue, 2 to 3 mm rings were cut from the center portion of the thoracic aorta with care to ensure the presence of an intact endothelium. In some rings the endothelium was purposely disrupted by passing a nylon cord through the lumen. All rings were maintained in an organ bath at 37° C with continuous bubbling of 95% O₂, 5% CO₂, in a modified Krebs Ringer buffer, pH 7.4, of the following composition (millimolar): 111 NaCl; 5 KCl; 1 NaH₂PO₄; 0.5 MgCl₂; 25 NaHCO₃; 2.5 CaCl₂; and 11.1 dextrose.

In experiments determining the effects of intimal integrity upon the AI dose-response relationship, care was taken upon removal of the aorta to minimize exposure of the adventitia to blood. This was done to limit potential absorption of plasma-converting enzyme by the aortic wall. Four ligatures were tied around the aorta in situ, two below the aortic arch and two above the mesenteric and renal arteries. The aorta was dissected free of connective tissue, severed between each set of ligatures, and thoroughly flushed with Krebs buffer. Remaining connective tissue was carefully removed, and 2 to 3 mm rings were cut.

Rings used for AI and All dose-dependent responses and velocity studies were suspended in a 10 ml organ bath from wire supports (0.5 mm) embedded in a glass rod and attached to a Grass FTO3C force transducer (Grass Instruments, Quincy, Massachusetts) coupled to a Brush 440 recorder (Brush 440-Gould Recording Systems, Cleveland, Ohio). These rings were originally placed under 2 g of passive isometric tension and allowed to equilibrate for 90 minutes. The buffer was changed every 30 minutes and the tension adjusted until a resting tension of 1.5 g was established. Before exposure to cumulative doses of angiotensins, rings were contracted to a plateau with an ED₁₀ concentration (0.5 to 1.0 × 10⁻⁷ M) of phenylephrine, and then relaxation was induced with A23187 or acetyl-B-methylcholine chloride (methacholine) to assess the integrity of the endothelium. Survey transmission and scanning electron micrographs were also obtained on rings and correlated with the relaxation responses to A23187 and methacholine.

Dose-dependent responses were induced by cumulative doses of AI (10⁻⁴ to 1 × 10⁻⁶ M) and All (3 × 10⁻¹⁰ to 3 × 10⁻⁸ M), while contraction velocities were determined following administration of a single maximal dose (10⁻⁷ M) for a 10-minute period. The AI was determined to be free of All and angiotensin III (AIII) contamination by thin layer chromatography on cellulose in butanol, acetic acid, water, and pyridine (15:3:12:10) and development by fluorescamine. Angiotensin I responses were obtained before and after the addition of 10 μg/ml teprotide. Experimental periods were separated by at least 45 minutes, with the buffer being changed every 15 minutes. Reproducible responses to the angiotensins were obtained for 2 consecutive cumulative dosage protocols.

Conversion studies were done with rings suspended on a wire triangle in 3 ml of buffer at 37° C and gassed with 95% O₂, 5% CO₂, An unlabeled All (10⁻⁶ M) trap was introduced 2 minutes before the addition of 3 × 10⁻⁶ M AI and tracer ¹²⁵I-Al (24 nci/nmol) or ¹²⁵I-All (40 nci/nmol). Aliquots (0.5 ml) of buffer were removed and placed in polypropylene tubes containing 10 μl of 1N HCl to decrease the pH to 5.7. Samples were collected at 3, 5, and 8 minutes and used to measure the amount of All produced. After the 8-minute sample was obtained, an additional 0.5 ml aliquot was removed and used to determine the total counts in a sample aliquot. The remaining media was drained and the ring washed three or four times (1 to 2 minutes) with warmed buffer until the counts in a 0.5 ml aliquot approached background. The second experimental period began with the addition of 30 μg/ml teprotide for 5 minutes, and then the protocol with ¹²⁵I-All was repeated.

Angiotensin I was separated from All on C₁₈ SEP-PAK columns (Waters Associates, Inc., Milford, Massachusetts) attached to a 10 ml reservoir. Columns were equilibrated with 10 ml of the modified Krebs Ringer buffer with the pH adjusted to 5.0 with 10N HCl. This was followed by 20 ml of 0.1 M sodium phosphate buffer (pH 5.7). The sample was loaded on the column and washed with 1 ml of 80% 0.1 M phosphate buffer, 20% acetonitrile (ACN). All the fluid was collected and discarded. Then 7.5 ml of 80% phosphate buffer, 20% ACN (pH 5.7), was passed through the SEP-PAK and fractions collected in polypropylene tubes for counting on a Beckman 4000 gamma counter (Beckman Instrument Company, Columbia, Maryland). This was followed by 5 ml of 25% 0.1 M phosphate buffer, 75% ACN (pH 5.7), which was also collected in fractions and counted. The columns were regenerated by washing with 100% ACN (5 ml).

Initially, using ¹²⁵I-Al and ¹²⁵I-All standards, 1 ml fractions of the eluate from the SEP-PAK were counted to determine the elution profiles for AI and All. Both incubated and nonincubated samples of AI and All were eluted to determine if incubation or buffer affected the elution profiles of the peptides. After the elution profiles were established, fraction volumes were increased to 2.5 ml.

Unpaired Student’s t tests were used to test the significance of the data. A p value less than 0.05 was considered to be statistically significant. ED₅₀ values for dose-dependent responses were obtained from probit plots of the data.
Materials
Phenylephrine (Sigma Chemical Company, St. Louis, Missouri) and acetyl-methylcholine chloride (Sigma) solutions were made fresh daily and diluted in buffer. Stock solutions of AI and All (U.S. Biochemicals, Cleveland, Ohio) were prepared in deionized water, frozen, and diluted into buffer after thawing. $^{125}$I-AI and $^{125}$I-All were purchased from New England Nuclear (Boston, Massachusetts), SQ20,881 ( teprotide) from Squibb (Princeton, New Jersey), and A23187 (calcium ionophore) from Calbiochem-Behring (La Jolla, California). Ionophore was dissolved in dimethylsulfoxide (DMSO) and frozen at -20° C; it was thawed and diluted in buffer before use.

Results
The elution profiles for AI and All standards are given in Figure 1. These were obtained by diluting a given quantity of $^{125}$I-AI or $^{125}$I-All to 3.0 ml in buffer, removing a 0.5 ml aliquot, acidifying with 1N HCl, and eluting the materials retained on the C$_{18}$ column. Then 1 ml fractions were counted, yielding the profiles plotted. The elution profiles for AI and All did not change after incubation with rings, although there was an increase in the amount of $^{125}$I that was not retained by the C$_{18}$ resin in the absence of ion pair reagent (ACN). No fragment of the $^{125}$I-II formed that was retained by C$_{18}$ resembling that of $^{125}$I-Al. If metabolites of AI (or All) were generated via enzymes other than ACE, the metabolites coeluted with the respective labeled peptide standard.

The integrity of the endothelium was assessed by applying either methacholine or the calcium ionophore A23187, both of which elicit endothelial-dependent relaxation$^{12, 13, 15, 16-18}$ after contraction with an agonist. An ED$_{50}$ (0.5-1.0 x $10^{-7}$ M) of the adrenergic agonist, phenylephrine, was used to contract the rings. When the contraction reached a plateau, 10$^{-6}$ M methacholine or A23187 was added; rings with an intact intima relaxed while those lacking an intimal layer did not. A representative tracing for intact rings and rings lacking an endothelial layer obtained in this laboratory has previously been published. In rings with excellent endothelial integrity established by electron microscopy, A23187 induced relaxation of tone stimulated by an ED$_{50}$ concentration of phenylephrine (n = 9) and methacholine (n = 12), 73% ± 6% and 53% ± 4%, respectively.

Figure 2 illustrates the effects of 10 μg/ml teprotide on AI dose response curves in intact rings and rings lacking intimal endothelium. The ED$_{50}$ values for AI in intact and endothelial-disrupted rings before (1.7 x $10^{-8}$ M) and after teprotide treatment were identical (3 x $10^{-7}$ M). It should be noted that in rings lacking an endothelium a longer time interval was required to generate the same amount of tension for a given AI concentration when compared to intact preparations. The AI dose response curves are presented in Figure 3. Disruption of the endothelium or the addition of teprotide had no effect on the ED$_{50}$ value for All (3.5 x $10^{-9}$ M) in intact and endothelial-disrupted rings.

The average contraction velocity for AI(10$^{-7}$ M) in intact rings was significantly greater (p < 0.001) than that of rings lacking an intima (Figure 4). The greatest average velocity in intact rings occurred at 5 minutes while, for endothelial-disrupted rings, the average velocity was greatest at 8 minutes. Pretreatment with 10
μg/ml teprotide for 5 minutes attenuated the contractile responses to AI such that only 260 ± 58 mg (n = 24) in tension was obtained after 10 minutes in intact rings, and 226 ± 73 mg (n = 16) tension in rings lacking an endothelium. This is compared to 2238 ± 323 mg (n = 24) and 1480 ± 303 mg (n = 16), respectively, before teprotide pretreatment. The fastest average contraction velocity for 10⁻⁷ M All occurred at 2 minutes in intact rings. Contraction velocity in response to All was significantly greater (p < 0.001) than that in response to the same concentration of AI. Contraction velocities induced by All were not altered by endothelial disruption or treatment with teprotide.

As seen in Table 1, when conversion of AI to All was measured with ¹²⁵I-AI, the rate of AI formation was most rapid at 3 minutes (18.77% = 5.6 × 10⁻⁷ M All). After the addition of 30 μg/ml teprotide, conversion was effectively inhibited at 3, 5, and 8 minutes (p < 0.05). When measuring the conversion of AI to All in intact rings compared to that in rings lacking an endothelium, there was no difference except at 3 minutes. After teprotide pretreatment, there was little or no conversion of ¹²⁵I-AI to All in intact rings or rings lacking an endothelium.

TABLE 1 Percentage of Conversion of ¹²⁵I-AI to All by Rabbit Aortic Rings with Intact (+ ENDO) or Disrupted (− ENDO) Endothelium with or without (Control) Teprotide (TEP) at 3, 5, and 8 Minutes

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>TEP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ ENDO</td>
<td>− ENDO</td>
</tr>
<tr>
<td>3</td>
<td>18.77 ± 4.24</td>
<td>6.73 ± 1.72</td>
</tr>
<tr>
<td>5</td>
<td>16.4 ± 5.9</td>
<td>10.3 ± 4.4</td>
</tr>
<tr>
<td>8</td>
<td>10.7 ± 2.11</td>
<td>11.3 ± 3.28</td>
</tr>
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*p < 0.05.
Discussion

Currently, high pressure liquid chromatography (HPLC) is the most precise method used for separating AI and All. It is a costly and time-consuming process requiring very small sample volumes. The use of C_18 SEP-PAK columns to separate AI and All quickly and efficiently could facilitate many research projects. As indicated in the results, we have successfully separated AI from All using the C_18 SEP-PAK and gravity flow. It is a relatively rapid, inexpensive procedure in which sample volume is dependent upon the size of the reservoir attached to the column. There are few potential problems — solutions must be the correct pH, adequate equilibration of the column is essential, and the established ratios of phosphate buffer to acetonitrile should be used.

The elution profiles for iodinated AI and All were not altered by incubation. When the AI standard was eluted, a small percentage (2.3%) of material eluted with the All solvent. The same was true when the All standard was eluted on the column, that is, 2.4% of the All showed an AI elution pattern. This can probably be attributed to impurities in the 125I-AI and 125I-All standards. The values for trace contaminants agreed closely with data we have obtained from thin layer chromatography of AI and All standards on cellulose in butanol, acetic acid, water, and pyridine (15:3:12:10).

When teprotide was added to the incubation media, a similar pattern evolved. There was 3.3% activity in the All fractions after teprotide pretreatment. Correcting for contaminants in the labeled material of 2.3% means that only 1% of the 125I-AI appeared in the All fraction in the presence of teprotide. These substances are probably degradative metabolites and impurities that adhere to the column in a manner similar to All. It is unlikely that this material was All, since the teprotide concentration was far in excess of the amount required to block ACE. Even if this actually reflected All being produced via another pathway (non-ACE), the amount of All would have been negligible in terms of the amount of peptide required for a contraction (Figure 3). Thus, we found no evidence for significant ACE activity in the intact rabbit aorta that resisted inhibition by teprotide. Velletri and Bean reported inhibition by teprotide. Velletri and Bean reported that the findings of Velletri and Bean. The enzyme activity was too great to reflect plasma ACE which may represent a site for peptide sequestration. This and the thick, muscular nature of the aortic wall suggests that conversion occurred more rapidly in intact rings. This was confirmed by the 3-minute timepoint in the study when conversion of radiolabeled AI was measured and when the AI dose-dependent contractions were observed.

Our findings reflected a large complement of ACE somewhere in the aortic wall and not limited to intimal endothelium. The importance of the endothelium appears to be related to the efficiency of the conversion of AI to All. In rings lacking an intact intima, the average contraction velocity for AI (10^{-7} M) at all times was significantly (p < 0.001) slower than in rings with an intact intima, suggesting that conversion occurred more rapidly in intact rings. This was confirmed by the 3-minute timepoint in the study when conversion of radiolabeled AI was measured and when the AI dose-dependent contractions were observed.

The incubation data indicated that the longer exogenous AI was exposed to rings lacking an endothelium, the more AI conversion in these rings mirrored the conversion in intact rings (at 5 and 8 minutes). This suggests that the AI concentration must equilibrate across the aortic wall, and the conversion velocity will increase until a saturating AI concentration has reached the sites of conversion. Disrupting the intima may expose the subendothelial connective tissue, which may represent a site for peptide sequestration. This and the thick, muscular nature of the aortic wall should impose a diffusion barrier impeding access of AI to ACE in the tunica media. This would increase the time required for AI to combine with the ACE and resultant accumulation of All. ACE localized in the media adventitia may not be as readily accessible to exogenous AI as enzyme in the intima. Thus, ACE activity in endothelial-disrupted rings showed an early lag phase (less than 5 minutes) that quickly increased to the same rate as that in intact rings. The velocity data corroborated the existence of a lag phase. The contractile velocity data indicated that the initial accumulation that occurred in the intact ring probably was responsible for the difference in the total tension achieved as a function of time and average velocities at 10 minutes.

Thus, conversion of AI to All occurred without intimal endothelium being present, although an initial lag
phase did occur in rings lacking an intact intima. Te-
protide did effectively inhibit conversion of AI to All,
as shown from velocity measurements after teprotide
and from the incubation data obtained with \[^{125}\text{I}-\text{AI}\].
ACE activity could not be used as an index of intimal
endothelial integrity.

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