Angiotensin II Increases Norepinephrine Release From Atria by Acting on Angiotensin Subtype 1 Receptors

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Norepinephrine stores in electrically driven guinea pig isolated atria were loaded with [3H]norepinephrine, and norepinephrine release was deduced from the radioactivity efflux. Electrical field stimulation of sympathetic nerve endings was applied during the refractory period of atrial contractions. The stimulation-induced release of norepinephrine was increased by angiotensin II (Ang II) (10^{-8} to 10^{-6} mol/L) in a concentration-dependent manner. The maximum observed effect was a 55% augmentation. The effects of 10^{-7} and 10^{-6} mol/L Ang II were abolished by 10^{-6} and 10^{-5} mol/L of the subtype 1 Ang II receptor antagonist losartan, respectively. Losartan by itself (10^{-6} mol/L) caused a 14% reduction of norepinephrine release. The subtype 2 Ang II receptor ligand PD 123319 (l-[[4-(dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid ditrifluoroacetate) in a concentration of 10^{-4} mol/L had no detectable influence on transmitter release and did not antagonize the effect of Ang II. Angiotensin I (10^{-6} and 10^{-5} mol/L) increased norepinephrine release maximally by 23%. This effect was antagonized by 10^{-5} mol/L losartan and did not appear in the presence of 10^{-5} mol/L of the converting enzyme inhibitor ramiprilat. These results suggest that Ang II increases norepinephrine release by an activation of subtype 1 receptors, whereas angiotensin I is converted to Ang II to become effective. (Hypertension. 1993;22:699-704.)

KEY WORDS • heart atrium • norepinephrine • angiotensin II • receptors, angiotensin • losartan

In plasma, angiotensin I (Ang I) is metabolized to the vasoconstricting octapeptide angiotensin II (Ang II) by a circulating converting enzyme. Additional amounts of Ang II are produced by local renin-angiotensin systems in a variety of tissues. In the heart, for instance, messenger RNA for the synthesis of renin has been detected, and a tissue-bound converting enzyme is also present, as well as Ang II, its precursor Ang I, and the metabolite angiotensin III. Such a local renin-angiotensin system may be responsible for a variety of physiological and pathophysiological effects. For instance, Ang II increases protein synthesis in chick heart cells. Thus, the local production of the peptide may contribute to the development of cardiac hypertrophy, which can be prevented or reversed by converting enzyme inhibitors in experimental and human hypertrophy. Other effects of Ang II are a release of catecholamines from the adrenal medulla and from dopaminergic neurons in the central nervous system and a facilitation of the release of norepinephrine from sympathetic nerve terminals in field-stimulated guinea pig, rat, and mouse atria. The positive inotropic effect of Ang II, which, according to Kuschinsky and Lüllmann and Theseyon and Klaus, does not appear in the presence of β-adrenergic receptor antagonists, may therefore be caused by an increase of the local norepinephrine concentration. However, a direct influence of the peptide on calcium channels has also been suggested.

The first aim of this study was to determine whether the prejunctional effect of Ang II on norepinephrine release is mediated by Ang II subtype 1 (AT₁) or subtype 2 (AT₂) receptors in the isolated guinea pig atrium. To this end, the AT₁-selective receptor antagonist losartan and the AT₂-selective receptor ligand PD 123319 (l-[[4-(dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid ditrifluoroacetate) were used. Further experiments with Ang I and the converting enzyme inhibitor ramiprilat suggest that a converting enzyme that continuously produces small amounts of endogenous angiotensin is active in the isolated atrium.

Methods

Experimental Procedure

Two hundred male Pirbright white guinea pigs were used for the study. They were bought from the Lippische Versuchstierzuchtanstalt, Extertal, FRG, had free access to Altromin standard diet and tap water until used, and weighed 300 to 500 g on the day of experiments. The animals were killed by a blow on the head, and the hearts were rapidly removed. The left atrium was dissected out and mounted in a 10-mL organ bath that contained Krebs-Henseleit solution of the following com-
position (mmol/L): NaCl, 117.6; KCl, 5.8; NaHCO₃, 25; NaH₂PO₄, 1.2; MgSO₄, 1.2; CaCl₂, 2.5; and glucose, 5.5 as well as 0.1 mmol/L Ca-EDTA and 0.06 mmol/L ascorbic acid as antioxidants. The solution was gassed with 95% O₂ and 5% CO₂. The atria were stimulated at a rate of 0.5 Hz through platinum electrodes in direct contact with the muscle with square pulses of 2-millisecond duration and 1.5 times threshold voltage (Stimulator T, Hugo Sachs, Hugstetten, FRG). The resting tension was adjusted to 10 mN, and the force of contraction was registered isometrically with a K 30 force-displacement transducer (Hugo Sachs) on a Helcroscope HE 16 (Hellige, Freiburg, FRG). After an equilibration period of 15 minutes, 10 μCi of 7-[³H]norepinephrine was added to establish a drug concentration between 5×10⁻⁸ and 10⁻⁷ mol/L in the loading bath (calculated from the specific activity of 10 to 20 Ci/mmol, see below). After a loading period of 1 hour, the atria were carefully rinsed with Krebs-Henseleit solution and transferred to a 20-mL organ bath for the release experiments.

During the release experiments, the atria were stimulated at a rate of 0.5 Hz as described above (continuous myocardial driving stimulation). The myocardial driving stimuli triggered a second Stimulator T that produced square pulses of 30-V strength and 0.2-millisecond duration. To evoke norepinephrine release from sympathetic nerve endings, these supramaximal stimuli (field stimuli) were applied 10 milliseconds after the driving stimulus, ie, during the myocardial refractory period, through two parallel platinum electrodes (1.5 cm long, 0.5 mm in diameter, 1 cm apart, one on each side of the muscle). For 1 hour, only myocardial driving stimulation was used, and the bathing solution was changed every 10 minutes to wash away all ³H]norepinephrine that had not been taken up into the storage vesicles. Thereafter, a single field stimulus (2 milliseconds, 30 V) was applied during each refractory period for 10 minutes. Atria that did not give a significant positive inotropic response to this depolarization were discarded after this test stimulation period.

For all other atria, the experiment proper began 15 minutes later. Cocaine (3×10⁻⁵ mol/L) and atropine (10⁻⁷ mol/L) were added to the organ bath to prevent neuronal reuptake of norepinephrine and cholinergic effects of electrical field stimulation, respectively. Norepinephrine release from sympathetic nerve endings was stimulated three times in each atrium by applying one 0.2-millisecond field stimulus during each myocardial refractory period. The stimulation periods lasted 5 minutes each and were separated by 15-minute intervals. The first and second stimulation periods (S₁ and S₂) served as individual controls, and the various test drugs were added 15 minutes before the third stimulation period (S₃).

Five minutes before the start and immediately after the end of S₁, S₂, and S₃, the organ bath was filled with fresh Krebs-Henseleit solution. Samples (1 mL) of the bathing fluid were collected immediately before the beginning and at the end of each stimulation period and again 5 minutes later for the determination of radioactivity efflux.

Calculations and Statistics

The stimulation-induced radioactivity efflux from the atrium into the organ bath was taken as an indicator of the stimulation-induced norepinephrine release. For measurement of the radioactivity in the bathing fluid, the 1-mL samples were mixed with 9 mL scintillation fluid (Hydroluma, Baker, Deventer, the Netherlands) and counted for 10 minutes in a scintillation counter (BF 5003, Laboratorium Dr Bertold, Wildbad, FRG). Results are given as disintegrations per minute (dpm) per milliliter bathing fluid. The basal, unstimulated outflow of radioactivity was determined in the samples from the 5-minute collection periods immediately before and after each stimulation period. To obtain the stimulation-induced radioactivity efflux, we subtracted the mean basal outflow from the total radioactivity measured in the sample from the stimulation period.

To compare the effects of the consecutive stimulation periods, we calculated the S₂-S₁ and S₃-S₁ ratios for the stimulation-induced radioactivity release for each experiment. Their means and associated standard errors (n=7 to 11 muscles per group) are presented in the figures. Friedman's analysis of variance with ranks was used to compare the predrug (S₂-S₁) release ratios from the different groups of atria. To evaluate drug effects, we compared the S₂-S₁ and S₃-S₁ ratios from the same group of atria with the U test for paired data. A value of P≤.05 for the two-tailed test was considered to indicate statistical significance throughout.

Drugs

The following drugs were used in the study: L-7-[³H]norepinephrine hydrochloride (10 to 20 Ci/mmol, Du Pont de Nemours, Dreieich, FRG); Ca-EDTA, ascorbic acid, cocaine hydrochloride (all E Merck, Darmstadt, FRG); atropine sulfate (Pharma Hameln, Hameln, FRG); Ang I, Ang II (Sigma Chemie, Deisenhofen, FRG); losartan (kindly donated by Du Pont, Wilmington, Del); PD 123319 (provided by Parke-Davis, Mich); and ramiprilat (donation from Hoechst AG, Frankfurt, FRG). All drugs were dissolved in double-distilled water. Stock solutions of the peptides (1 mg/mL) were stored at −22°C until use. All other solutions were prepared daily, and microliter amounts were added to the organ bath.

Results

Predrug Control Values for Basal Outflow and Stimulated Release of Radioactivity

Even when the sympathetic nerve endings were not depolarized by field stimulation, there was a continuous efflux of radioactivity from the atria. This basal outflow is thought to consist mainly of inactive norepinephrine metabolites. In 22 groups of atria, the mean unstimulated efflux measured for the 5-minute period immediately preceding S₁ ranged between 87±8 and 178±54 dpm/mL (lowest and highest value, respectively). It decreased by approximately 10% to 25% during the time course of the experiments and was not significantly influenced by any of the drugs under study.

Field stimulation released additional amounts of radioactivity, and this stimulation-induced release can be regarded as a reliable indicator of an exocytotic release of norepinephrine from sympathetic nerve terminals. For the first stimulation period (S₁), mean release rates between 116±16 and 263±69 dpm/mL were obtained (highest and lowest mean and SEM,
FIG 1. Bar graph shows influence of angiotensin II (ANG II) on radioactivity release from left atria caused by three consecutive field-stimulation periods (S₁ to S₃). Means of S₂-S₁ release ratio (open columns) and S₃-S₂ release ratio (filled columns) are shown for each group of atria (n=8 to 9). Vertical bars indicate associated SEM. Angiotensin II was added 15 minutes before S₁. *Significant difference (P<0.05, two-tailed) between S₃-S₁ and S₂-S₁ ratio (U test for paired data). Drug concentrations are in moles per liter.

respectively). Field stimulation also produced a significant positive inotropic effect, increasing the force of contraction by between 7.93±1.38 and 17.70±2.36 mN (highest and lowest mean value measured for S₁). Although no drugs were added before S₁, less radioactivity was released during the second stimulation period than during S₁ in most experiments, so that mean S₂-S₁ release ratios between 0.85±0.08 and 0.98±0.07 were calculated (Figs 1 through 5).

Control Experiments

In control experiments, when no drug was added before S₁, there was a further small decrease of the stimulation-induced release of radioactivity. But the resulting mean S₂-S₁ ratios in five groups of atria (rang-

Effects of Angiotensin II

Ang II increased the stimulation-induced release of radioactivity in a concentration-dependent manner (Fig 1). The effect became apparent at a concentration of 3×10⁻⁸ mol/L and reached its maximum at a concentration of 10⁻⁶ mol/L. However, because of its large variability, the influence of 10⁻⁶ mol/L was not significantly different from the effect of 10⁻⁷ mol/L, and concentrations beyond 10⁻⁶ mol/L were not tested. The S₂-S₁ release ratio of 1.28±0.19 obtained with 10⁻⁷ mol/L underestimates the maximum drug effect because

FIG 2. Bar graph shows influence of angiotensin II (ANG II) in combination with losartan (LOS) on radioactivity release from left atria caused by three consecutive field-stimulation periods (S₁ to S₃). Means of S₂-S₁ release ratio (open columns) and S₃-S₂ release ratio (filled columns) are shown for each group of atria (n=9). Vertical bars indicate associated SEM. Drugs were added 15 minutes before S₁. *Significant difference (P<0.05, two-tailed) between S₂-S₁ and S₃-S₂ ratio (U test for paired data). Drug concentrations are in moles per liter.
the release during \( S_2 \) was smaller than during \( S_3 \) (see above). Compared with \( S_2 \), Ang II caused a 55% increase of the stimulation-induced release. The effect of Ang II was accompanied by a small (12%) augmentation of the positive inotropic effect of field stimulation, but the slight influences of the test drugs on atrial contractility are not further described or discussed.

The nonpeptidic AT\(_1\)-specific receptor antagonist losartan reduced the stimulatory influence of Ang II on transmitter release (Fig 2). A concentration of \( 10^{-6}\) mol/L losartan was sufficient to suppress completely the effect of \( 10^{-7}\) mol/L Ang II, whereas \( 10^{-5}\) mol/L was needed to abolish the influence of \( 10^{-6}\) mol/L of the peptide. By itself, \( 10^{-7}\) mol/L losartan had no effect on transmitter release (data not shown), but \( 10^{-6}\) mol/L caused a 14% reduction of the stimulation-induced release of radioactivity (Fig 2).

In contrast to losartan, the AT\(_2\)-specific receptor ligand PD 123319 in concentrations up to \( 10^{-4}\) mol/L had no detectable influence on the effect of Ang II and was also without effect when applied alone (Fig 3).

**Effects of Angiotensin I**

The precursor of Ang II, Ang I, also increased the stimulation-induced release of radioactivity (Fig 4). However, the concentrations of the decapeptide needed to produce a significant change were approximately 10 times greater than those of the octapeptide. Although no clear-cut effect was seen in some preliminary experiments with \( 10^{-7}\) mol/L (not shown), \( 10^{-6}\) and \( 10^{-5}\) mol/L produced a 23% and 18% increase, respectively.

The effect of \( 10^{-6}\) mol/L Ang I was abolished by \( 10^{-5}\) mol/L losartan (Fig 4) and did not appear when \( 10^{-6}\) mol/L of the converting enzyme inhibitor ramiprilat was applied simultaneously (Fig 5). Ramiprilat by itself did not modify the stimulation-induced release of radioactivity.

**Discussion**

It has been concluded from in vivo experiments that Ang II increases the release of norepinephrine in the heart\(^{25}\); the effect has also been demonstrated directly in isolated heart tissues.\(^{14,20}\) The present results are in line with previous studies that have demonstrated a 50% increase of transmitter release by \( 10^{-8}\) mol/L Ang II in guinea pig atria,\(^12\) a doubling by \( 3 \times 10^{-7}\) mol/L in rat atria,\(^13\) and a 50% increase by \( 10^{-8}\) to \( 10^{-7}\) mol/L in mouse atria.\(^14\) Obviously, the renin-angiotensin system modulates sympathetic tone not only by acting on the central nervous system\(^{11}\) or by increasing the release of catecholamines from the adrenal medulla,\(^10\) but also by a local effect on sympathetic nerve endings in the tissues. As autoinhibition of norepinephrine release by stimulation of prejunctional \( \alpha_2 \)-adrenergic receptors was not excluded, the true maximum of this local stimulatory effect may even have been underestimated in our experiments and in the earlier in vitro studies.\(^{12,14}\)

Clearly, the influence of Ang II on norepinephrine release is complex and depends on several factors, including the concentration of the peptide, the presence of AT\(_1\) and AT\(_2\) receptors, and the effect of converting enzyme inhibitors.
release in the presence of \(\alpha\)-adrenergic receptor antagonists deserves further investigation.

In previous investigations,\textsuperscript{12,13} the effect of Ang II could be prevented by the nonselective angiotensin receptor antagonist saralasin. In our experiments, the effect of Ang II was abolished by losartan, a competitive Ang II antagonist with a high selectivity for AT\(_1\) receptors.\textsuperscript{19,27} We did not construct complete concentration-response curves because the maximum increase of norepinephrine produced by the peptide was relatively small, but the results shown in Fig 2 are compatible with the assumption of a competitive antagonism between losartan and Ang II. This suggests that in guinea pig atria the influence of Ang II on the release of norepinephrine is mediated by prejunctional AT\(_1\) receptors. Apparently, AT\(_2\) receptors are not involved, because the ligand PD 123319, which is selective for AT\(_2\) receptors,\textsuperscript{20} had no effect. AT\(_2\) receptors are present in the heart and account for up to 30% of the total angiotensin receptor population.\textsuperscript{19,28-29} Their physiological role is still uncertain, but AT\(_2\) receptors located on human cardiac myocytes have recently been shown to stimulate collagen synthesis.\textsuperscript{30}

The precursor of Ang II, Ang I, was also able to increase the stimulation-induced release of norepinephrine. The antagonism by losartan shows that this effect also is caused by a stimulation of AT\(_1\) receptors. Ang I has a low affinity to angiotensin receptors,\textsuperscript{31} but it is unlikely that it was the active compound in our experiments because its effect was not seen in the presence of the converting enzyme inhibitor ramiprilat. We conclude that Ang I must be converted to Ang II, which then acts as the receptor stimulant, as has already been suggested from experiments in guinea pig and rat atria.\textsuperscript{2,4,14} and rat Langendorff-perfused hearts.\textsuperscript{32} In these studies, Ang I was found to be 10 to 20 times less effective than Ang II, whereas both compounds were roughly equipotent in increasing the beating frequency of isolated guinea pig right atria.\textsuperscript{33} In our experiments we needed 10-fold greater concentrations of the decapetide to produce roughly the same increase of transmitter release as with the octapeptide. Only approximately 7% of the administered dose of Ang I is metabolized to Ang II in perfusion experiments with isolated organs.\textsuperscript{3} This small conversion rate seems to be a reasonable explanation for the lower efficacy of Ang I but makes it difficult to understand why in our experiments with isolated organs losartan was needed to antagonize the effect of Ang I. As judged from the experiments with Ang II (Fig 2), \(10^{-6}\) mol/L losartan should have been sufficient. We cannot explain this discrepancy at the moment.

The small reduction of norepinephrine release caused by losartan is an interesting finding. The drug is lipophilic (octanol/water partition coefficient of 7.77 at physiological pH; unpublished results) and, in principle, might act like a local anesthetic to inhibit membrane depolarization and subsequent exocytosis at the sympathetic nerve endings. However, the effect was fully overcome by increasing concentrations of Ang II (Fig 2), which makes such an unspecified influence unlikely. We suggest, therefore, that losartan antagonizes, at AT\(_1\) receptors, the stimulating effect of small amounts of endogenous Ang II that are produced continuously by the tissue converting enzyme. In contrast to our findings, no reduction of the transmitter release was observed in guinea pig atria in the presence of saralasin.\textsuperscript{12} But as saralasin is a partial agonist, the remaining intrinsic activity of this compound may have prevented the expected reduction. Perhaps more important, ramiprilat, in a concentration sufficient to inhibit the converting enzyme, did not reduce the transmitter release in our experiments, and several converting enzyme inhibitors, including captopril, were found to be ineffective by others.\textsuperscript{12,32} But this too does not necessarily contradict our hypothesis. An admittedly speculative explanation can be suggested for the lack of the effect of the converting enzyme inhibitors. The converting enzyme is a rather unspecific dipeptidase that also inhibits the inactivation of bradykinin.\textsuperscript{34,35} Bradykinin is known to stimulate norepinephrine release from nerve terminals,\textsuperscript{36} and thus the stimulating influence of an increased bradykinin concentration and the inhibitory effect of a reduced Ang II concentration could cancel each other out. The fact that in pithed rats converting enzyme inhibitors do not change the stimulation-induced release of catecholamines\textsuperscript{37,38} may even increase the norepinephrine spillover rate in conscious spontaneously hypertensive rats\textsuperscript{39} supports this idea. If Ang II produced in the tissue causes a significant modification of the norepinephrine release via prejunctional AT\(_1\) receptors in the isolated atrium, a similar effect may occur also in vivo. Because of its implications for the local regulation of sympathetic tone, this idea clearly deserves further investigation.

In conclusion, our results have shown that in field-stimulated guinea pig atria, Ang II applied to the bathing solution increases the release of norepinephrine by an activation of prejunctional AT\(_1\) receptors. Ang I is converted to Ang II to produce a similar effect. In addition, a continuous production of endogenous Ang II may be responsible for a certain degree of AT\(_1\) receptor activation in the untreated atrium.

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