Angiotensin II Directly Stimulates Release of Atrial Natriuretic Factor in Isolated Rabbit Hearts

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**Background.** Previous studies have shown that infusion of angiotensin II (Ang II) increases plasma concentrations of atrial natriuretic factor (ANF) in vivo. This phenomenon has been considered secondary to the effects of Ang II on cardiac and systemic hemodynamics. The present study was designed to assess whether Ang II may exert a direct stimulatory effect on ANF release from the heart independent of changes in hemodynamics.

**Methods and Results.** Isolated rabbit hearts were perfused in the Langendorff mode. Heart rate, coronary flow, and atrial and left ventricular (LV) volumes were kept constant. After stabilization, Ang II was infused intracoronary at increasing doses (10⁻¹¹ to 10⁻⁸ M) in nine hearts and at a single dose of 10⁻¹⁰ M in 10 hearts. Each infusion lasted for 5 minutes and was followed by a 10-minute washout period. Four hearts received vehicle alone for 80 minutes. Ang II induced a dose-dependent increase in coronary perfusion pressure and in LV developed pressure. ANF release, measured by radioimmunoassay on the extracts of the cardiac effluent, also increased during Ang II infusion and returned to the basal values during the 10-minute washout period. In the control group, coronary perfusion pressure, LV developed pressure, and LV end-diastolic pressure did not change appreciably over the observation period, whereas ANF release progressively decreased during perfusion.

**Conclusions.** Ang II can directly stimulate cardiac release of ANF in isolated rabbit hearts independently of changes in hemodynamics. (Circulation 1993;87:192–198)

**KEY WORDS** • angiotensin • atrial natriuretic factor

Atrial natriuretic factor (ANF) is a cardiac hormone that exhibits potent diuretic, natriuretic, vasorelaxant, and renin- and aldosterone-inhibiting effects and appears to play an important role in the regulation of body fluid volume and blood pressure. This hormone is typically released from the heart in response to atrial stretch, although several studies have revealed that plasma levels of ANF in vivo can also be increased by vasoconstrictor agents such as phenylephrine, vasopressin, epinephrine, and angiotensin II (Ang II). These latter findings would suggest that humoral factors might also regulate ANF release via direct stimulation of atrial tissue. Those studies are not conclusive, however, since any stimulatory effect that might occur cannot be separated from the hemodynamic actions of those agents, and therefore increased ANF release might simply be the consequence of increased atrial distension. In fact, earlier studies performed both in experimental animals and in humans suggested that the hemodynamic changes could entirely account for the increased secretion of ANF associated with Ang II infusion. On the other hand, recent studies by Volpe and coworkers showed that systemic infusions of low doses of Ang II stimulated ANF secretion in dogs in the setting of controlled volume expansion and minimal hemodynamic changes. Furthermore, recent studies by Volpe et al. showed that Ang II is able to specifically promote ANF release in the rabbit, even when its pressor action is prevented by the concomitant administration of sodium nitroprusside.

The present study was designed to determine whether Ang II has a direct stimulatory effect on cardiac ANF secretion, independent of changes in hemodynamics. The effects of this hormone on ANF release were studied in isolated Langendorff-perfused rabbit hearts in which heart rate, coronary flow, and atrial and ventricular volumes were kept constant.

**Methods**

**Isolated Heart Preparation**

Female New Zealand White rabbits (body weight, 1.5–2.0 kg) were anticoagulated with heparin (1 ml i.p.) and anesthetized with sodium pentobarbital (30 mg/kg i.p.). The chest was opened and the heart was rapidly isolated, with care taken to avoid excision of atrial tissue. The aorta was cannulated, and retrograde perf-
fusion was started by means of a constant-flow roller pump (Masterflex, Cole-Parmer Instrument Co., Chicago, Ill.). The perfusate was a modified Krebs-Ringer bicarbonate buffer containing (in mM) sodium chloride 117, calcium chloride 3.0, EDTA 0.5, magnesium sulfate 1.0, sodium bicarbonate 24.0, potassium chloride 6.0, and glucose 16.7, pH 7.4. The buffer was maintained at 37°C and equilibrated with 95% O₂/5% CO₂, and it was not recirculated. The flow rate was adjusted to the metabolic needs of each heart so that an initial perfusion pressure of 80 mm Hg could be obtained. Coronary flow was then kept constant throughout the experiment. The coronary circulation perfused the atrial myocardium, which then drained into the general effluent. Venous flow from the coronary sinus drained outside through the pulmonary artery by way of the right atrium and right ventricle. In addition, the aorta freely emptied outside the heart through the venae cavae and pulmonary veins, which were cut free. Thus, both atria were in an empty, flaccid state throughout the experiment. Cardiac venous effluent was collected at selected time points to measure coronary flow rates and to quantify ANF release. Effluent samples were collected by drip from the entire heart to include also the fraction of the venous flow that does not drain into the coronary sinus. Coronary perfusion pressure was continuously monitored via a side arm in the perfusion cannula connected to a Statham P23dB pressure transducer. A side arm in the perfusion line allowed delivery of the drug directly to the heart. Right atrial pacing at 180 beats per minute was achieved by means of an epicardial electrode sutured on the right atrial appendage and connected to a Harvard stimulator (Harvard, Research Stimulator). To assess contractile function, a latex balloon was inserted into the left ventricle through the mitral orifice, secured by means of a ligature that included the mitral annulus, and connected to a pressure transducer. The balloon was initially inflated with a volume of saline sufficient to produce an end-diastolic pressure of 10 mm Hg, which is on the plateau of the Frank-Starling curve for this preparation. All subsequent measurements of developed pressure, calculated as the difference between peak systolic and end-diastolic pressures, were made at this same end-diastolic volume. Left ventricular (LV) and coronary perfusion pressures were recorded on a Gould 2400 four-channel direct-writing recorder (Gould Instruments Co., Cleveland, Ohio).

Experimental Protocol

After 30 minutes of stabilization, basal hemodynamic measurements were obtained. Cardiac effluent (10 ml) was collected, placed immediately on ice, and stored at –20°C until assayed. Ang II (Peninsula Laboratories Europe, Merseyside, UK) was dissolved in appropriate amounts of saline and administered in a group of nine hearts at increasing concentrations of 10⁻¹¹, 10⁻¹⁰, 10⁻⁹, and 10⁻⁸ M via a side arm in the aortic cannula by means of an infusion pump (Harvard, South Natick, Mass.) at a flow rate 1/₁₀₀₀ of that of each heart. Each infusion lasted for 5 minutes and was followed by a 10-minute washout period. All concentrations are expressed as final concentrations in the perfusate. Hemodynamic parameters and ANF release were measured at 10-minute intervals during the equilibration period, at the end of each 5-minute infusion period, and at the end of each 10-minute washout period. For technical reasons, it was not possible to measure ANF concentration in the cardiac effluent for the 10⁻⁹ M dose in two of nine hearts.

In 10 additional hearts, after stabilization, a single dose of Ang II (10⁻¹⁰ M) was infused for 5 minutes; this was followed by a 10-minute washout period.

Parallel experiments (n=4) were performed to determine the hemodynamic stability of the experimental preparation and to evaluate the spontaneous release of ANF from the heart. For this purpose, after 20 minutes of stabilization, hemodynamic parameters were measured, and effluent samples were collected at 10-minute intervals during administration of vehicle alone for 80 minutes.

At the end of the experiment, the heart was removed, the aorta and the pulmonary artery were dissected, and the heart was blotted and weighed.

Radioimmunoassay for Atrial Natriuretic Peptide

The concentration of ANF in the samples from coronary effluent was measured by radioimmunoassay as previously described,¹⁴ using rabbit antiserum (RAS 8798, Peninsula Laboratories), iodinated human ANF (2,000 Ci/mmol, Amersham, Arlington Heights, Ill.), and human ANF-(99-126) (Bissendorf Peptide GmbH, FRG) as a standard. A 100% cross-reactivity with the rat 28-residue peptide has been shown for the antibody used in our assay. In addition, the rat ANF sequence is identical to that of rabbit ANF. ANF was extracted from the perfusate by use of C-18 cartridges (Millipore, Milford, Mass.). The peptide retained on C-18 was eluted by means of 80% aqueous CH₃CN. The lyophilized eluates were reconstituted in radioimmunoassay buffer (phosphate buffer 0.1 M, pH 7.4). Bound/free separation was carried out with 1.5% charcoal–dextran. Nonspecific binding was about 4%. Recoveries determined from each sample by adding to it a small amount of radiolabeled ANF ranged from 59% to 85%. Intra-assay and interassay coefficients were 6.6% and 10.5%, respectively. The radioimmunoassay sensitivity was 3 pg per tube. The concentrations of ANF were corrected for the coronary flow, and ANF release was expressed as pg·min⁻¹·g wet weight⁻¹.

Analysis of Data

Data are expressed as mean±SEM. Distribution of the data was tested by the Bartlett test. One-way ANOVA for repeated measures was applied to analyze the responses in the Ang II and control groups. For ANF, data were available in only seven hearts for the 10⁻¹⁰ M dose; this dose was omitted from the analysis. After ANOVA, nonparametric post hoc selected contrasts across dependent variables for within-group effect were used.¹⁵

Results

In the group of control hearts, coronary perfusion pressure, LV developed pressure, and LV end-diastolic pressure did not change appreciably over the 80-minute perfusion period (Table 1).

Infusion of Ang II (10⁻¹¹ to 10⁻⁸ M) consistently induced a dose-dependent coronary vasoconstriction, as indicated by the significant increase of coronary perfusion pressure. This phenomenon was not reversible during the 10-minute washout period between two
consecutive infusions, since baseline levels progressively tended to increase (Figure 1, panel A). LV developed pressure, indexed as the difference between LV systolic pressure and LV end-diastolic pressure, also increased in a dose-dependent manner during Ang II infusion in all hearts. However, the effects of Ang II on contractility subsided upon discontinuation of drug infusion (Figure 1, panel B).

LV end-diastolic pressure showed a progressive increase over the experimental period ($p=0.0001$ by ANOVA). However, selected contrasts between the values of LV end-diastolic pressure for each dose of Ang II and the corresponding baseline did not show statistical significance (Figure 1, panel C).

Isolated rabbit hearts showed a sustained release of ANF under control conditions, with marked variations among individual hearts (Figure 2). ANF release in unstimulated hearts progressively decreased during perfusion ($p=0.002$ by ANOVA) (Figure 2, left panel).

In the hearts that were to be infused with Ang II, basal release of ANF in the perfusate also tended to decrease during the 30-minute equilibration period: mean values were 1,516±285 pmol·min$^{-1}$·g$^{-1}$ at 10 minutes, 1,631±385 pmol·min$^{-1}$·g$^{-1}$ at 20 minutes, and 1,311±229 pmol·min$^{-1}$·g$^{-1}$ at 30 minutes ($p=NS$).

A large variability in ANF release among individual hearts was also observed with respect to the effects of Ang II. Absolute ANF responses to Ang II in individual animals are shown in Figure 2, right panel. Ang II infusion was followed by a transient increase in ANF release that subsided upon discontinuation of the drug infusion. This phenomenon was observed in seven of nine hearts. ANOVA showed significant variation of ANF release in this group ($F=2.79, p=0.03$). Selected contrasts of ANF concentrations for each dose of Ang II showed significant differences with the correspondent baseline for 10$^{-10}$ and 10$^{-3}$ M (both $p<0.05$). Figure 3 depicts the mean percent changes of ANF levels at each time point in the control group (left panel) and at each Ang II dose (right panel). It is evident that Ang II exerted a noticeable influence on ANF release from the perfused hearts, because increases rather than decreases were observed. ANF released in the perfusate significantly increased in the group of 10 hearts during a single intracoronary infusion of 10$^{-10}$ M Ang II and returned to baseline after a 10-minute washout period (Table 2).

**Table 1. Hemodynamic Variables Measured During Infusion of Vehicle Alone in Four Control Hearts**

<table>
<thead>
<tr>
<th>Variable</th>
<th>30 Minutes</th>
<th>40 Minutes</th>
<th>50 Minutes</th>
<th>60 Minutes</th>
<th>70 Minutes</th>
<th>80 Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPP (mm Hg)</td>
<td>72.3±4.0</td>
<td>73.5±4.2</td>
<td>74.8±4.5</td>
<td>73.3±4.9</td>
<td>74.5±5.0</td>
<td>76.8±5.4</td>
</tr>
<tr>
<td>LVDP (mm Hg)</td>
<td>80.5±7.0</td>
<td>79.5±6.8</td>
<td>79.5±7.2</td>
<td>80.5±7.6</td>
<td>79.0±7.3</td>
<td>78.8±7.0</td>
</tr>
<tr>
<td>LVEDP (mm Hg)</td>
<td>11.8±0.6</td>
<td>11.5±0.5</td>
<td>10.8±0.9</td>
<td>11.3±1.1</td>
<td>11.0±1.0</td>
<td>11.5±0.5</td>
</tr>
</tbody>
</table>

CPP, coronary perfusion pressure; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure. Values are mean±SEM.

**Discussion**

In the present study, infusion of Ang II significantly increased cardiac release of ANF. Since our experiments were performed on isolated perfused hearts, in which heart rate, coronary flow, and atrial and LV volumes were kept constant and other neural and humoral influences could be eliminated, this finding demonstrates that Ang II can directly stimulate cardiac release of ANF independently of its effects on hemodynamics. Previous animal and human studies have reported increased plasma levels of ANF during infusion of Ang II in vivo. However, Ang II administration was constantly associated with marked hemodynamic changes, suggesting that increased ANF secretion might have occurred as a consequence of changes in atrial distension, i.e., through a hemodynamic mechanism, rather than through direct receptor stimulation. In fact, changes in plasma ANF in rats infused with several vasoconstrictors, including arginine vasopressin and Ang II, correlated with changes in right atrial pressure or systemic blood pressure.

The effects of Ang II on ANF secretion in an experimental model that allowed control of hemodynamic influences have been previously evaluated by Dietz, who observed no stimulatory effects on ANF secretion in a rat heart–lung preparation. One explanation for the divergent results with respect to the present study may relate to the experimental models. Recent studies have documented that ANF released into the right atrium can be removed or degraded as it passes through the lungs, at least in humans. If this mechanism were also operating in rats, it might obscure an increase in ANF secretion in the heart–lung preparation. Similarly, the lack of stimulation of ANF release reported by Lachance and Garcia in atrial slices incubated in the presence of Ang II might be ascribed to the fact that the process of tissue mincing might expose and/or release endogenous peptidases, which may inactivate any excess ANF produced.

Our data are in agreement with the results of a recent study by Volpe and coworkers, who examined the effects of low doses of Ang II on plasma ANF levels in conscious and anesthetized dogs under controlled volume expansion and simultaneous converting enzyme inhibition to prevent spontaneous fluctuation of blood volume and endogenous Ang II levels. The findings indicated that low doses of Ang II can increase plasma ANF levels by a mechanism largely independent of hemodynamic changes. Similar results were obtained in a more recent study in which Ang II was administered to rabbits during the simultaneous infusion of sodium nitroprusside to counteract its pressor effects.

A possible limitation of our study is that no clear dose–response relation between Ang II concentrations and ANF release was observed, although Ang II consistently raised ANF levels in the effluents from a large majority of the hearts. Since buffer-perfused hearts lack the substrates for peptide synthesis, it might be hypothesized that this phenomenon is related to progressive depletion of the endogenous pool of amino acids for
ANF resynthesis, which will tend to blunt the response to multiple infusions of Ang II. This speculation is directly supported by the observation that in parallel experiments, the concentrations of ANF in the cardiac effluent of control hearts progressively and markedly decreased over time. Furthermore, in those hearts that received Ang II, basal ANF release tended to decrease during the stabilization phase, and there was also a tendency for ANF concentrations in the cardiac effluent to decrease with each consecutive washout period. We believe that this progressive decrease in ANF release is not a sign of deterioration of the preparation, because control hearts proved to be hemodynamically stable throughout the 80-minute observation period. On the other hand, the increased ANF levels measured in the cardiac effluent after Ang II infusions were not the result of fluctuations over baseline, but they were temporally linked to each Ang II infusion.

To further demonstrate the direct relation between Ang II infusion and cardiac ANF release and to eliminate possible artifacts deriving from the approach of gradually increasing the dose of Ang II in the same heart preparation, we performed 10 additional experiments in which a single dose of Ang II was infused to each heart for 5 minutes. We chose $10^{-10}$ Ang II concentration as a single dose to minimize the confounding hemodynamic effects produced by higher doses of the drug. These experiments clearly showed a causal and temporal relation between Ang II infusion and ANF release. Since in the present study we collected the effluent from the entire heart, we cannot exclude that the stimulated release of ANF was at least in part produced by ventricles.

Ang II infusion induced a dose-dependent increase of coronary perfusion pressure, indicating a constrictive effect of Ang II on the coronary arteries. We considered the possibility that the increase in coronary perfusion pressure induced by Ang II affects ANF release. However, the increase in coronary perfusion pressure recorded with the highest dose of Ang II that we used in our experiment (i.e., about 15 mm Hg) is relatively small and is not high enough to exert any metabolic effect, since in our model coronary flow was kept constant. In principle, it is still possible that, although total coronary flow remained constant, relatively small changes in coronary resistances might have affected transmural distribution of flow. We consider this possibility unlikely, on the basis of previous observations from our laboratory, which demonstrated that a 30–mm Hg increase in coronary perfusion pressure was not accompanied by detectable changes in transmural perfusion in this experimental model. Furthermore, the increases in ANF release in our experiments were not temporally linked to the increases in coronary perfusion pressure. In fact, Ang II induced a dose-dependent increase of coronary perfusion pressure, which was not reversible during the 10-minute washout period between two consecutive infusions, since baseline levels progressively tended to increase. In contrast, Ang II infusion was followed by a transient increase in ANF release that subsided upon discontinuation of the drug infusion. Thus, ANF release did not increase (and actually decreased) during the intervals between two consecutive Ang II infusions, in spite of the progressive increase in coronary perfusion pressure.

Similarly, we believe that the progressive increase in LV end-diastolic pressure observed in our experiments did not affect ANF release. Although in vivo increase in LV end-diastolic pressure is accompanied by increased distension of both left atrium and ventricle, this phenomenon cannot occur in the isolated Langendorff-perfused heart. In fact, 1) in this experimental model,
left atrial pressure is not influenced by changes in LV end-diastolic pressure. In this preparation, left atrial and LV cavities are not in communication because of the ligature around the mitral annulus, which secures the latex balloon into the LV cavity. Furthermore, the left atrium is in an empty state throughout the experiment, since the pulmonary veins are cut free, and no fluid accumulates. 2) The isolated Langendorff-perfused heart is an isovolumic preparation. This volume is then left unchanged throughout the experiment. Therefore, any subsequent increase that may occur in LV end-diastolic pressure is not caused by changes in LV volume but is related to increased resting (i.e., diastolic) tension of LV myocardium, which may exert some force around the balloon. 3) In our experiments, no temporal relation was found between the increase in LV end-diastolic pressure and ANF release.

A slight but significant increase of LV developed pressure was observed after Ang II infusion, consistent with the positive inotropic effects of Ang II observed in most animal species as well as on human cardiac muscle. This inotropic response to Ang II is most likely related to an increase in the permeability of the cell membrane to Ca2+ during the action potential. An increase in Ca2+ influx through the voltage-dependent Ca2+ channel, as well as receptor-mediated Ca2+ mobilization, seems also to be involved in the mechanism of ANF secretion by a variety of stimuli. Therefore, it could be hypothesized that Ang II may induce ANF secretion through the receptor-mediated Ca2+ mobilization. At the same time, some evidence seems to indicate that ANF secretion can be stimulated via activation of the protein kinase C/phosphatidylinositol pathway. In
TABLE 2. Atrial Natriuretic Factor Released in the Perfusate in 10 Hearts During a Single Intracoronary Infusion of Angiotensin II

<table>
<thead>
<tr>
<th>Heart</th>
<th>ANF (mM·min⁻¹·g⁻¹)</th>
<th>10⁻¹⁰ M</th>
<th>10-Minute washout</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>501</td>
<td>589</td>
<td>554</td>
</tr>
<tr>
<td>2</td>
<td>371</td>
<td>489</td>
<td>434</td>
</tr>
<tr>
<td>3</td>
<td>229</td>
<td>499</td>
<td>296</td>
</tr>
<tr>
<td>4</td>
<td>1,200</td>
<td>1,971</td>
<td>1,280</td>
</tr>
<tr>
<td>5</td>
<td>694</td>
<td>1,041</td>
<td>845</td>
</tr>
<tr>
<td>6</td>
<td>1,489</td>
<td>1,600</td>
<td>978</td>
</tr>
<tr>
<td>7</td>
<td>4,047</td>
<td>4,709</td>
<td>3,724</td>
</tr>
<tr>
<td>8</td>
<td>1,509</td>
<td>1,992</td>
<td>1,475</td>
</tr>
<tr>
<td>9</td>
<td>1,339</td>
<td>1,886</td>
<td>1,078</td>
</tr>
<tr>
<td>10</td>
<td>453</td>
<td>447</td>
<td>715</td>
</tr>
</tbody>
</table>

Mean±SEM 1,183±353 1,522±409* 1,137±310

**ANF, atrial natriuretic factor; Ang II, angiotensin II.**

*p=0.002.

The phorbol ester 12-O-tetradecanoylphorbol-β-acetate, a protein kinase activator, is able to induce a dose-dependent increase in ANF secretion by cultured rat cardiocytes. Furthermore, ANF release from atrial myocytes can be increased by stimulation of muscarinic cholinergic, α₁-adrenergic, and vascular-type vasopresin receptors, all of which share as a common feature the activation of the phosphoinositide system. Since a similar coupling of receptor stimulation to inositolphosphate production has been demonstrated to occur after Ang II stimulation of cardiac muscle, it might be speculated that Ang II stimulates cardiac ANF secretion through a dual mechanism, involving the above-mentioned receptor-mediated Ca²⁺ mobilization as well as the activation of protein kinase C.

Within the dose ranges we used, a significant increase in ANF release was always observed at 10⁻¹⁰ M Ang II. This dose is very close to plasma Ang II concentration seen in patients with congestive heart failure, i.e., 7×10⁻²⁵ M. In humans, increased ANF secretion has typically been observed in the setting of volume expansion and/or increased atrial stretch. It should be pointed out, however, that significantly elevated plasma ANF levels have been reported in pathological situations associated with decreased body fluid volume such as severe preeclampsia. This observation supports the speculation that ANF secretion might be directly stimulated by other mechanisms even in the absence of volume expansion. Whether Ang II is the actual mechanism involved in preeclampsia is unclear: there are few studies in which plasma Ang II levels have been measured in hypertensive pregnancy, and the results of these studies are inconclusive. Interestingly, Symonds and Broughton-Pipkin reported a highly significant correlation between diastolic blood pressure and plasma Ang II levels in primigravida. In the same study, the authors also found that preeclamptic women had higher levels of Ang II despite lower plasma renin activity levels and suggested that Ang II is secreted from the placental bed.

The direct stimulatory effect of Ang II on cardiac ANF secretion observed in the present study, together with the notion that ANF may exert inhibitory effect on Ang II-induced aldosterone secretion by adrenal cells in vitro and in vivo, and may reduce plasma renin activity and renin secretory rate, suggests the possible existence of a negative-feedback hormonal mechanism between the heart and the kidney, which might contribute to the control of body fluid volume and blood pressure. Such a mechanism might be of a special relevance to pathological situations characterized by high Ang II plasma levels, such as congestive heart failure and renal vascular diseases.

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