Protection of the Arterial Internal Elastic Lamina by Inhibition of the Renin-Angiotensin System in the Rat

Wei Huang, François Alhenc Gelas, Mary J. Osborne-Pellegrin

Abstract—Spontaneous rupture of the internal elastic lamina (IEL) occurs in some arteries of the rat during growth and aging. Inbred, normotensive, Brown Norway (BN) rats are particularly susceptible to rupture of the IEL, especially in the abdominal aorta (AA). Preliminary experiments showed that different angiotensin-converting enzyme (ACE) inhibitors protect against rupture of the IEL in the BN rat to a greater extent than hydralazine, suggesting a role of the renin-angiotensin system (RAS) in this phenomenon. To explore this possibility, we have treated male BN rats from 4.5 to 14 weeks of age with either enalapril or losartan (both at 1, 3, and 10 mg · kg$^{-1}$ · d$^{-1}$) or with the calcium antagonists mibefradil (at 3, 10, 30, and 45 mg · kg$^{-1}$ · d$^{-1}$) and amlodipine (at 30 mg · kg$^{-1}$ · d$^{-1}$). Systolic blood pressure (SBP) was measured weekly, and at the end of treatment we (1) recorded body and heart weights, (2) measured various parameters of the RAS in plasma, (3) quantified interruptions in the IEL on “en face” preparations of AA, and (4) quantified elastin, collagen, and cell proteins in the media of the thoracic aorta. Results showed that enalapril and losartan similarly decrease SBP and rupture of the IEL in the AA, suggesting that enalapril inhibits the latter via a decrease in the production of angiotensin II (Ang II) and not via another effect on ACE. The decrease in IEL rupture and in SBP, as well as the modifications in the parameters of the RAS, were all dose dependent. Mibefradil had little effect on the RAS and, at the highest doses, decreased SBP to an extent similar to that for enalapril at 3 mg · kg$^{-1}$ · d$^{-1}$ but did not significantly inhibit IEL rupture. Amlodipine decreased SBP, increased plasma renin concentration, and was without effect on IEL rupture. All treatments at the highest doses had a hypertrophic effect on the aortic media but differed in their effects on the heart, with enalapril and losartan decreasing and mibefradil and amlodipine increasing heart weight, suggesting that the inhibition of IEL rupture may be related to a cardiac hypertrophic effect. All these results, taken together, suggest that Ang II plays a role in the rupture of the IEL that is, in part, independent of SBP. (Circ Res. 1998;82:879-890.)

Key Words: Brown Norway rat ■ enalapril ■ internal elastic lamina ■ losartan ■ mibefradil ■ systolic blood pressure

Rupture of the IEL occurs spontaneously in rat arteries, to different degrees depending on the artery and the strain studied. The formation of IIEL is influenced by many factors, including age, sex, and hemodynamic factors such as blood pressure and flow. Although it appears that local factors determine IEL rupture within different arteries of any one rat, the overall susceptibility to this lesion is genetically determined. Indeed, we have previously reported that the inbred BN strain is particularly susceptible to IEL rupture and is the only strain among those we have studied to present significant numbers of ruptures in its AA.

The reason for this genetically determined susceptibility has not been elucidated at the present time. Aortas of BN rats present lower lysyl oxidase activity, higher elastase activity, and a lower elastin/collagen ratio than do aortas of the LE strain of rats, who are particularly resistant to IEL rupture. All these characters can conceivably contribute to increased susceptibility to IEL rupture. In addition, the BN rat, when rendered hypertensive, shows poor survival and, unlike the LE rat, is susceptible to cerebrovascular hemorrhage.

In the present study, using the normotensive BN rat as a model, we have explored the possible pharmacological prevention of IEL rupture by testing the effect of blood pressure–lowering drugs on this phenomenon. In pilot experiments, we compared the effects of imidapril, an ACE inhibitor, and hydralazine, a drug with direct vasodilatory action, on rupture of the IEL in different arteries. In view of the potent inhibitory effect of ACE inhibition on IEL rupture, in the final series of experiments we have studied in detail the effects of an ACE inhibitor (enalapril) and another blocker of the RAS (losartan, an antagonist of the Ang II type 1 receptor) and compared them with the effects of two calcium antagonists (mibefradil and amlodipine). These drugs, with the exception of amlodipine, were studied at several dose levels, and their effects on rupture of the IEL in the AA, parameters of the RAS, heart weight, and the composition of the thoracic aorta were determined. We observed that inhibitors of the RAS exhibited a potent protective effect against IEL rupture, an effect that may be, at least in part, independent of their blood pressure–lowering effect.
Materials and Methods

Animals

Inbred male BN rats were supplied by IFFA CREDO, Domaines des Oncins, L’Arbresle, France. They were received in our laboratory at 4 weeks of age, and experimentation was started after a few days of stabilization. The procedure followed for the care and euthanasia of studied animals was in accordance with the European Community Standards for the care and use of laboratory animals.

Drugs

The ACE inhibitors used in the present study were as follows: imidapril (Tanabe), enalapril (Merck Sharp & Dohme), and captopril (Sigma). Hydralazine was supplied by Sigma; losartan, by DuPont-Merck, R&D; and the calcium antagonists mibefradil and amloidipine, by Hoffmann-La Roche SA.

Experimental Protocol

Pilot Experiment

Rats were divided into appropriate groups and treated from 4.5 to 24 weeks of age. Eight rats were used as controls, 8 were treated with imidapril at 3 mg kg⁻¹ d⁻¹ in powdered chow, 8 were treated with imidapril at 6 mg kg⁻¹ d⁻¹ in powdered chow, and 7 were treated with hydralazine at 20 mg kg⁻¹ d⁻¹ in the drinking water. Chow containing imidapril was freshly mixed once a week, and the hydralazine solution in water bottles was renewed daily. All rats were kept under identical conditions, and their SBPs and body weights were recorded periodically (at 10, 12, 14, and 18 weeks of age). At the end of the experimental period, when aged 24 weeks, rats were anesthetized with sodium pentobarbital (Nembutal, 40 mg/kg IP, Abbott) after their body weights were recorded, and experimentation was started after a few days of stabilization. The procedure followed for the care and euthanasia of studied animals was in accordance with the European Community Standards for the care and use of laboratory animals.

Final Experiments

In view of the results of the pilot experiment, a second series was designed to study the inhibitory effect of ACE inhibitors on IEL rupture in more detail, with particular attention paid to blood pressure changes, especially at the beginning of treatment. In addition, comparison was made with the Ang II receptor antagonist, losartan, and also with two calcium antagonists, mibefradil and amloidipine, antihypertensive drugs that do not inhibit the RAS. The experimental period was shortened, with the rats being treated from only 4.5 to 14 weeks of age.

In experiment I, the effects of enalapril, at increasing doses, were compared with those of losartan. Three groups of 8 rats were treated with enalapril at doses of 1, 3, and 10 mg kg⁻¹ d⁻¹ (E1, E3, and E10 groups, respectively) in chow, and three other groups of 8 rats were treated with losartan at doses of 1, 3, and 10 mg kg⁻¹ d⁻¹ (L1, L3, and L10 groups, respectively) administered in the drinking water. Eight untreated rats served as controls. SBP, heart rates, and body weights were recorded weekly. In the middle of treatment (at 9 weeks of age) and at the end of treatment (at 14 weeks of age), 1 mL of blood was sampled from the jugular vein under anesthesia (50 mg/kg IP ketamine and 4 mg/kg IP xylazine) and collected on heparin. Plasma was frozen for assay of parameters of the RAS. At the end of treatment, after blood sampling, the thoracic aorta was rapidly dissected out, cleaned of blood and adipose tissue in ice-cold saline, frozen in liquid nitrogen, and stored at −80°C while awaiting the quantification of elastin, collagen, and cell proteins. After this, a catheter was placed in the aorta, above the renal arteries for the perfusion of the AA and the iliac arteries for the quantification of IIEL by histological examination of “en face” preparations. In addition, heart weight was recorded.

In experiment II, 30 rats were divided into five groups of 6. One group was untreated and served as a control, one group was treated with E3 (for comparison with experiment I), and three groups were treated with mibefradil at doses of 3, 10, and 30 mg kg⁻¹ d⁻¹ (M3, M10, and M30 groups, respectively) added to chow. The length of treatment and the parameters measured were identical to those of the first experiment using enalapril and losartan.

A third supplementary experiment (experiment III) was performed using a higher dose of mibefradil to try to decrease SBP to levels attained by E10 and L10 in experiment I and to test the effects of another long-acting calcium antagonist, amloidipine. Forty rats were divided into five groups of 8. One group was untreated and served as a control, one group was treated with E3, one group was treated with M30 (for comparison with experiments I and II), one group received M45, and the final group received A30. All drugs were administered in the chow. The length of treatment and the parameters measured were identical to those in experiments I and II.

Blood Pressure Measurements

SBP was measured in conscious rats, under standardized conditions routinely used in our laboratory, by using a tail cuff and pulse transducer (BP recorder 8006, Apleex) after 20 minutes under a specially adapted thermostated heating element set at 32°C. Heart rates were also recorded.

Quantification of IEL on En Face Preparations

The method used was based on one used previously to study IEL rupture in the caudal artery and has recently been adapted to study the aorta.7 Under ketamine/xylazine anesthesia, after blood sampling and removal of the thoracic aorta, the mesenteric artery was ligated to prevent perfusion of the mesenteric bed, and a catheter (PE-100) was placed in the aorta above the renal arteries in the direction of blood flow. The arteries distal to the catheter were perfused at a rate of 6 mL/min with a Braun perfusion pump as follows: (1) PBS containing 1% procaine for 1 minute to rinse and dilate the arteries, (2) buffered formalin for 10 minutes to fix the arteries, (3) distilled water for 1 minute, (4) orcein for 2 minutes to stain the IEL, (5) differentiation of orcein with 70% ethanol, followed by distilled water to rinse, (6) Groat’s hematoxylin for 1 minute to stain the endothelial nuclei and those of smooth muscle cells at sites where the IEL is absent, and (7) 0.1 mol/L phosphate buffer, pH 7.5, containing 5% sucrose for 5 minutes to rinse.

After this perfusion, the AA, the proximal 1 cm of the iliac arteries, and, in some cases, the renal and caudal arteries were dissected out, opened longitudinally under a dissecting microscope, and held open, luminal surface uppermost, under sucrose on paraffin wax in a Petri dish, using entomological pins. En face preparations were then washed, dehydrated, cleared, and mounted on slides, with the luminal surface uppermost.

With this technique, ruptures in the IEL, which have been previously studied in detail by light and electron microscopy,1,2,5 appear as dark blue-gray transverse bands due to the absence of elastin, which is stained pink elsewhere, and to the intense staining of underlying smooth muscle cell nuclei, which are not stained in areas where the IEL is present. They may thus be quantified with

Selected Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>A30</td>
<td>amlodipine at 30 mg kg⁻¹ d⁻¹</td>
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<tr>
<td>AA</td>
<td>abdominal aorta</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin-converting enzyme</td>
</tr>
<tr>
<td>Ang I, Ang II</td>
<td>angiotensin I and II</td>
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<td>BN</td>
<td>Brown Norway</td>
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<tr>
<td>E1, E3, E10</td>
<td>enalapril at 1, 3, and 10 mg kg⁻¹ d⁻¹</td>
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<tr>
<td>IEL</td>
<td>internal elastic lamina</td>
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<td>IIEL</td>
<td>interruption(s) of the IEL</td>
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<td>L1, L3, L10</td>
<td>losartan at 1, 3, and 10 mg kg⁻¹ d⁻¹</td>
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<td>LE</td>
<td>Long Evans</td>
</tr>
<tr>
<td>M3, M10, M30, M45</td>
<td>mibefradil at 3, 10, 30, and 45 mg kg⁻¹ d⁻¹</td>
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<td>PRA</td>
<td>plasma renin activity</td>
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<td>PRC</td>
<td>plasma renin concentration</td>
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<td>RAS</td>
<td>renin-angiotensin system</td>
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<td>SBP</td>
<td>systolic blood pressure</td>
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References

1. With this technique, ruptures in the IEL, which have been previously studied in detail by light and electron microscopy, appear as dark blue-gray transverse bands due to the absence of elastin, which is stained pink elsewhere, and to the intense staining of underlying smooth muscle cell nuclei, which are not stained in areas where the IEL is present. They may thus be quantified with...
ease under the light microscope. For all arteries considered, the total number of ruptures (ranging in length in the circumferential direction from 250 to 2000 μm, all being between 130 and 250 μm in width) was recorded, irrespective of size. Segments considered were as follows: for the AA, between the origin of the left renal artery and the iliac bifurcation; for the left renal artery, between its origin and the renal hilum; for the iliac arteries, the first centimeter after their origin; and for the caudal artery, a length of ~7 cm from the base of the tail. Results are expressed as total numbers of IEL per artery, except for the caudal artery, where they are expressed per centimeter of artery.

Measurement of Parameters of the RAS in the Plasma

PRA and Concentration

PRA was determined by radioimmunoassay of Ang I generated by the incubation of plasma. Results are expressed as picomoles of Ang I formed per milliliter of plasma per hour of incubation. PRC was determined in conditions similar to those for PRA but after adding an excess of exogenous angiotensinogen (present in the plasma of binephrectomized rats) to saturate the renin-substrate reaction.

Plasma Angiotensinogen Concentration

Plasma angiotensinogen concentration was determined by radioimmunoassay of Ang I generated during incubation of plasma with an excess of exogenous renin purified from rat submaxillary glands, in order to exhaust circulating angiotensinogen. The number of molecules of Ang I generated corresponds with the number of molecules of angiotensinogen available for the reaction. Plasma angiotensinogen concentration is expressed as picomoles per milliliter of plasma.

ACE Activity

Plasma ACE activity was measured in plasma from enalapril-treated, losartan-treated, and control rats using the fluorometric assay described previously. The method is based on the conversion of the substrate analoge hippuryl-His-Leu (5 mmol/L in 0.1 mol/L phosphate buffer, pH 8.3, containing 0.3 mmol/L NaCl; Sigma) to hippurate and His-Leu, which is quantified spectrofluorometrically by formation of a fluorescent adduct with o-phthalaldehyde (Sigma). The fluorescence at 500 nm was measured using an excitation wavelength of 365 nm (F-2000, Hitachi spectrofluorometer). Plasma ACE activity was expressed as nanomoles hippuryl-His-Leu cleaved per milliliter of plasma per minute of incubation. Different concentrations of His-Leu (Bachem) incubated with o-phthalaldehyde and treated in the same conditions were used as standards.

ACE activity was measured in plasma before dialysis and also after dissociation and removal of the ACE inhibitor by dialysis in order to determine the concentration of ACE in these plasma. For this purpose, the plasma of enalapril-treated rats was dialyzed (0.3 mL, Slide-A-Lyzer; cutoff, 10,000 D; Interchim) against 10 mmol/L potassium phosphate buffer (pH 8/10 μmol/L EDTA for 24 hours (EDTA chelates zinc after dissociation of the complex and prevents reassociation) and then against the same buffer without EDTA for 36 hours to eliminate the EDTA. This procedure of dialysis against buffer without chloride completely dissociates the enzyme-inhibitor complex, which has a very short half-life in the absence of chloride ions. Plasma of control and losartan-treated rats were dialyzed in parallel. Plasma ACE activity was then measured in diayed plasma, as described above, in the presence of zinc.

Quantification of Elastin, Collagen, and Cell Proteins in the Thoracic Aorta

Thoracic aortas were thawed and manipulated on ice under a dissecting microscope. The media was separated from the adventitia, and the length of media studied was recorded. Elastin, collagen, and cell protein content of individual thoracic aortic mediae were determined without homogenization, using a method based on that described by Wolinsky as follows: After delipidation in acetone/diethyl ether (1/1 [vol/vol]) and drying, the dry weight was recorded using a Sartorius R 160P balance (precision, 0.01 mg). Cell proteins were extracted by solubilizing cell membranes by gentle agitation in 0.3% SDS for 12 hours. Cell proteins were assayed in the decanted solution using the method of Lowry et al and expressed as milligrams per centimeter of aortic media. The extracellular proteins, other than elastin, were solubilized by three 15-minute extractions in 1 mL of 0.1N NaOH in a boiling water bath. Medial elastin content was quantified by determining the dry weight of the residue and expressed as milligrams per centimeter of aortic media. The extra-cellular proteins, including collagen, present in the NaOH were evaporated to dryness and hydrolyzed in 6N HCl in vacuum-sealed vials for 24 hours at 110°C. Hydroxyproline was assayed in the hydrolysate using a colorimetric assay according to Woessner. Collagen was quantified from hydroxyproline on the basis of the assumption that collagen contains 12.77% hydroxyproline by weight and expressed as milligrams per centimeter of aortic media.

Statistical Analysis

All results are expressed as mean±SD, except for the Fig 6, where SEM is used. Statistical comparison between groups was performed using either ANOVA followed by the Fisher test or, in cases in which the distribution was unknown, the Kruskal-Wallis test followed by the Mann-Whitney test. ANCOVA was used to adjust values of IEL in the AA for arterial pressure. These analyses were performed using Statview 4.5 and Super Anova statistical software (Abacus Concepts Inc).

Results

Pilot Experiment

Results of the first experiment comparing imidapril and hydralazine are summarized in Table 1. It can be seen that although all three drugs inhibit rupture of the IEL in the AA and iliac arteries, imidapril is more effective than hydralazine. In addition, imidapril inhibits rupture in the renal artery, whereas hydralazine is without effect. Only imidapril (3 mg·kg⁻¹·d⁻¹) had a slight effect on IEL rupture in the caudal artery. This lesser effect of hydralazine does not appear to be due to a lesser lowering of blood pressure, since hydralazine lowered SBP to an extent similar to that for imidapril (3 mg). No significant effects on heart weight were noted for either treatment compared with the control condition, but in the 3 mg imidapril group, heart weight was significantly lower than in the hydralazine group. The beneficial effects of ACE inhibition on IEL rupture in the AA and renal artery were confirmed in another experiment (results not shown) using enalapril (3 mg·kg⁻¹·d⁻¹) and captopril (20 mg·kg⁻¹·d⁻¹).

Final Experiments

In view of the results of the preliminary experiments, the second (final) series was designed to study more closely the blood pressure changes induced by the drugs used, especially at the beginning of the treatment, a period not studied in the preliminary experiments, and to establish dose-response curves. The experimental period was reduced to 10 weeks of treatment (from 4.5 to 14 weeks of age), making it impossible to study the renal artery, where rupture in the IEL occurs later in life. The caudal artery was also eliminated from study because of the absence of effect of the drugs on IEL rupture in this artery. In this series, we thus focused our attention on IEL rupture in the AA and iliac arteries, the SBP changes throughout the period of treatment, and the modifications in parameters of the RAS. The quantification of elastin, colla-
Gen and cell protein content of the thoracic aortic media was added to the protocol.

Because the parameters measured gave similar results in the first two experiments of this series, both for the control groups and for the group treated with E3, to facilitate comparison, we present the results of these two experiments on the same figures. The results of the third experiment are presented in tabular form.

Experiments I and II

**Systolic Blood Pressure**

Figure 1 shows the mean values of SBP throughout the experimental period for each treatment. Mean SBP was significantly lowered compared with control SBP in rats treated with all doses of enalapril and with L3, L10, and M30. For all these treatments, SBP had started to decrease by the second week of treatment and, with the exception of E1 and L3, remained significantly lower than control values throughout the experimental period. Almost identical falls in SBP were obtained with E3 and M30.

Heart rate was not significantly modified by enalapril or losartan. Only M3 significantly reduced heart rate from the fifth week of treatment onward, whereas with M10 and M30 no alteration in heart rate was observed (control, 372±7.1 bpm; M3, 347±13.4 bpm [P≤0.05]; M10, 356±16 bpm [P=NS]; and M30, 368±20 bpm [P=NS]).

**IEL Rupture**

The effects of the different treatments on IEL rupture in the AA are shown in Figure 2. Enalapril and losartan inhibit the formation of AA IIEL in a dose-dependent manner, with the lowest doses (E1 and L1) being without effect and the highest doses (E10 and L10) inhibiting by 80% or more. In experiments I and II, E3 had similar effects on both SBP and AA IIEL, demonstrating the reproducibility of our model. For mibefradil, only the highest dose (M30), which significantly decreased SBP, tended to have a slight inhibitory effect on AA IIEL formation, but this effect was not statistically significant. Thus, although E3 and M30 decreased blood pressure to similar extents (Figure 1), the effects of these two treatments on AA IIEL were significantly different (P≤0.01).

The numbers of IIEL in the iliac arteries were also affected by these different treatments in a manner similar to those in the AA. The total number of IIEL in both iliac arteries taken together in control rats was 6.71±3.09. Values for E3 and E10 were 2.93±1.7 (P≤0.0001) and 1.00±0.2 (P≤0.0001), respectively; those for L3 and L10 were 3.29±3.09 (P≤0.002) and 1.75±1.49 (P≤0.0001), respectively. M30 was without effect on IIEL in the iliac arteries (6.67±3.88), as were E1 (6.38±2.97) and L1 (7.25±1.75).

**Table 1. Effects of Imidapril and Hydralazine on Spontaneous Rupture of the IEL in the BN Rat**

<table>
<thead>
<tr>
<th></th>
<th>Control (n=8)</th>
<th>Imidapril 3 mg (n=8)</th>
<th>Imidapril 6 mg (n=8)</th>
<th>Hydralazine (n=7)</th>
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<tr>
<td>Total No. IIEL in AA</td>
<td>61±15</td>
<td>30±7</td>
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<tr>
<td>Total No. IIEL in both iliac arteries</td>
<td>17.0±3.0</td>
<td>7.0±3.5§</td>
<td>4.4±2.4¶</td>
<td>11.3±4.0†</td>
</tr>
<tr>
<td>Total No. IIEL in left renal artery</td>
<td>2.60±1.30</td>
<td>1.10±0.09§</td>
<td>0.62±0.70&quot;</td>
<td>2.57±1.70</td>
</tr>
<tr>
<td>No. IIEL/cm caudal artery</td>
<td>7.38±0.80</td>
<td>6.30±0.62*</td>
<td>6.84±1.10</td>
<td>6.90±0.79</td>
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<td>Body weight at 22 wk, g</td>
<td>351±25</td>
<td>317±30¶</td>
<td>293±19‡</td>
<td>304±18‡</td>
</tr>
<tr>
<td>Mean SBP, mm Hg</td>
<td>121±5</td>
<td>112±4*</td>
<td>107±2†</td>
<td>111±2*</td>
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<tr>
<td>Heart weight, g/100 g body wt</td>
<td>0.25±0.02</td>
<td>0.24±0.02§</td>
<td>0.25±0.03</td>
<td>0.27±0.02</td>
</tr>
</tbody>
</table>

Values are mean±SD.

*P≤0.03, †P≤0.005, and §P≤0.0001 compared with values of the control group; ¶P≤0.03, ||P≤0.005, and ¶¶P≤0.0005 compared with values of the hydralazine group, using a one-way ANOVA followed by the Fisher test.

**Figure 1.** Mean systolic arterial pressure (mm Hg) during the period of treatment (from 4.5 to 14 weeks of age) in male BN rats. Groups are as follows: control group (C1 and C2); enalapril group (E1, E3, and E10); losartan group (L1, L3, and L10); and mibefradil group (M3, M10, and M30) (n=8 for experiment I and n=6 for experiment II). Values are mean±SD. *P≤0.05, **P≤0.01, and ***P≤0.001 compared with control group, using a one-way ANOVA followed by the Fisher test. Note that the difference between E3 and M30 in experiment II is not statistically significant (NS).
Figure 3 illustrates a typical rupture in the IEL in the AA of a male control rat as it appears in a longitudinal section of paraffin-embedded orcein-stained AA (Figure 3a) and as viewed in an en face preparation (Figure 3b). The clean break in the IEL is readily visible in both preparations, as is the appearance of such a rupture as a dark transverse band in the en face preparation.

En face preparations of AA of rats in the different treatment groups, which were used for the quantification of IIELs, are illustrated in Figure 4a through 4e at a lower magnification than in Figure 3b. It can be observed that not only are the numbers of IIEL reduced by enalapril and losartan but also their size. M30 has a much lesser effect on lesion number and size than does E3 (Figure 4, compare panels b and d). En face preparations of iliac arteries of control and E10-treated rats are shown in Figure 4f and 4g.

Parameters of the RAS

**PRA, PRC, and Angiotensinogen**

PRA at the end of the experimental period as a function of the dose of enalapril, losartan, and mibefradil is shown in Figure 5A. There was a dose-dependent increase in PRA for E1, E3, L1, L3, and L10, but there was no difference between E3 and E10, probably because of the exhaustion of angiotensinogen.

As expected, mibefradil had no effect on PRA, except at the highest dose (30 mg·kg⁻¹·d⁻¹), where a small increase was observed. PRC as a function of dose was determined for enalapril and losartan, as shown in Figure 5B, where a dose-dependent increase can be observed for both compounds, with enalapril increasing PRC more than losartan. PRC was not determined for mibefradil because of the small effect of this drug on PRA.

Values of PRA and PRC in the middle of treatment were not different from those at the end of treatment for enalapril and losartan, and the slight increase in PRA noted at the end of treatment for mibefradil was not observed at the middle of treatment (values not shown).

Plasma angiotensinogen concentrations at the end of treatment, as a function of dose, for the three compounds are shown in Figure 5C. As expected, enalapril and losartan decreased angiotensinogen in a dose-dependent manner, whereas mibefradil was without effect. Middle-of-treatment values were not different from end-of-treatment values (values not shown).

There was a significant correlation between the parameters of the RAS and the values of IIEL in the AA (PRA, \( r = -0.67 \), \( P = 0.001 \); PRC, \( r = -0.64 \), \( P = 0.001 \); and angiotensinogen, \( r = 0.70 \), \( P = 0.001 \)).

**ACE Activity**

ACE activity, before dialysis, in plasma taken at the end of treatment, is shown in Figure 5D. It can be seen that enalapril inhibits ACE activity in a dose-dependent manner, whereas losartan has no effect. It is of interest that E1 inhibits ACE activity by 53% in the present conditions of measurement, whereas it has only a small effect on SBP. Seventy percent of ACE inhibition is achieved with E3, and 89% is achieved with E10.

After dialysis of the above plasmas, ACE activity in control and losartan-treated plasma was the same as before.
dialysis, whereas for the enalapril-treated rats, a 3.5-fold increase in activity was observed for E1 and a 4-fold increase was observed for E3 and E10 (control, 255±22 nmol·mL⁻¹·min⁻¹; L10, 263±24 nmol·mL⁻¹·min⁻¹; E1, 907±103 nmol·mL⁻¹·min⁻¹; E3, 1022±128 nmol·mL⁻¹·min⁻¹; and E10, 1001±108 nmol·mL⁻¹·min⁻¹) The difference between enalapril-treated rats and control or losartan-treated rats was highly significant (P<0.001), and the difference between E1

![Figure 4](http://www.ahajournals.org) En face preparations of arteries of male BN rats, aged 14 weeks, stained in situ with orcein and Groat’s hematoxylin to illustrate IEL, which appear as dark transverse bands (arrows). Bar=1 mm. a through e, AA. Panels are as follows: a, control (showing 12 IEL); b, E3 (showing 4 IEL); c, E10 (showing 2 IEL); d, M30 (showing 10 IEL); and e, L10 (showing 2 IEL). f and g, iliac arteries. Panels are as follows: f, control (showing 4 IEL); g, E10 (devoid of IEL).

![Figure 5](http://www.ahajournals.org) Parameters of the RAS as a function of dose in plasma of male BN rats aged 14 weeks, after 10 weeks of treatment by enalapril, losartan, and mibefradil. Values are mean±SD. A, PRA (*P<0.001 compared with control). B, PRC (**P<0.01 and ***P<0.001 compared with control). C, Plasma angiotensinogen concentration (*P<0.05 and **P<0.001 compared with control). D, Plasma ACE activity (*P<0.001 compared with control and losartan values). □ indicates enalapril; ▲, losartan; and ◆, mibefradil. Statistical significance was estimated by a one-way ANOVA followed by the Fisher test.
and the higher doses of enalapril was also significant (P ≤ 0.02). These results indicate an increase in the number of ACE molecules in plasma, which is already maximal at the 3 mg dose of enalapril.

**Effects on Body and Heart Weight**

Body and heart weights are shown in Table 2. Enalapril at 3 and 10 mg had an inhibitory effect on body weight gain, as did L10 (but to a much lesser extent). Heart weight (expressed per 100 g body weight) was significantly decreased compared with the control value only by E10 and L10. In contrast, M30 caused a significant increase in heart weight compared with the control and E3 values. Thus, for an equivalent fall in SBP, effects of E3 and M30 on heart weight were significantly different.

**Effects on Composition of the Aortic Media**

Treatment effects on the aortic media are summarized in Table 2. The dry weight of the aortic media, expressed per aortic length, was significantly reduced in the rats having received E10, L10, and L3, indicating a hypotrophic effect of these treatments on the aortic wall. The hypotrophic effect of E10 and L10 was due to the simultaneous decrease in aortic elastin, collagen, and cell proteins. The effect on cell proteins was the most significant and was observed, to a lesser extent, also with E3 and L3. This result indicates a hypotrophic effect of inhibition of the RAS on the aortic smooth muscle cells, which probably causes a decrease in their production of extracellular matrix at the highest doses of enalapril and losartan.

M30 also showed a tendency to decrease aortic dry weight and cell proteins, but this effect, which was probably related to the fall in SBP, was smaller than with inhibition of the RAS and did not reach statistical significance. Since E1 and L1 were without effect on any parameter studied, the results are not shown.

**Experiment III**

The results of experiment III are summarized in Table 3. As in the previous experiments, M30 and E3 both decreased SBP compared with the control value, and only E3 decreased IEL rupture. M45 had only a slightly greater effect on SBP than did M30, but it had no effect on IEL rupture. These results demonstrate that it is not possible to decrease blood pressure using mibebradil to levels reached with E10 and L10 in the

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Values are mean±SD.

**TABLE 3. Effects of High Doses of Calcium Antagonists Compared With E3**

| Control (n=8)    | E3 (n=8) | M30 (n=9) | M45 (n=7) | A30 (n=8) |
|---------------------------------------------------------------|
| Body weight at 14 wk, g                  | 259±27  | 227±22†  | 267±18¶  | 256±23§  | 285±17¶ |
| Heart weight, g/100 g body wt            | 0.254±0.017 | 0.242±0.006  | 0.265±0.008§  | 0.306±0.041†¶ | 0.288±0.008§¶ |
| Heart rate, bpm                             | 388±16  | 386±15  | 398±15  | 406±8*  | 429±12¶ |
| PRC, ng·mL⁻¹·h⁻¹                               | 5.8±1.6  | 7.7±2.24†  | 10.7±4.3¶‡ | 16.6±8.3¶  | 69.0±18.9¶ |
| Mean SBP, mm Hg                              | 141±8  | 130±6†  | 133±8*  | 131±6†  | 134±4* |
| Total No. IEL in AA                           | 22.8±3.0 | 10.8±5.6‡  | 21.9±5.5‡  | 25.0±7.7¶  | 24.0±8.3¶ |
| Dry weight of media, mg/cm                  | 1.542±0.069 | 1.302±0.092†  | 1.465±0.088¶  | 1.423±0.079§¶  | 1.534±0.116|| |
| Elastin in media, mg/cm                     | 0.704±0.030 | 0.584±0.040¶  | 0.688±0.038§  | 0.682±0.022|| 0.739±0.044|| |
| Collagen in media, mg/cm                    | 0.175±0.013 | 0.152±0.011†¶ | 0.159±0.008§¶ | 0.152±0.016* | 0.174±0.015§ |
| Cell proteins in media, mg/cm               | 0.384±0.077 | 0.341±0.033*  | 0.357±0.028*  | 0.335±0.028†  | 0.341±0.028† |

Values are mean±SD.

*P<0.05, †P<0.01, and ‡P<0.001 compared with values of the control group; §P<0.05, ||P<0.01, and |||P<0.001 compared with values of the E3 group, using a one-way ANOVA followed by the Fisher test, except for aortic parameters expressed as mg/cm, for which the Kruskal-Wallis test followed by the Mann-Whitney U test was used.
normotensive BN rat. M45 is the maximal dose tolerated by rats, and indeed, one rat died after 1 month of treatment with M45. A30 also decreased SBP compared with control SBP but did not inhibit IEL rupture.

As observed in experiments I and II, E3 had a greater hypotrophic effect on the aortic media than did M30. However, M45 showed a significant hypotrophic effect, which was due to a decrease in cell proteins and collagen. This result obtained with M45 allows us to dissociate the inhibitory effect on IEL rupture from this aortic hypotrophic effect since M45 was without effect on IEL rupture. In contrast, the effect on heart weight was very different between E3 and M30, M45, and A30. In this experiment, enalapril showed a tendency to decrease heart weight, whereas M45 and A30 significantly increased heart weight compared with the control condition. There was a significant difference between heart weights of E3-treated rats and all the calcium antagonists–treated groups.

M45 increased PRC compared with the control condition, but A30 caused a marked increase, which approached that induced by E3. Heart rates were not different from control values except for the A30 group, for which values were significantly elevated.

Figure 6 shows the relationship between SBP and AA IIEL in all rats treated with enalapril, losartan, and mibefradil at all doses from experiments I, II, and III. Because of the slightly different values of SBP and IIEL numbers in control groups from the three experiments, values are presented as a percentage of the mean of the appropriate control values for each experiment. It can be seen that for enalapril and losartan there is a significant correlation between SBP and IIEL in the AA ($r=0.65, P=0.0001$) and that mibefradil inhibited IIEL formation to a much lesser degree than enalapril and losartan for the same decrease in SBP and in a non–dose-dependent manner. Furthermore, ANCOVA was performed on the data from experiment II to test the difference between drugs after adjustment on the level of SBP. This adjustment confirmed our conclusion that part of the effect of the inhibition of the RAS on the rupture of the IEL is independent of SBP. Taken together, all the present results suggest that Ang II plays a role in the formation of IIEL, which is, at least in part, independent of its effects on blood pressure.

Discussion

The object of the present study was to investigate the effects of various blood pressure–lowering drugs with different mechanisms of action on the spontaneous rupture of the IEL. More specifically, we have explored the effects of blockade of the RAS on this phenomenon to determine whether the beneficial effect of ACE inhibition is related to the decrease in Ang II production or its effects on other peptides, such as bradykinin, and to evaluate the role played by blood pressure lowering. The present study was conducted in normotensive rats of the BN strain with a high genetically determined susceptibility to IEL rupture. This study enters into the much larger field of investigation of possible “arterioprotective” effects of certain antihypertensive drugs, such as ACE inhibitors, and their relation with the decrease in arterial pressure induced. Indeed, in other models of study, a vasculoprotective effect of ACE inhibitors, which may be in part independent of the fall in arterial pressure, has been reported.

Our pilot experiment revealed the potent inhibitory effect of chronic ACE inhibition on IIEL formation in the AA and iliac and renal arteries compared with treatment by the vasodilator hydralazine, despite a similar decrease in SBP. These observations suggested that the inhibition of the formation of IIEL by ACE inhibition was partly independent of the fall in SBP and could be the result either of a decrease in the production of Ang II or of an effect related to other substrates or products of ACE. Indeed, a well-known physiological function of ACE is to transform Ang I to Ang II, but this enzyme also inactivates bradykinin and can also act on other peptide substrates. Therefore, we compared losartan treatment with enalapril treatment to determine whether the effects of ACE inhibition on IIEL rupture are due to a decrease in the production of Ang II or to some other effect. Both treatments decrease the action of Ang II: enalapril, by inhibiting its production; losartan, by antagonizing its binding to the Ang II type 1 receptor. We observed that both treatments diminished IIEL to a similar extent and in a dose-dependent manner. Thus, it appears that the protective effects of ACE inhibition are mediated mainly via decreased Ang II production and not by some other effect. Both treatments also decreased SBP in a dose-dependent manner, except that E3 was slightly more effective than L3.

In this experiment, in addition to the decrease in SBP and IIEL, the modification of the parameters of the RAS reflecting the suppression of Ang II action (PRA, PRC, and angiotensinogen) were also dose dependent, and IIEL numbers correlated with both SBP and the parameters of the RAS, further suggesting that the suppression of Ang II action was the main factor involved in the protective action of these drugs.

A second experiment was designed to investigate whether the decrease in IIEL induced by enalapril and losartan was
mainly due to the decrease in SBP or to other effects of Ang II independent of the change in blood pressure. For this purpose, we compared, in dose-response curve studies, the effects of mibefradil, a calcium antagonist, which has little or no effect on the RAS, with the effects of enalapril and losartan. We chose mibefradil, rather than another calcium antagonist, because it is long acting and thus covers the 24-hour period efficiently, even if the rats ingest it only at night. It is also of interest because it blocks both L- and T-type calcium channels, with a more selective blockade of T-type channels, unlike the majority of calcium antagonists.19 Our observation that M30 decreased IIEL formation significantly less than did E3 for a similar fall in SBP (see Figure 6) suggests, as did the preliminary data obtained with hydralazine, that Ang II plays a role in the formation of IIEL that is at least partly independent of the SBP.

In the third experiment, we increased the dose of mibefradil to 45 mg·kg⁻¹·d⁻¹ to try to obtain a greater fall in SBP to compare with E10 and L10, and we also tested amlodipine, another long-acting calcium antagonist, which blocks only L-type channels. Our results confirmed the lack of inhibitory effect of calcium blockade on IIEL rupture despite a fall in SBP equivalent to that for E3 and also showed that it is not possible with mibefradil or amlodipine to obtain SBP values equivalent to those for E10 and L10 in this model.

Taken together, our results with different antihypertensive drugs suggest that although IIEL are decreased in part by the lowering of blood pressure, for enalapril and losartan there is an additional effect. Previous studies have shown that rupture of the IEL is probably the result of an interaction of hemodynamic forces on an arterial wall whose fragility varies between individuals. The fragility of the arterial wall may be determined genetically, as is the case for the BN rat, or can be induced by, for example, administration of β-aminopropionitrile.20 Moreover, greatly increased hemodynamic stress induces rupture of the IEL in arteries that do not develop them spontaneously, and deoxycorticosterone acetate–salt hypertension increases IIEL rupture in the AAs of BN rats compared with age-matched normotensive control rats.21 In the present study, all the drugs used decrease SBP, but it appears that enalapril and losartan have, in addition, other hemodynamic effects not shared by hydralazine, mibefradil, and amlodipine and/or a direct cellular effect (independent of the SBP) that decreases arterial fragility.

In our experiments, arterial pressure was measured in the conscious rat by using the tail-cuff method. This gives values of SBP but provides no index of diastolic pressure, mean arterial pressure, or pulse pressure. Although, in general, all these parameters are fairly well correlated, further hemodynamic studies are required to investigate whether the parameters not measured here may be involved in IIEL rupture. The drugs may also have differential effects on sympathetic activation; eg, hydralazine is known to stimulate sympathetic activity.

It appears that decreased arterial distensibility may be involved in aortic IIEL rupture in the BN rat, since rupture occurs only in the abdominal part, which is more rigid and less distensible than the thoracic part. Indeed, the elastin/collagen ratio, which is normally regarded as a fairly good index of arterial distensibility in large arteries, passes from 1.09 in the total thoracic aorta to 0.52 in the AA in the adult BN rat.2 This increased rigidity of the AA, common to all mammalian species, contributes to the increased pulse pressure detected in the abdominal segment compared with the thoracic segment.23 Pulse pressure may itself play a role in inducing IIEL rupture. The fact that the BN rat presents a lower aortic elastin/collagen ratio compared with another strain of rat that is resistant to IIEL rupture in the AA also supports the hypothesis that low aortic distensibility may be a contributing factor to IIEL rupture.

It was thus possible that the drugs used in these experiments had differential effects on aortic distensibility and pulse pressure, as has previously been suggested.24 However, the results of our biochemical determinations on the thoracic aorta did not support such a hypothesis, since the IIEL-inhibiting treatments did not increase elastin/collagen ratios, nor did mibefradil and amlodipine decrease it. Clearly, further studies are required to elucidate the mechanism of the protective effect of RAS inhibition on IIEL rupture.

Enalapril exhibited an inhibitory effect on body weight gain in the BN rat, which was observed to a much lesser extent for losartan. A similar effect of enalapril on body weight gain was observed in the Wistar rat by Keeley et al., but the effect was transitory, and the difference compared with control rats did not reach statistical significance. Thus, it appears that the BN rat is more susceptible to this effect. Our previous studies suggested that growth rate may be a factor influencing IIEL rupture in the caudal artery of the Wistar rat.4 In the present experiments, we cannot eliminate the possibility that growth inhibition may contribute to the inhibition of IIEL rupture, since E3 inhibited body growth significantly more than did M30, M45, and A30. However, growth cannot be a major determinant of IIEL rupture in the AA, since losartan, which affected body weight gain much less than did enalapril and had a slightly lesser SBP-lowering effect at the 3 mg dose, was as effective as enalapril in inhibiting aortic IIEL rupture. In addition, in the pilot experiment, hydralazine inhibited body weight gain as much as imidapril, decreased SBP to a similar extent, but inhibited IIEL formation to a significantly lesser degree.

We quantified aortic cell proteins since Ang II has been reported to have trophic25 and mitogenic27 effects on arterial smooth muscle cells “in vitro.” Ang II induces proto-oncogenes and growth factor gene expression in cultured vascular smooth muscle cells.26,29 But whether Ang II has direct effects on vascular hypertrophy “in vivo,” independent of the arterial pressure, remains controversial, although some recent studies strongly suggest the existence of such effects.30–32

In the present study, all the treatments that had hypotrophic effects on the thoracic aortic media had blood pressure–lowering effects, although RAS inhibitors were more effective than mibefradil for the same fall in SBP. Our observation that inhibition of the RAS has a hypotrophic effect on the arterial wall in the growing normotensive rat is in accord with previous studies by Keeley et al.22 Thus, the RAS appears to play a role in normal vascular development and growth. Moreover, prolonged ACE inhibition retards the intimal and medial thickening that occurs with age in the adult normo-
tensive rat, in the hypertensive rat, such a treatment inhibits medial hypertrophy, although it is not clear how much of this effect is related to the fall in blood pressure. Recently, infusion of Ang II after vascular injury has been reported to result in a marked enhancement of vascular smooth muscle cell proliferation, and ACE inhibitors have been shown to reduce neointimal formation after vascular injury.

It has been suggested that the growth-stimulating effect of Ang II on the left ventricle and the arterial wall is mediated by the Ang II type 1 receptor. In the present study, we observed little difference between the effects of enalapril and losartan on all the parameters studied, except that losartan was sometimes slightly less effective than enalapril. L10, like E10, decreased heart weight and aortic dry weight, and enalapril and losartan at 10 and 3 mg decreased cell content of the thoracic aortic media, suggesting that all the cardiovascular trophic effects of Ang II that we have inhibited are mediated by the Ang II type 1 receptor. However, some recent studies have suggested that the Ang II type 2 receptor may also be involved in the trophic effects of Ang II, but the extent of its involvement remains unclear.

In our experiments, inhibitors of the RAS, at high doses, had a hypotrophic effect on the heart, whereas calcium antagonists had, or tended toward, a hypertrophic effect. The exact mechanism involved is unclear, but this may be related to a sympathetic stimulation induced by calcium antagonists and not by RAS inhibitors. Indeed, hydralazine, which is known to cause reflex stimulation of the sympathetic nervous system, also tended to increase heart weight, which, in our preliminary experiment, was significantly greater than that after treatment with the ACE inhibitor, imidapril. In the hypertensive rat, it has been shown that enalapril is more effective than mibefradil in inducing regression of cardiac hypertrophy. In addition, ACE inhibition decreases heart weight in normotensive rats, whether young and still growing or already adult, compared with untreated control rats. It is possible that part of the difference between the effects of ACE inhibition and calcium antagonism on heart weight reflects a combination of the removal of the trophic effect of Ang II on the heart and the absence of reflex sympathetic stimulation following the fall in SBP in the case of ACE inhibition, effects that are not shared by calcium antagonists. Since this effect on the heart was the only effect observed that showed a marked difference between the two classes of drugs that differed in their effect on IEL rupture, it is possible that the inhibition of IEL rupture by RAS inhibitors may be related in some way to this hypotrophic, or a lack of hypertrophic, effect on the heart. More studies are required to explore this possibility.

Our results suggest that ACE inhibition induces ACE synthesis. The plasma concentration of ACE was increased markedly by enalapril, at all doses used, whereas no difference was observed with losartan. This result is in accord with a previous findings in our laboratory. The mechanism of this induction is unknown, but the lack of effect of losartan suggests that Ang II is not implicated in the regulation of ACE gene expression in vivo.

In another model, the minipig subjected to an atherogenic diet, ACE inhibition has been reported to prevent alteration of elastic laminae by specifically inhibiting fragmentation of dense elastic laminae in the AA. The mechanisms of this effect are unknown but may be related in some way to our present observations.

Our results suggest that in normotensive BN rats the RAS and ACE may play a role in the development of IEL. The RAS has not been extensively studied in BN rats compared with other strains. It is of interest, however, that the BN strain is the one that, among those studied so far, presents the highest circulating ACE levels. The significance of the high plasma ACE levels in the BN rat is unknown but reminds one of subjects with the ACE gene deletion polymorphism, who have higher plasma and tissue ACE levels and appear to be at greater risk for some cardiovascular diseases. Indeed, although the BN rat does not appear to develop any known cardiovascular disease as long as it remains normotensive, when it becomes hypertensive, it is more susceptible to cerebrovascular hemorrhage than another strain of rat, the LE, which is resistant to IEL rupture and has lower circulating ACE levels. In addition, the BN rat has been recently shown to be genetically predisposed to renal hypertensive damage.

In conclusion, we have shown in the present study that inhibition of the RAS protects against rupture of the IEL in the BN rat more effectively than do hydralazine and the calcium antagonists, mibefradil and amlodipine, for equivalent decreases in SBP. The similar effects of enalapril and losartan in our model suggest that a decrease in the action of Ang II via the Ang II type 1 receptors is involved in this additional inhibitory effect, which appears to be independent of SBP. The mechanism of this apparent “arterioprotective” effect has not been elucidated by our studies of the effects of these drugs on aortic composition but may possibly be related to their hypotrophic, or lack of hypertrophic, effects on the heart. Further investigation is required to determine exactly how Ang II action is involved.

Interruptions or gaps in the IEL exist in human arteries and have been the object of a few studies. They are present in most large and some medium-sized muscular arteries from an early age in normal subjects and have been described in detail by Meyer et al., but their significance and the factors involved in their formation are unknown at present. Other groups have shown that various defects in the IEL, including fragmentations and interruptions, occur with age in different human arteries, are more pronounced in male than in female subjects, and may be related to the subsequent development of intimal thickening, arteriosclerosis, and atherosclerosis. In addition, rupture and fragmentation of the arterial elastic laminae are common features of arteries of patients with various heritable connective tissue diseases. It is premature at the present time to extrapolate our present findings in the BN rats to humans, with possible therapeutic implications. However, it appears to us that the BN rat can be compared with human subjects with subclinical forms of certain inborn connective tissue disorders, as IEL occur in both muscular and elastic arteries. The benefit of treatment with β-blockers to decrease stress on the aortic wall in
patients with Marfan’s syndrome has been reported. In view of our results demonstrating a protective effect of inhibitors of the RAS, a class of drugs widely used in clinical practice, on hemodynamically induced arterial damage in a normoten-
sive rat with hereditary arterial fragility, it is possible that RAS inhibition could also be used for such preventive treatment. Our observations in the BN rat point out the need for a clinical investigation aiming at the comparison of the effects on cardiovascular events of antihypertensive drugs of different classes.

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