Effects of Angiotensin II and Some Analogues on Vascular Permeability in the Rabbit

By Abel Lazzarini Robertson and Philip A. Khairallah

ABSTRACT

Recent investigations in the rat have shown that endothelial cell contraction should be included among the multiple actions of angiotensin II (AII) in large arteries. In the current study, after intravenous injections (60 mg/kg) of the azo dye Evans blue, a segment of the rabbit abdominal aorta was isolated between temporary ligatures and injected with $1 \times 10^{-10}$ g of AII diluted in Ringer's solution. Diffuse increased permeability of the aortic endothelium occurred within 60 seconds only in areas exposed to AII. Although injections of Ringer's solution alone produced no blueing, surface staining and scanning electron microscopy of the aortic endothelium after AII administration showed endothelial cell contraction, widening of the interendothelial junctions, and surface sudanophilia. Similar studies were carried out to determine changes in dermal vascular permeability. Of all the substances tested, AII, prostaglandin E$_2$ ($1 \times 10^{-10}$ - $1 \times 10^{-8}$ g), and serum triglycerides ($1 \times 10^{-9}$ g) produced the most marked vascular response characterized by the appearance, within 15 minutes, of a bluish wheal or papule. Injections of angiotensin I or norepinephrine at concentrations of $10-100 \times 10^{-9}$ g or of Ringer's solution alone did not induce skin changes. Synthetic peptides, competitive antagonists to the myotropic and vasopressor activities of AII, blocked extravasation of Evans blue dye injected simultaneously with the octapeptide, but antihistaminics did not inhibit its effects. We concluded that the multiple actions of AII in blood vessels are related not only to the control of blood pressure but also to the regulation of the permeability and the function of the vascular wall as a whole.

KEY WORDS

arterial permeability

capillary permeability

endothelial contraction

atherosclerosis

prostaglandins

serum triglycerides

interendothelial gaps

guinea pigs. More recently, we presented evidence that AII directly contracts endothelial cells of the rat aorta and coronary arteries, leading to a widening of the interendothelial space separating adjacent cells (3). This widening of the gap between cells is associated with increases in vascular permeability to plasma macromolecules.

The present study was designed to further evaluate this action of AII, to extend the evaluation to dermal vessels, and to delineate the effects of synthetic peptides, competitive antagonists of AII, on vascular permeability.

Methods

Randomly bred New Zealand white female rabbits, weighing 6-8 lb, were used for all experiments. For evaluation of aortic endothelial
permeability, fasting rabbits were anesthetized with sodium pentobarbital, 24–30 mg/kg, iv, and subsequently slowly given 60 mg/kg of Evans blue dye1 (0.5% aqueous solution, equivalent to 0.452% anhydrous salt) in the marginal ear vein. Fifteen minutes thereafter, a midline laparotomy was performed without delay, and the abdominal aorta was exposed in its full length. After temporary ligation of all collaterals with no. 0 black silk loops, umbilical tapes were used to isolate a 2.5-cm segment of the abdominal aorta which was carefully marked with adventitial sutures proximally and distally. Then 0.2 ml of Ringer’s solution alone or of Ringer’s solution containing All or some of its analogues was injected through a 28-gauge needle into the isolated segment. Sixty seconds later the umbilical tape ligatures were released, and the rabbit was killed by injection of an air embolism. The thoracic and the abdominal aortas were then opened longitudinally, and representative specimens were taken for light and electron microscopy of both the proximal aorta and the isolated segment, using the adventitial markers as guidelines. The remaining aortic endothelial surface was photographed and processed for surface staining, using modifications of the technique proposed by Duff et al. (4). This procedure is based on the argyrophilic properties of the interendothelial spaces. It was supplemented in our study by use of fat stains to demonstrate localization of surface lipids. The endothelial aortic surface was mounted under tension on small Masonite boards and fixed in a 1% solution of phosphate-buffered gluteraldehyde for 30 minutes at 4°C. The excess fixative was then rinsed off with distilled water, and a 0.25% aqueous solution of silver nitrate was carefully dripped for 70 seconds onto the tilted endothelial surface with a small pipette. The sample was then rinsed in distilled water and exposed perpendicularly to the rays of a mercury-arc ultraviolet light at a distance of exactly 8 inches for an average time of 5.5 minutes. The light source had a built-in filter giving 70% transmission at 250–375 nm. The exposure time and the distance of the endothelial surface from the ultraviolet light are critical when reproducible results are desired. The endothelial surface was then counterstained in saturated acetone-ethanol solutions containing Sudan IV or Oil Red “0,” this procedure was followed by immersion in 70% propylene glycol for 3 minutes with constant agitation. For microscopic evaluation, the specimens were temporarily mounted in glycerine and photographed using incident slit illumination at both low and medium magnifications.

For electron microscopy, the specimens were processed by dehydration in ascending concentrations of ethanol followed by embedding in epoxy resins. Sections used for specimen orientation were 1μ thick; they were stained with toluidine blue and examined by phase-contrast microscopy. Thin sections selected on the basis of orientation of the endothelial lining perpendicular to the plane of fixation were stained with lead citrate and uranyl acetate and examined at 60–80 kv in an Elmiskop 1 electron microscope.

For evaluation of the effects of vasoactive agents on dermal capillary permeability, the following method was used. The backs of adult rabbits or guinea pigs were carefully shaved 24 hours before the test to avoid false positive reactions due to skin inflammation. Fasting animals were anesthetized and injected with Evans blue dye as described above. Fifteen minutes later, each vasoactive agent was injected intradermally at a total volume of 0.2 ml. A well-developed bluish papule rapidly appeared. The intensity of the reaction was qualitatively determined by measuring the size of the papule with a caliper. Ink markings on the skin were used to identify the injection sites. After a maximum skin reaction had occurred, usually after 60 minutes, the animals were killed by injection of an air embolism, and the skin was carefully removed. The degree of bluing of individual test sites was compared with that resulting from the injection of a standard dose (10 × 10−6 g) of All in the same animal. Similar injections of Ringer’s solution alone were used as control tests of skin sensitivity. The severity of blueing was read in all cases by the same observer and graded from negative to +3 on an arbitrary scale based on the surface area involved: the maximum diameter of the area of blueing was used for estimation of the response (Table 1). By and large, estimation of the severity of blueing was more reliable if the test was read from the dermal side of the skin, since the thickness of epidermal layers and their transfluence varied considerably between animals.

The following vasoactive agents were used in this study: prostaglandins E, and F2,8-Ala-AlII (5),8 4-His-6-Tyr-AlII (6),3 4-Phe-8-Ala-AlII (7),8 1-succinic acid (Suc)-4-Phe-8-Ala-AlII (7),3 and 4-Phe-8-Tyr-AlII (8).8

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1 Supplied by General Diagnostics, Warner-Lambert, Morris Plains, N. J.
2 Supplied by Dr. J. E. Pike, The Upjohn Company, Kalamazoo, Michigan.
3 Synthesized in our laboratory by Dr. M. C. Khosla in collaboration with Drs. F. M. Bumpus and R. R. Smeby.
Results

In experiments with temporary isolation of an aortic segment in vivo, a diffuse increase in the permeability of the aortic endothelium to Evans blue dye occurred only in areas exposed to AII. As shown in Figure 1, the proximal aortic segment of the same rabbit showed no blueing during the time of these observations. When a similar volume of Ringer’s solution instead of AII was injected under identical experimental conditions, no blueing occurred in the isolated segment or in the rest of the aorta. Intra-aortic injections of 1.0 ng of AII without isolation of an aortic segment produced diffuse endothelial blueing not only of the whole aorta but also of its major branches.

Surface staining was associated with consistent morphological changes in the endothelial cells exposed to AII (Fig. 2). Rounding and shortening of the endothelial cells and apparent widening of the interendothelial spaces was accompanied by increased surface sudanophilia and by deposition of fibrin strands. Irregularities and discontinuities of cellular boundaries were prominent in the AII-treated surface, but they were absent in proximal segments of the same aorta or following control injections of Ringer’s solution. Surface defects, suggesting endothelial cell lysis, or gross discontinuities of the endothelial surface were not found. Scanning electron microscopy confirmed these observations and showed the rounded cytoplasm of endothelial cells projecting into the vascular lumen with deposition of fibrin strands and blood cells on the endothelial surface (Fig. 3). Histological sections perpendicular to the aortic intima showed that surface blueing following injection of AII in the rabbit aorta was often accompanied by the appearance of platelet aggregates and the trapping of serum lipids above and below the endothelial surface (Fig. 4). These platelet aggregates consisted of a few distinct platelets, often mixed with other blood cells and fibrin.

Results of our studies on dermal capillary permeability in rabbits, in the presence of several vasoactive agents, are summarized in Table 1. Intradermal injections of up to 100 ng of angiotensin I did not produce significant increases in capillary permeability to Evans blue dye for up to 180 minutes following the injection. AII, on the other hand, in concentrations as low as 0.1 ng produced severe capillary leakage after 60 minutes or longer. Norepinephrine injected at concentrations of 10–100 ng had no significant effects. Prostaglandin E1 at 0.1–10 ng produced the most rapid and severe changes in capillary permeability, whereas prostaglandin F2α in similar concentrations induced no response. Serum triglycerides at concentrations of 1 µg or
higher also produced overt increases in capillary permeability, but injections of Ringer's solution alone produced no changes. When commercial antihistaminics such as Benzdryl (dyphenhydramine HCl) at concentrations of 10–100 μg were injected simultaneously with AII, they did not inhibit its effect on dermal capillary permeability.

Two of the angiotensin II analogues, 1-Suc-4-Phe-8-Tyr-AII, in dose ratios between 250 to 1 and 500 to 1, and 4-Phe-8-Tyr-AII, in dose ratios between 10 to 1 and 200 to 1, completely inhibited at 30 minutes but only partially inhibited at 60 minutes the increase in capillary permeability induced by AII.

As shown in Table 1, none of the other analogues of AII increased by themselves vascular permeability to Evans blue dye. 8-Ile-AII and 8-Ala-AII, as well as 4-Phe-8-Ala-AII, at dose ratios between 5 to 1 and 10 to 1 inhibited completely the action of AII for 30 minutes. Figure 5 shows areas of skin blueing in a rabbit 30 minutes after injection of AII alone and after injection of 8-Ile-AII mixed with AII at ratios of 100 to 1, 10 to 1, and 1 to 1, respectively. The simultaneous injection of the analogue completely inhibited the vascular leakage induced by AII under these experimental conditions.

When observed under a dissecting microscope, the areas of epidermal blueing were accompanied by interstitial edema of the underlying dermis (Fig. 6). Ultrastructural evaluation of the endothelial lining of vessels
Scanning electron micrograph of thoracic aorta of rabbit fixed in vitro 30 seconds after injection of 0.1 ng of AII. Note the ridges corresponding to contracted endothelial cells and the blood cells and fibrin strands attached to the vascular surface. Gold-coated specimen.

**TABLE 1**

**Rabbit Skin Tests for Studying the Effects of Vasoactive Agents on Dermal Vascular Permeability**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Average dose</th>
<th>Size of papule (mm)</th>
<th>30 min</th>
<th>60 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin I</td>
<td>10–100 x 10^{-4} g</td>
<td>2</td>
<td>2</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>1–10 x 10^{-6} g</td>
<td>2–4</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>10–100 x 10^{-6} g</td>
<td>2</td>
<td>4–6</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Prostaglandin E_{1}</td>
<td>0.1–10 x 10^{-6} g</td>
<td>2</td>
<td>4–6</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Prostaglandin F_{1a}</td>
<td>1–100 x 10^{-6} g</td>
<td>2</td>
<td>4–6</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1 x 10^{-4} g</td>
<td>2</td>
<td>4–6</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Ringer’s solution</td>
<td>0.2 ml</td>
<td>2</td>
<td>4–6</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

**Angiotensin II Analogues**

1-Suc-4-Phe-8-Tyr-AII:AII | 500:1 to 250:1 | 2–4
4-Phe-8-Tyr-AII:AII       | 200:1 to 10:1  | 2–4

**Analogue Inhibitors of Angiotensin II**

8-Ile-AII     | 1–100 x 10^{-6} g | 1:1
8-Ala-AII     | 1–100 x 10^{-6} g | 1:1
4-His-6-Tyr-AII | 10–100 x 10^{-6} g | 1:1
4-Phe-8-Ala-AII | 1–100 x 10^{-6} g | 1:1
8-Ile-AII:AII | 10:1 to 1:1     | 2–4
8-Ala-AII:AII | 10:1 to 5:1     | 4–6
8-Ala-AII:AII | 1:1             | 2–4
4-Phe-8-Ala-AII:AII | 10:1 to 5:1 | 4–6
4-Phe-8-Ala-AII:AII | 1:1             | 4–6

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FIGURE 4

Microscopic section of the abdominal aorta of a rabbit treated similarly to the one shown in Figure 1. Note the presence of clumps of blood cells on the endothelial surface (E) at the site of platelet attachment to the endothelial cells as well as in the adjacent subendothelial space (arrows). L = lumen. Sudan IV, hematoxylin stains.

FIGURE 5

Dermal surface of dorsal skin of the rabbit after injection of All (solid circles) at concentrations, from left to right, of 10 ng, 1 ng, and 0.1 ng, respectively. Broken circles show response to All in combination with 8-Ile-All. Note complete inhibition of response. Unstained preparation.

and capillaries showed widening of intercellular spaces and subendothelial edema.

Discussion

As a local hormone, the octapeptide All has been shown to have multiple biological actions. Recently we showed (3) that left intraventricular injections of the peptide at concentrations of 0.1-10 ng are able to contract arterial endothelial cells, which have some of the ultrastructural characteristics of smooth muscle cells. It appears that these contractile responses of arterial endothelial cells start within approximately 30 seconds after injection, reach a maximum within 2 minutes, and are terminated within 8 minutes. The contraction of the endothelial cells induces widening of the intercellular spaces, and temporary tortuous gaps measuring over 1,000 Å appear between the endothelial cells. The term interendothelial gap has been proposed to differentiate these spaces from the intercellular junctions found in normal endothelium. Their main functional significance is that of allowing temporary leakage of circulating macromolecules into the perivascular tissues.

The azo dye, Evans blue, was used in our study to measure vascular permeability following acute widening of the interendothelial spaces. Areas of spontaneous blueing following intravascular injection of Evans blue dye were shown by Packham et al. (9) to correspond in swine to sites of increased vascular permeability. 

Circulation Research, Vol XXI, December 1972
ANGIOTENSIN II AND VASCULAR PERMEABILITY

Typical example of an area of increased vascular permeability after injection of 0.1 ng of AII in the dermis. Note that the dye (Evans blue) diffused as indicated by the arrows without disturbing other small vessel branches (C) in the subcutaneous panniculus (SV). Unstained specimen, scale at the bottom is marked off in millimeters.

Uptake of labeled serum albumin. These observations were recently extended by Somer and Schwartz (10) with the demonstration that similar regions in the young pig also show increased accumulation of free tritiated cholesterol and correspond anatomically to sites where atheromatous lesions first appear in older animals. These authors described the presence of subendothelial edema and occasional platelet clumps at the sites of blueing as a possible indication of spontaneous focal changes in arterial permeability. Based on ultrastructural observations on the effects of AII and other vasoactive agents on the permeability of the rat aorta and the coronary endothelium mentioned above, we decided to apply the Evans blue technique to the qualitative demonstration of acute increases in permeability in the rabbit aorta following local administration of the octapeptide. As shown, the effects of AII injected into a temporarily isolated aortic segment were quite dramatic and restricted to the endothelial surface exposed to the vasoactive agent. Aortic endothelial surfaces proximal or distal to the vasoactive agent rather than to hemodynamic or hypoxic changes induced by temporary suspension of blood flow was demonstrated by two control experiments: (1) blueing did not occur in the isolated segment if it was injected with Ringer’s solution alone and (2) diffuse blueing of the distal aorta and its branches was found when the aortic flow was not temporarily obstructed during injection.

The significance of these sudden changes in aortic permeability in relation to induction of vascular disease seems manifold. Relative to atherogenesis, for example, it has been proposed that a metabolic or structural barrier may exist in normal arterial intima against the influx of serum cholesterol (11). Sudden changes in aortic endothelial permeability such as those shown in this study following injection of AII may provide temporary openings of this barrier and facilitate initiation of self-perpetuating metabolic alterations in the arterial cell that lead to atheroma. Whether the increased concentration of AII found in some patients with renovascular hypertension (12) explains their higher incidence of severe atherosclerosis should be studied further.

The morphological changes found in rabbit aortic endothelium exposed to AII in this study agreed with previous observations in the rat (3). Surface staining showed rounding of the endothelial cells as well as qualitative widening of the interendothelial functions in the creation of endothelial gaps. Recently, Lassen and Trap-Jensen (13) calculated the size of interendothelial slits that would be needed to explain current data on transepithelial exchange of small hydrophobic molecules such as $^{51}$Cr-EDTA and found, using Fick’s law of diffusion, that the slits could be

Circulation Research, Vol. XXXI, December 1972
extremely small—well within the range of 40 Å found in normal endothelium. Larger molecules such as albumin or lipoproteins have diameters above 100 Å; it is possible that temporary endothelial contraction and widening of interendothelial spaces to gaps even less than 1,000 Å in width (beyond the resolution of the light microscope [2,500 Å]) could suffice for leakage of these macromolecules into the subendothelial spaces. The diffuse and severe endothelial response induced by AII in the rabbit aorta, shown in our study by scanning electron microscopy, would be therefore more than adequate to produce massive permeability changes.

In the rat, it has been found that the effect of AII is enhanced and prolonged by release of vasoactive agents, particularly serotonin, following the degranulation of circulating platelets temporarily attached to the exposed subendothelial basement membrane, shown by Majno (14) to be rich in collagen as a result of endothelial cell contraction (3). These findings suggest that platelets may play an important role in maintenance of abnormal focal endothelial permeability and indirectly be involved in the pathogenesis of vascular disease.

Serum triglycerides were tested in this study following observations by Varess et al. (15) suggesting increased permeability of rat aorta to colloidal iron particles following 9 weeks on an atherogenic diet. These authors found a good correlation between deposition of lipid droplets and iron particles in the aortic wall. Their study followed observations by Friedman and Byers (16) showing accumulation of Evans blue dye in aortic lesions of cholesterol-fed rabbits and rats.

In our observations, serum triglycerides alone also acted as powerful stimuli for increased aortic endothelial permeability. Whether this finding was a direct effect of this lipid fraction on endothelial permeability or whether it was histamine-mediated needs to be determined.

The effect of the prostaglandins on aortic endothelial permeability deserves further study since the most effective compound tested in this study, prostaglandin E₁, is a vasodilator instead of a vasoconstricting agent.

In contrast to the observations of Bisset and Lewis (2), our findings indicated that dermal capillary permeability to Evans blue dye occurred in rabbits at sites of intradermal injections of AII, but this response was minimal in guinea pigs. It should be emphasized, however, that significant individual variations in the response to some drug concentrations were often found in our rabbits, not only for AII but for all other vasoactive agents tested. For that reason, each animal was tested with a standard concentration of AII and individual results for all other agents tested were compared with the responses to the standard. Under these control conditions, rabbits responded to AII doses one-hundred times lower than those reported by Bisset and Lewis (2). These doses fall closer to accepted physiological ranges. We are aware that the qualitative visual method used to evaluate stain responses by measuring the diameter of the area of blueing at predetermined times may have suffered from unavoidable inaccuracies.

The effects of AII on vascular permeability are still not fully understood. That the effects of the peptide on endothelial cells are not histamine-mediated seems probable since antihistaminics did not prevent its action. The relatively slow onset of effects, some occurring after 30 minutes, others after 60 minutes or longer (Table 1), was unexpected. The assumption is made that the main action of the peptide on the dermal vascular wall is contracting the endothelial cells and widening the interendothelial spaces, leading to plasma leakage. Such phenomena should start in a few seconds after injection, reach a maximum in about 2 minutes, and rapidly disappear thereafter. The length of induction of gross vascular leakage would suggest that other factor(s) may continue the short-lived effect of AII. A possible explanation now under investigation in our laboratory is that, similar to our previous observations in the arterial endothelium (3), attachment, contraction, and degranulation of circulating platelets
may, through the release of serotonin or other platelet-related vasoactive agents, initiate a vicious circle.

The finding that the effects of AII can be partially or totally inhibited by some of its analogues that have competitive antagonistic activities may be of pharmacological significance. Inhibition of the increased vascular permeability induced by AII with these analogues resembles their effects at similar relative doses on the myotropic or vasopressor activity of the octapeptide AII (17). Furthermore, inhibition of the effects of AII on dermal vascular permeability should provide a simple and effective testing system for the development of future antagonists.

These studies suggest that AII and other vasoactive agents have complex and possibly multiple sites of action in the blood vessels related not only to the control of peripheral blood pressure but also to the permeability and the function of the vascular wall as a whole. These agents may play lasting and important roles in the initiation of vascular diseases ranging from atherogenesis to intravascular thrombosis and peripheral edema.

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References

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