β-Adrenergic–Induced Local Angiotensin Generation in the Rabbit Hind Limb Is Dependent on the Kidney

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Evidence was sought for β-adrenergic–induced increase in femoral vascular angiotensin production in sham-operated and nephrectomized rabbits. Systemic blood pressure and right femoral blood flow were monitored in anesthetized rabbits. Arterial and femoral venous plasma angiotensin II (Ang II) and angiotensin I (Ang I) were measured by radioimmunoassay after high-performance liquid chromatography. Isoproterenol, 1 and 10 nmol/min, was infused intrafemorally, reducing femoral vascular resistance by 47±5% and 60±6% in the sham-operated group, and by 50±6% and 63±4% in the nephrectomized group, respectively. The hemodynamic effect of isoproterenol was blocked by 2 μmol/kg propranolol injected intravenously plus 0.2 μmol/min infused intrafemorally, indicating that the effect was β-adrenergically mediated. In the sham-operated group, arterial Ang II and Ang I levels were increased, respectively, by 85±16% and 103±23% with the low dose of isoproterenol, and by 121±13% and 563±126% with the high dose of isoproterenol. The apparent femoral Ang II secretion rate was increased by 3.2-fold and 4.4-fold, and the apparent femoral Ang I secretion rate increased by 4.3-fold and 21.2-fold, with the low and high dose of isoproterenol, respectively. Propranolol abolished or markedly attenuated the increased arterial angiotensin levels and the increased femoral angiotensin secretion rates. Neither the low nor the high dose of isoproterenol caused any increase in plasma levels or the apparent femoral secretion rates of the angiotensins in the nephrectomized group. Low plasma levels of Ang I and Ang II remained in the nephrectomized group, representing some locally generated angiotensins. These results indicate that isoproterenol, by acting on β-adrenergic receptors, caused an increase of systemic and local angiotensin production, which depends on the presence of the kidney. (Hypertension 1991;17:1010–1017)
ify that effects induced by isoproterenol were β-adrenergically mediated.

Methods

Animal Preparation

Eighteen male New Zealand White rabbits weighing 3.2–4.0 kg were divided into two groups of nine each. One group was bilaterally nephrectomized, and the other group was sham operated. Both operations were carried out under aseptic conditions while the rabbits were anesthetized by intravenous injection of 30 mg/kg sodium pentobarbital. Nephrectomy and sham operation were performed through bilateral flank incisions using a retroperitoneal approach. Tight ligatures were tied around the renal pedicle, and the kidney was removed after cutting between the ties. Incisions were closed, and the animal was treated with 100,000 units penicillin and 125 mg dihydrostreptomycin. The kidneys were exposed briefly in an operation similar to the above, and the incisions were closed during sham operation. After anesthesia was induced, but before the surgical procedure, a 3 ml blood sample was withdrawn from the central ear artery for determination of control plasma renin activity (PRA), blood urea nitrogen, and hematocrit. Three additional rabbits were used to estimate the in vitro formation of angiotensins in plasma.

Acute Experiment

Twenty-four hours after the operation, the animal was used in an acute experiment. Anesthesia was induced by sodium pentobarbital (30 mg/kg) and maintained during the entire experiment by infusion (6 mg/kg/hr) into a catheterized jugular vein with small adjustments made depending on the depth of anesthesia. The left carotid artery and jugular vein were cannulated with PE-50 tubing. Mean arterial blood pressure (MAP) was monitored with a Statham P23AA pressure transducer (Gould-Statham, Oxnard, Calif.) attached to the catheter in the carotid artery. The jugular vein catheter was for intravenous administration of drugs and reinjection of blood cells after separation of plasma with centrifugation at 7,710g. Three additional branches of the right femoral artery were ligated, and the right femoral blood flow (FBF) was monitored continuously with a blood flow probe (5 mm in circumference, Carolina Medical Electronics, Inc., King, N.C.) placed on the most rostral portion of the femoral artery and connected to a Carolina electromagnetic flowmeter. A 25-gauge needle attached to PE-50 tubing was inserted in a distal location of the femoral vein for venous sampling. MAP and FBF were recorded on a polygraph (Grass Instrument Co., Quincy, Mass.). Three milliliters of blood from the left carotid artery was withdrawn again for determination of PRA, blood urea nitrogen, and hematocrit to obtain preexperi-mental values. Radioimmunoassay for PRA was conducted as described previously.

Determination of Plasma Angiotensin I and II

For angiotensin determinations, 4 ml was collected of both arterial and femoral venous blood with chilled plastic syringes containing 0.3 ml of a cocktail solution of 6 mg/ml o-phenanthroline, 40 mg/ml disodium EDTA, 0.1 mg/ml pepstatin A (solubilized in ethanol), and 0.5 mg/ml captopril; the blood samples then were immediately centrifuged at 4°C. In vitro generation of Ang I and Ang II was prevented by inclusion of these inhibitors in the sampling syringes. The plasma was kept frozen at −80°C before analysis of angiotensins. Ang I and Ang II were extracted on 1-ml Bond Elut columns (disposable solid phase column, Analytichem International, Harbor City, Calif.) by the method of Nussberger et al.21 with some modifications. Glass and plastic tubes were rinsed with a solution of 0.1 M Trizma base and 0.5% bovine serum albumin and then dried. The 1-ml Bond Elut cartridge containing 100 mg phenylislyl silica was rinsed with 1 ml HPLC-grade methanol, then 2 ml glass distilled water. Two milliliters of plasma (kept on ice) was passed through the column by vacuum (Vac Elut, Analytichem) and then washed with 1 ml water. The column was eluted with 1.5 ml methanol into conical 2.5-ml polypropylene tubes. The eluate was evaporated to dryness on a Speed-Vac evaporator (Savant Instruments, Inc., Hicksville, N.Y.) and then reconstituted with 140 μl mobile phase that consisted of 58% buffer A and 42% buffer B for separation of the peptides by high-performance liquid chromatography (HPLC). After centrifugation, the solution was injected into a 100-μl loop coupled to an HPLC column (Partisil 10 ODS-3 C18 column, 4.6×250 mm, Whatman Inc., Clifton, N.J.). An adaptation of the method of Chappell et al was used.22 The solvent system consisted of buffer A, 0.15% heptfluorobutyric acid (Sequanol grade, Pierce Chemical Co., Rockford, Ill.) (vol/vol) in water, and buffer B, 80% acetonitrile (Fisher Scientific Co., Fair Lawn, N.J.) in 0.15% heptfluorobutyric acid. Mobile phase flow rate was 1.0 ml/min. A programmed gradient was used to separate the angiotensin peptides. For elution of Ang II and angiotensin-(2–8) heptapeptide (Ang III), a 42–44% (buffer B) concave gradient (curve 4 of Beckman System Gold, Beckman Instruments, Inc., San Ramon, Calif.) was performed over 8 minutes. To elute Ang I and des-[Asp³]Ang I, a linear gradient (curve 0) of 44–48% (buffer B) was used for 15 minutes. One-minute fractions were collected from 8 to 19 minutes and then evaporated to dryness. Fractions from 8 to 13 minutes were reconstituted to 120 μl with Trizma buffer for Ang II assay, and fractions from 14 to 19 minutes were reconstituted to 250 μl with Trizma buffer for Ang I assay. Retention times for Ang II, Ang III, and Ang I were 10.5, 12.0, and 15.5 minutes, respectively, based on the injection of 250-pg stan-
standards in the same volume and composition of solvent as the samples, as well as radioimmunoassay afterward. For radioimmunoassay of Ang I or Ang II, 100 µl of each fraction was used. Antisera for both Ang I and Ang II were used. Fractions taken from 10 to 11 minutes were considered to constitute true Ang II, and fractions from 15 to 16 minutes, true Ang I. The Ang I antiserum does not cross-react with Ang II, 10-500 pg/ml plasma. Cross-reactivity of the Ang II antiserum with Ang I, 1.25-50 ng/ml plasma, was 0.1-0.2%. Cross-reactivity of the Ang II antiserum was 100% for Ang III. The overall recovery of 15,000 cpm 123I-Ang II (representing 4.5 pg Ang II) added to 2 ml rabbit plasma, run through the entire procedure including column extraction and HPLC, was 78±2% (mean±SD, n=6). The overall recovery of 10,000 cpm 123I-Ang I added to 2 ml rabbit plasma was 74±3% (mean±SD, n=5). Values were not adjusted based on this percentage of recovery.

To estimate the amounts of angiotensins that may have been formed in the blood itself flowing through the femoral vascular bed, experiments were performed in three normal rabbits. After a control blood sample was taken from the carotid artery, isoproterenol was infused infrafemorally in the dose of 10 nmol/min for 5 minutes, and then 20 ml systemic arterial blood was collected and immediately divided into five tubes. Tube 1, containing the cocktail, was placed on ice, and tubes 2-5 were incubated at 37°C for 10, 30, 60, and 120 seconds, respectively; then 0.3 ml of cocktail was added to each tube, mixed well, and immediately centrifuged at 4°C. Extraction, HPLC separation, and radioimmunoassay of Ang I and Ang II of these samples were the same as described above.

Calculation of the Apparent Femoral Angiotensin I and II Secretion Rates

To know the net production of Ang I and Ang II in the femoral vascular bed, the intrafemoral Ang I and Ang II extraction ratios in the rabbit had to be determined. 123I-Ang I or 125I-Ang II (1 µCi) was infused into the left carotid artery for 5 minutes, and blood samples were taken every 10 minutes from the left femoral vein and right femoral artery. Sampling began at 15 minutes when the arterial tracer level reached a steady state. Centrifugation, column extraction, and HPLC separation were as described above. The radioactivity of the labeled angiotensins was measured by gamma counting (Biogamma, Beckman Instruments). Fractions from 13 to 14 minutes represented 123I-Ang II, and from 19 to 20 minutes 125I-Ang I, based on the injection of standard 125I-Ang I (Du Pont, Wilmington, Del.) or 123I-Ang II (Amer sham, Arlington Heights, Ill.) onto the HPLC column and the measurement of their radioactivity individually. The arterial-venous difference of the radioactivity is equivalent to the amount degraded in the femoral vascular bed. Therefore, extraction ratios (ERs) are calculated by the following equations:

Ang II ER = (FA 125I-Ang II – FV 125I-Ang II)/FA 125I-Ang II

Ang I ER = (FA 125I-Ang I – FV 125I-Ang I)/FA 125I-Ang I

where FA is femoral arterial and FV is femoral venous amounts (counts per minute).

On the basis of the extraction ratios of Ang I and Ang II, assumed to remain constant in these experiments, the apparent femoral Ang I and Ang II secretion rates (SRs) are determined according to the following equations, where Ang I and Ang II concentrations are in picograms per milliliter:

Ang II SR (pg/min) = [(FV Ang II – (1-ER)FA Ang II)] × femoral plasma flow (ml/min)

Ang I SR (pg/min) = [(FV Ang I – (1-ER)FA Ang I)] × femoral plasma flow (ml/min)

Experimental Protocol

Approximately 20 minutes was allowed for MAP and FBF to stabilize after completion of the surgical procedure. Then, the first arterial and femoral venous samples (A1 and V1) were taken for control angiotensin determinations. Ten minutes after the sampling, a low dose of isoproterenol (1 nmol/min) was infused directly into the femoral artery to stimulate local angiotensin production. Despite being given intra-arterially, the doses of isoproterenol decreased blood pressure because of overflow from the limb. Five minutes after the start of isoproterenol infusion, the second arterial and femoral venous samples (A2 and V2) were taken, and isoproterenol infusion was stopped. Fifteen minutes later, a high dose of isoproterenol (10 nmol/min) was infused intra-arterially for 5 minutes, and the third pair of samples (A3 and V3) was collected. Thirty minutes after the isoproterenol infusion was stopped, a bolus injection of propranolol (2 µmol/kg) was given intravenously and also continuously infused intra-arterially at 0.2 µmol/min to block β-receptor-mediated effects. Ten minutes later, the fourth pair of samples was taken, and again the isoproterenol infusions were given and sampling was repeated. After the withdrawal of each pair of blood samples and centrifugation, the resuspended blood cells were returned to the rabbit through the jugular vein catheter.

Statistical Analysis

Data are expressed as mean±SEM except where indicated. Statistical significance was determined by one-way analysis of variance (ANOVA) with repeated measures, two-factor ANOVA with repeated measures, and paired t test where appropriate. A
Results

Plasma Renin Activity, Blood Urea Nitrogen, and Hematocrit

The control values of PRA in the two groups were similar (Table 1), but 24 hours after nephrectomy, PRA decreased to 0.1±0.03 ng Ang I/ml/hr, which indicates effective elimination of the systemic renin-angiotensin system in the nephrectomized group. PRA remained unchanged after sham operation in the other group. At the end of the experiment, PRA was greatly increased in the sham-operated but not significantly changed in the nephrectomized group. Blood urea nitrogen increased from 21±1 to 73±3 mg/dl by nephrectomy but was unchanged after sham operation (from 20±1 to 18±1 mg/dl). There was no significant change of hematocrit after either sham operation or nephrectomy.

Hemodynamic Effect

Control MAP and FBF were lower in the nephrectomized than in the sham-operated rabbits (78±3 versus 91±2 mm Hg and 12±1 versus 16±2 ml/min, respectively). Isoproterenol given intra-arterially increased FBF and, because of overflow into the systemic circulation, lowered MAP in a dose-dependent manner (Figure 1). The low dose of isoproterenol (1 nmol/min i.a.) increased FBF by 15±2 and 14±2 ml/min, lowered MAP by 6±2 and 14±2 mm Hg, and decreased femoral vascular resistance (FVR) by 47±5% and 50±6% in the sham-operated and nephrectomized group, respectively. Intra-arterial isoproterenol in the dose of 10 nmol/min caused greater effects, increasing FBF by 17±3 and 17±2 ml/min, lowering MAP by 20±4 and 25±2 mm Hg, and therefore decreasing FVR by 60±6% and 63±4% in the sham-operated and nephrectomized group, respectively. These effects of isoproterenol were blocked by the intravenous administration of propranolol.

Plasma Angiotensin I Concentration

Control arterial and femoral venous Ang I concentrations were, respectively, 116±28 and 125±25 pg/ml in the sham-operated group and increased to 250±37 and 318±62 pg/ml with the low dose of isoproterenol and to 742±125 and 1,236±268 pg/ml with the high dose of isoproterenol. The effect of isoproterenol was attenuated or abolished by propranolol. Control arterial and femoral venous Ang I concentrations were 36±5 and 24±4 pg/ml, respectively, in the nephrectomized group, and neither isoproterenol nor propranolol had any effect on these Ang I concentrations.

![Figure 1. Effect of isoproterenol (Isop.) on mean arterial blood pressure (MAP) and femoral blood flow (FBF) in sham-operated and nephrectomized rabbits. Results are mean±SEM.](http://hyper.ahajournals.org/doi/10.1016/0001-2267(78)90016-0)
Plasma Angiotensin II Concentration

Isoproterenol also increased plasma Ang II levels in a dose-dependent manner, and propranolol abolished this effect. The control arterial and femoral venous Ang II concentrations were, respectively, 52±10 and 51±9 pg/ml in the sham-operated group and were increased to 96±11 and 93±16 pg/ml by the low dose of isoproterenol and to 113±21 and 104±17 pg/ml by the high dose of isoproterenol. The control arterial and femoral venous Ang II concentrations were 23±3 and 25±3 pg/ml, respectively, in the nephrectomized group. Again, isoproterenol and propranolol did not exert any effect on plasma Ang II levels in the nephrectomized rabbits.

The Ang I and Ang II formation in plasma taken from the three rabbits treated with the high dose of isoproterenol was 0.87±0.09 and 0.36±0.12 pg/ml/sec (mean±SD), respectively, at 37°C incubation.

Apparent Femoral Angiotensin II Secretion Rate

The extraction ratio of 125I-Ang II was 71±4% (mean±SD, n=6) and was used to calculate the Ang II secretion rate. As shown in the top panel of Figure 2, the Ang II secretion rate in the sham-operated group was increased from 294±41 pg/min to 953±138 and 1,281±212 pg/min by 1 and 10 nmol/min of isoproterenol, respectively. Propranolol abolished the effect induced by isoproterenol, but it did not reduce significantly the basal Ang II secretion rate. In the nephrectomized group, the basal Ang II secretion rate was 94±10 pg/min and was not significantly increased by the doses of isoproterenol before or after propranolol. Propranolol had no significant effect on Ang II secretion rate.

Apparent Femoral Angiotensin I Secretion Rate

Based on the 125I-Ang I extraction ratio of 77±7% (mean±SD, n=5), the Ang I secretion rate was calculated according to the equation in the "Methods" section. The bottom panel of Figure 2 shows the effects of isoproterenol and propranolol on the Ang I secretion rate. In the sham-operated group, isoproterenol in the dose of 1 and 10 nmol/min increased the Ang I secretion rate from 856±197 pg/min to 3,690±471 and 18,155±2,615 pg/min, respectively. Propranolol significantly attenuated this effect. The basal Ang I secretion rate in the nephrectomized group was only 79±19 pg/min and was not influenced by isoproterenol or propranolol.

Discussion

The arterial concentration of Ang I and Ang II in this study reflects the overall circulating level to which all vascular beds are exposed. Femoral venous Ang I and Ang II represent two portions of the angiotensins, a smaller one delivered from the arterial blood and a greater one formed inside the femoral vascular bed. Campbell23 has reported that 60–90% of the Ang II presented to the femoral vascular bed is metabolized in one passage. A recent study in human subjects with essential hypertension reported that 59±3% of 125I-Ang I was metabolized across the leg.24 The authors concluded that a major fraction of Ang I in circulating plasma is produced locally, rather than in circulating plasma itself. Our own data demonstrate that 77±7% (SD) of the arterially delivered 125I-Ang I and 71±4% (SD) of the arterially delivered 125I-Ang II are degraded when passing through the femoral vascular bed in the rabbit. Based on these extraction ratios, only 23±3% of the arterial Ang I and 29±2% of the arterial Ang II reach the femoral vein, and most of the angiotensins present in the femoral venous plasma are generated by the tissues in the femoral vascular bed. The apparent femoral Ang I and Ang II secretion rates were calculated to represent the net production per unit time of the angiotensins in the vascular bed. This locally formed Ang II reflects de novo generation but
also may represent conversion from arterially delivered Ang I through the action of tissue angiotensin converting enzyme in the vascular bed. Considering very little Ang I and Ang II formation in the plasma itself (see “Results”) and the short transit time (approximately 10 seconds) of the blood through the femoral vascular bed, there was only a minimal contribution of plasma-formed angiotensins to the femoral secretion rates.

The intent of our study was to examine whether local β-adrenergic receptor stimulation in the hind limb vascular bed would induce Ang II generation independent of the systemic renin-angiotensin system. Bilateral nephrectomy 24 hours before the acute experiment essentially eliminates renally derived renin and theoretically would allow detection of purely locally generated Ang II. In the sham-operated group, isoproterenol given intra-arterially caused hind limb vasodilation, hypotension, and increases in arterial and venous Ang I and arterial and venous Ang II. The Ang I and Ang II hind limb secretion rates also were markedly increased by isoproterenol.

We first will consider the effect of β-adrenergic receptor stimulation on the angiotensin secretion rates. Either of two explanations can be offered to account for the increase in angiotensin secretion rate. First, β-adrenergic stimulation of local Ang II synthesis mediated by hind limb vascular renin and angiotensin converting enzyme may account for this effect. Second, renal renin release caused by β-adrenergic stimulation acting in concert with hind limb angiotensin converting enzyme may form Ang II locally and result in the increase of secretion rate. Previous investigations using the isolated perfused rat mesenteric artery have demonstrated β-adrenergic stimulation of Ang II production, at least under in vitro conditions. However, when local Ang II generation in the isolated perfused rat hind limb was studied, prostaglandin but not β-adrenergic stimulation was suggested to be a mediator. Similarly, in the present study, in the absence of the kidneys, β-adrenergic stimulation did not cause an increase in Ang II secretion rate. Thus, the local synthesis of Ang II is not governed by β-adrenergic stimulation in the femoral vascular bed. The finding that was most striking was the powerful influence the kidney had on local angiotensin production. With the kidneys intact, isoproterenol-induced release of renal renin was associated with a marked increase in hind limb angiotensin generation, suggesting the need of renally derived renin for local angiotensin production.

It also is of interest that the increase in Ang I secretion rate far surpassed that of Ang II, suggesting that the renin reaction was highly efficient in this vascular bed.

Although many studies have provided evidence for the local generation of angiotensin in vascular beds, the underlying mechanism for local angiotensin synthesis is poorly understood. Some in vitro studies using isolated perfused vascular beds, isolated blood vessels, and cultured cells have suggested the role of tissue-synthesized renin in the production of local angiotensins. With the use of an RNAase protection technique, the specific renin mRNA has been detected in many tissues, such as aorta, adrenal gland, heart, and lung. Two in vivo studies have reported the persistence of vascular reninlike activity after bilateral nephrectomy. However, other studies have demonstrated that bilateral nephrectomy removes all detectable reninlike activity from the arterial wall and that most of the reninlike activity within the aortic wall is derived from plasma renin. There has been a long debate over the relative importance of the two sources of renin. The results from our study not only demonstrate the local production of angiotensins in the femoral vascular bed, which was increased by isoproterenol, but also show that the β-adrenergic-induced increase of local and systemic angiotensin generation is dependent on the presence of the kidney. The most likely renal factor involved here is renin, but this has to be verified by administration of renin in the nephrectomized animal. However, our results strongly support the hypothesis that renin released by the kidney is taken up by vascular tissues, where it acts on angiotensinogen to form Ang I and ultimately Ang II.

Isoproterenol, in a dose-dependent manner, increased systemic and local generation of Ang I and Ang II. This effect was abolished by propranolol, indicating that it was β-adrenergically mediated. A low level of local angiotensin generation was present even 24 hours after bilateral nephrectomy. At this point in time, 98% of the PRA had been removed, but the basal femoral Ang II secretion rate still accounted for 32% of the normal value. However, this Ang II production was not increased by isoproterenol. The mechanism by which isoproterenol caused renal renin release is uncertain. This may have occurred as a result of direct β-adrenergic receptor stimulation of juxtaglomerular cells or may have resulted from stimulation of juxtaglomerular cells indirectly through baroreceptor reflex activation of renal nerve activity due to the reduction in blood pressure.

As would be expected, isoproterenol, in a dose-dependent manner, caused an acute increase in FBF and fall in MAP and FVR in both groups of rabbits, although it increased the systemic and local Ang II generation at the same time. The hemodynamic effect of isoproterenol was blocked by propranolol, indicating that it was β-adrenergically mediated. Despite the β-adrenergic-induced increase in Ang II production, the effect of isoproterenol on FVR was unaffected. The decrease in FVR due to isoproterenol was equivalent in the sham-operated and nephrectomized rabbits. This result is consistent with our previous finding that FVR in the rabbit is only weakly affected by the renin-angiotensin system directly. The fact that the control MAP in the nephrectomized group was lower than that in the sham-operated group suggests that the systemic re-
nin-angiotensin system supports blood pressure under the conditions of these experiments.

It has been reported that aortic mRNA for angiotensinogen is increased 48 hours after bilateral nephrectomy and as a result the activity of the tissue renin-angiotensin system may be stimulated. As seen in this study, nephrectomy removed 98% of the PRA, but the arterial and femoral Ang I and Ang II concentrations were very low. These results do not provide evidence for a greater local femoral angiotensin generation after nephrectomy. The fact that plasma Ang I and Ang II concentrations in the nephrectomized group did not decrease in proportion to the PRA suggests that the residual angiotensins may be derived from the tissue renin-angiotensin system. Other reports have indicated persistence of Ang II in nephrectomized humans and animals.6–8 Our results lend further support for the presence of a local renin-angiotensin system in vivo; however, the actual physiological function of this system is not known.

In summary, our in vivo study has demonstrated that β-adrenergic agonist stimulates the systemic and local hind limb production of Ang I and Ang II and that this effect is dependent on the presence of the kidney. This suggests that the femoral vascular bed mainly uses renal renin to produce angiotensins locally. It appears that this locally generated Ang II does not influence the hind limb vasodilator response to isoproterenol. Local angiotensin generation still occurs after nephrectomy, but at a much diminished rate.

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