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Research Article

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Prejunctional Angiotensin II Receptors

Facilitation of Norepinephrine Release in the Human Forearm

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Abstract

To determine if peripheral angiotensin II (Ang II) prejunctional receptors facilitating NE release exist in humans, we used [^{3}H]NE kinetic methodology to measure forearm NE spillover during intrabrachial arterial Ang II infusions in eight normal male subjects. We used the following protocol to optimize conditions for demonstrating these receptors: (*a*) lower body negative pressure (−15 mmHg) to increase sympathetic nerve activity to skeletal muscle; and (*b*) intraarterial nitroprusside to maintain a high constant forearm blood flow (~ 10 ml/min · 100 ml) to maximize the proportion of neuronally released NE that spills over into the circulation. During lower body negative pressure, the following were infused intraarterially for three consecutive 20-min periods: saline, Ang II (4 ng/min), and Ang II (16 ng/min). During the Ang II infusions, forearm venous NE increased significantly from 173 to 189 and 224 pg/ml ($P < 0.01$), and forearm NE spillover increased from 384 to 439 and 560 ng/min · 100 ml ($P < 0.05$ for high Ang II). Forearm NE clearance was unchanged. During low and high dose Ang II, the plasma venous Ang II concentrations were 25 and 97 pM, respectively. Since normal subjects increase plasma Ang II from 4 to 20–22 pM with exercise, standing, or diuretic administration, and patients with severe congestive heart failure can have a plasma Ang II of ~ 25 pM at rest, we suggest that Ang II might facilitate NE release in severe congestive heart failure, especially under conditions of stress. (*J. Clin. Invest.* 1994. 93:684–691.) Key words: congestive heart failure • angiotensin-converting enzyme inhibitors • [^{3}H]-norepinephrine kinetics • norepinephrine spillover

Introduction

Excessive activation of the sympathetic nervous system (SNS)[1] and renin-angiotensin system is well documented in congestive heart failure (CHF) and may contribute to progression of the disease (1–5). Plasma NE has been used as a marker for SNS activity and correlates with increased peroneal sympathetic efferent nerve traffic measured by microneurography (6, 7). [^{3}H]NE kinetic studies have demonstrated that the elevated plasma NE is equally contributed to by an increase in NE spillover and a decrease in NE clearance (8–10). Plasma renin activity is also elevated but is more variable depending on the severity of heart failure, and the degree of compensation, treatment, and volume status (11–17). Circulating angiotensin II (Ang II) levels are difficult to measure accurately, but they are also elevated in advanced CHF (12, 14).

Interaction of the SNS and renin-angiotensin system has been demonstrated to occur at multiple levels (18–20). One site of interaction is the peripheral sympathetic neuroeffector junction, where NE release is thought to be facilitated by prejunctional Ang II receptors (21–26). It has been widely proposed that one of the mechanisms for the efficacy of angiotensin-converting enzyme (ACE) inhibitors in CHF is the inhibition of the effect of Ang II to facilitate NE release, especially in the heart (5, 14, 15, 17). Prejunctional Ang II receptors have been demonstrated in tissue preparations (21–23) and in animal models (24–27), but have been very difficult to demonstrate in humans.

Webb et al. (28) demonstrated that brachial artery infusion of subpressor doses of Ang II augmented the sympathetically mediated vasoconstriction induced by lower body negative pressure (LBNP). They concluded that there was a prejunctional effect because there was no change in forearm blood flow with Ang II infusion alone, and the vasoconstrictor effect of intraarterial NE was not enhanced by coadministration with Ang II. However, this technique is indirect, since their measurements were limited to the postjunctional effects of the drugs.

Recently, Goldsmith and Hasking (29), using a more direct [^{3}H]NE kinetic technique to measure NE release, were unable to demonstrate increased systemic NE spillover by intravenous infusion of Ang II during rest or 60° upright tilt. Failure to demonstrate enhanced NE release may have been related to multiple factors: (*a*) systemic (intravenous) infusion of Ang II may not result in high enough Ang II concentrations; (*b*) the measurement of systemic NE kinetics from venous blood may not be a sensitive enough technique; (*c*) an increase in perineuronal NE concentration secondary to Ang II may cause negative feedback inhibition to decrease further NE release via stimulation of prejunctional alpha$_2$ receptors; (*d*) the additional NE released through the facilitation by Ang II would be subject to the avid neuronal uptake system which may mask the primary effect; and (*e*) a decrease in regional blood flow caused by Ang II may retard the diffusion of NE out of the tissue and may also mask an effect.

In light of the inconclusive and indirect data regarding the existence and activity of prejunctional Ang II receptors in humans, and the potential importance of this system as a target

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1. *Abbreviations used in this paper:* ACE, angiotensin-converting enzyme; Ang II, angiotensin II; CHF, congestive heart failure; CL, clearance; CVP, central venous pressure; DHBA, dihydroxy benzylamine; FBF, forearm blood flow; Fex, fractional extraction; FPF, forearm plasma flow; LBNP, lower body negative pressure; SNS, sympathetic nervous system; SO, spillover.

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for therapy of CHF, we performed this study to test the hypothesis that these receptors do exist in the human forearm and can facilitate the release of NE. The study was designed to circumvent the problems mentioned above. To enhance the ability to demonstrate a facilitatory effect of Ang II, a state of heightened sympathetic tone was first induced by cardiopulmonary baroreceptor unloading produced by LBNP (−15 mmHg). Cardiopulmonary baroreceptor unloading increases sympathetic nerve activity to skeletal muscle (30–33). We used a regional infusion of Ang II into the brachial artery to achieve plasma Ang II concentrations calculated to be at both physiologic and pathologic levels. We used [^{3}H]NE kinetic methodology to examine the effects of Ang II on forearm as well as systemic NE spillover and clearance (34). We also maintained a markedly increased forearm blood flow by the simultaneous intraarterial infusion of nitroprusside to facilitate NE washout into the circulation (35) and to minimize the masking of an effect by local modulating factors such as neuronal NE reuptake and prejunctional alpha$_2$ autoinhibition.

Methods

Subjects. We studied 12 normal male volunteers with a mean age of 22.8±0.8 yr (±SEM) (range 20–31 yr). No subject enrolled in the study had a history of hypertension, cardiac disease, or thyroid disease. No medication or substance that might have altered SNS activity was being used by any volunteer. The protocol was reviewed and approved by the clinical investigation committee of The Milton S. Hershey Medical Center. Informed consent was obtained for each individual.

Instrumentation. Subjects were comfortably positioned supine in an LBNP chamber. Polyethylene wrap was used to obtain an airtight seal at the level of the iliac crests. All studies were performed in a temperature controlled, partially darkened, quiet room. Using the modified Seldinger technique and 2% Xylocaine anesthesia (Astra Pharmaceutical Products, Inc., Westboro, MA), a 10.8-cm, 20-gauge catheter (Arrow International, Reading, PA) with an in-line 0.018-in spring wire was positioned in the brachial artery of the nondominant arm for sampling arterial blood and for infusion of Ang II and nitroprusside. A 1.25-in 20-gauge venous cannula was inserted antegrade in the ipsilateral antecubital vein, and the tip was positioned in the brachial vein 10 cm above the forearm crease. This was used for sampling of mixed venous brachial blood from the study forearm. After inflation of a wrist cuff (see below), the sample was reflective of blood draining the entire forearm, except for cutaneous veins emptying into the cephalic vein (36). The contralateral antecubital vein was cannulated with a 30-cm 16-gauge catheter (Arrow International), and the tip was positioned in the thorax for infusion of [^{3}H]NE and for intermittent measurement of central venous pressure. Since there was a continuous infusion through the intraarterial catheter, systemic blood pressure and heart rate were measured by an automated sphygmomanometer (Dinamap; Critikon, Tampa, FL) on the dominant arm. The electrocardiogram was monitored continuously by chest electrodes. Respirations were monitored with a pneumograph. Forearm blood flow in the study arm was measured by strain-gauge plethysmography (see below).

Protocol. After complete instrumentation, the subjects rested quietly for 30 min. When the subject achieved stable baseline hemodynamic parameters, simultaneous arterial and venous blood samples were obtained for determination of baseline plasma NE and to provide plasma for determination of [^{3}H]NE recovery by alumina (Al_2O_3) adsorption (9, 36). [^{3}H]NE was prepared for each subject just before use by diluting sterile pyrogen-free L-[ring 2,5,6-^{3}H]NE (Dupont New England Nuclear, Boston, MA) of high specific activity (~ 45 Ci/mmol) in 0.9% saline containing ascorbic acid (2 mg/ml). The [^{3}H]NE intravenous infusion was initiated with a bolus of 15 μCi/m^2 over a period of 5 min, followed by a constant infusion of 0.7 μCi/min · m^2 for 90 min. The initial 30-min infusion of [^{3}H]NE was to achieve a steady state concentration of plasma [^{3}H]NE. Intraarterial infusion of sodium nitroprusside (Roche Laboratories, Nutley, NJ) was begun simultaneously to achieve a forearm flow of ~ 10 ml/min · 100 ml forearm. The gain of the plethysmograph was initially fixed so that this flow would be equivalent to a measured angle of ~ 45°. The nitroprusside was adjusted through the remainder of the study to maintain this level of flow.

After the initial 30-min equilibration period, LBNP (−15 mmHg) was begun and maintained constant through three consecutive 20-min study periods. During these three study periods, the following intraarterial infusions were administered to eight subjects at flow rates of 400 μl/min: control, LBNP alone (0.9% saline); low Ang II (4 ng/min); and high Ang II (16 ng/min). Four subjects received only intraarterial saline infusions (400 μl/min) during the three study periods. The Ang II concentrations selected were based on pilot studies and were designed to achieve calculated plasma Ang II levels in the physiological exercise and CHF ranges. Forearm volume, measured by water displacement, was 1,164±27 ml. Ang II (Peninsula Laboratories, Inc., Belmont, CA) was prepared fresh for each study from an individual aliquot of stock solution (1 μg/ml) by dilution with 0.9% saline. Aliquots of sterile stock solution were stored at −70°C after preparation.

During the last 10 min of each study period, the wrist cuff was inflated to suprasystolic pressure to occlude blood flow to the hand. 2 min later a venous blood sample was drawn for measurement of plasma NE and [^{3}H]NE. Forearm blood flow was then measured, and arterial and venous blood samples were drawn for measurement of NE, [^{3}H]NE, and Ang II. The volume of blood withdrawn was replaced with an equal volume of saline to prevent changes in blood volume, which might produce a change in central venous pressure and the level of cardiopulmonary baroreceptor afferent nerve activation.

LBNP technique. Isolated cardiopulmonary baroreceptor unloading was produced by applying −15 mmHg pressure to the lower body, which was enclosed in a negative pressure airtight chamber. The pressure within the chamber was continuously monitored with a pressure transducer (American Edwards Laboratories, Irvine, CA). This was positioned ~ 15 cm below heart level so the transducer was always exposed to a positive catheter pressure. It has been previously demonstrated that LBNP of −15 mmHg produces a decrease in central venous pressure without associated alterations in mean arterial pressure, aortic pulse pressure, or heart rate, thereby avoiding significant activation of arterial (carotid and aortic) baroreceptors (30).

Forearm blood flow technique. Forearm blood flow (milliliters per minute · 100 ml) was measured in the study arm by the venous occlusion technique using a mercury-in-silastic single strand strain-gauge plethysmograph (37). The arm was extended with the elbow and wrist supported and the midforearm at midheart level. The strain-gauge was externally calibrated at a force of 10 *g* and was positioned on the nondominant forearm 10 cm below the olecranon process. All flow measurements were performed with the wrist cuff inflated to suprasystolic pressure (240 mmHg) using intermittent venous occlusions to 50 mmHg.

Analysis of NE and [^{3}H]NE. All blood samples were immediately placed in prechilled tubes with 1/50 vol of a solution containing 90 mg/ml EGTA and 60 mg/ml of reduced glutathione. The tubes were immediately chilled and centrifuged, the plasma was stored in aliquots and frozen (−70°C) within 15 min of blood drawing, and analyses for NE and [^{3}H]NE were performed within 1 mo.

Plasma NE concentration was determined by HPLC with electrochemical detection after alumina adsorption and extraction with perchloric acid as described previously by our group (38). All plasma samples for each individual were run the same day in duplicate. Duplicate extracted samples with known concentrations of NE and the internal standard dihydroxybenzylamine (DHBA) were run simultaneously for determination of standard recoveries. NE and DHBA were separated by reverse-phase HPLC using an 8-cm HR-80 column (ESA, Inc., Bedford, MA) packed with 3-μm spherical octadecylsilane. Mobile phase was delivered at 1.5 ml/min by a solvent delivery module

(model 5700; ESA, Inc.) and contained the following in 1 liter: methanol (30 ml), 1-heptanesulfonic acid (0.25 g), Na_2EDTA (0.09 g), and monobasic sodium phosphate (6.9 g) adjusted to pH 3.2. NE and DHBA were measured coulometrically using a series of three conditioning/detector cells (models 5021 and 5011; ESA, Inc.) set at the following potentials: +0.35, +0.10, and −0.26 V. Peak heights were determined by an integrator (model SP 4270; Spectra-Physics Analytical, San Jose, CA).

The [^{3}H]NE concentration was determined from separate duplicate 4-ml plasma samples by alumina adsorption as previously described (9). A sample of the perchloric acid extract was added to scintillation vials containing 10 ml scintillation cocktail (BCS; Amersham Corp., Arlington Heights, IL). Samples were counted in a liquid scintillation spectrometer (model 6800; Beckman Instruments, Inc., Fullerton, CA) after dark adaptation. Quench correction was by H number.

A portion of the [^{3}H]NE infusate was frozen at the end of each study and was stored for analysis at the time the plasma alumina procedure was performed. The infusate sample was used to determine the [^{3}H]NE recovery during the alumina procedure. A portion of the infusate was added to duplicate 4-ml baseline plasma samples that were extracted and counted (9).

To ensure that the [^{3}H]NE in plasma samples was stable during storage, we spiked a plasma pool with [^{3}H]NE infusate and prepared multiple aliquots which were frozen and stored under the same conditions as study samples. Four aliquots were not frozen but were subjected to immediate alumina adsorption and HPLC separation. The peaks for NE and 3,4-dihydroxyphenylglycol (DHPG) were collected and counted. Four samples were thawed and analyzed similarly after storage for 10, 22, and 40 d. The [^{3}H]NE disintegrations per minute (dpm) per milliliter of plasma were not significantly different for the four time periods ($P = 0.72$ by ANOVA). Taking the [^{3}H]NE radioactivity in the fresh samples as 100%, the [^{3}H]NE in the 10-, 22-, and 40-d samples was 95.5, 96.4, and 98.6%, respectively. The [^{3}H]-DHPG in the samples at the four time points was 0.93, 1.04, 0.91, and 0.98% of the [^{3}H]NE radioactivity.

NE kinetic calculations. Systemic NE clearance (liters per minute · square meters) and spillover (nanomoles per minute · square meters) were determined by a modification of the technique described by Esler et al. (34). At steady state, systemic NE clearance is equal to the steady state [^{3}H]NE infusion rate divided by the actual [^{3}H]NE plasma concentration. Systemic NE spillover is calculated as the product of NE clearance and plasma NE concentration. Since NE kinetics calculated from brachial venous plasma samples reflect a significant contribution from the forearm circulation and would reflect a local Ang II effect, separate calculations of systemic kinetics were made using arterial and venous samples. Data for clearance and spillover were normalized to body surface area or 100 ml of forearm.

To insure that a steady state was present at the end of each study period, the radioactivity (dpm) of the two plasma venous samples were compared by the Student's paired t test and were found not to be significantly different. For the 20- and 30-min control samples, this was 1,033±45 and 1,042±58 dpm, $P = 0.86$. For LBNP alone, the data were 1,140±19 and 1,177±52 dpm, $P = 0.35$; for LBNP plus Ang II (4 ng/min), 1,131±64 and 1,235±49, $P = 0.20$; and for LBNP plus Ang II (16 ng/min), 1,243±58 and 1,297±68, $P = 0.21$.

Although [^{3}H]NE rerelease occurs from sympathetic nerve terminals, the contribution to the overall pool for our purposes was considered to be negligible. To determine if the [^{3}H]NE taken up by sympathetic neurons could be rereleased and could contribute significantly to the forearm venous or arterial plasma NE and radioactivity, three additional subjects received a 30-min infusion of [^{3}H]NE. Three forearm venous and arterial plasma samples were taken over the last 10 min of infusion (period A). The [^{3}H]NE infusion was terminated, and three more samples were drawn 20, 25, and 30 min later (period B). Three more samples were drawn during 15 min of LBNP at −15 mmHg (period C), then again during 15 min of LBNP at −40 mmHg (period D). LBNP increased both forearm venous and systemic arterial NE (venous: A, 198; B, 242; C, 281; D, 511 pg/ml. arterial: A, 169; B, 171; C, 200; D, 306 pg/ml). LBNP did not increase plasma radioactivity (venous: A, 732; B, 108; C, 75; D, 71 dpm. arterial: A, 2,737; B, 92; C, 78; D, 74 dpm).

Forearm NE kinetic calculations. To evaluate the local effect of intraarterial Ang II on neuronal NE release, regional (forearm) NE kinetic calculations were performed. Forearm NE clearance and spillover were calculated from the arterial and venous plasma NE concentrations, the fractional extraction of [^{3}H]NE (Fex[^{3}H]NE), and forearm plasma flow (FPF) (34). Fex[^{3}H]NE was derived from the [^{3}H]NE radioactivity of arterial and venous blood as follows: $\text{Fex}[^3\text{H}]\text{NE} = ([^3\text{H}]\text{NE}_A - [^3\text{H}]\text{NE}_V)/[^3\text{H}]\text{NE}_A$. FPF was derived from the forearm blood flow (FBF) and hematocrit as follows: FPF = FBF · (1 − hematocrit). The equations for forearm NE spillover and clearance are the following:

$$\text{Forearm NE spillover} = \{(\text{NE}_V - \text{NE}_A) + (\text{NE}_A \cdot \text{Fex}\,[^3\text{H}]\,\text{NE})\} \cdot \text{FPF, and}$$

$$\text{Forearm NE clearance} = \text{Fex}\,[^3\text{H}]\text{NE} \cdot \text{FPF.}$$

Measurement of plasma Ang II concentration. Blood for Ang II analysis was collected into prechilled glass tubes containing 0.5 ml of inhibitor solution (2% ethanol, 0.025 M phenanthroline, 0.125 M Na_2EDTA, 2 g/liter neomycin). The blood was immediately chilled and centrifuged, and the plasma was stored at −70°C. Frozen plasma was shipped on dry ice to Lausanne by overnight air to measure specifically Ang II [angiotensin-(1-8) octopeptide as described previously (39)]. Briefly, the procedure involves extraction of peptides from plasma by reversible adsorption to phenylsilyl-silica. Then, the Ang II was isolated by isocratic reversed-phase HPLC and was quantified by radioimmunoassay. The detection limit of this assay is 0.4 pM.

Statistics and data analysis. All NE kinetic data represent the average of two separate venous blood samples and a single arterial sample obtained during the last 5 min of each study period. All hemodynamic data were averaged from multiple measurements obtained during the last 7 min of each study period. Forearm blood flow data were collected during the last 5 min of each study period and were averaged from at least eight high quality flow curves.

All statistical analyses were performed using a one- or two-way ANOVA for repeated measures. Determination of significance between measurements from baseline and each intervention were made using the Student-Newman-Keuls posthoc analysis (40), when a significant F value was obtained. All data are expressed as mean±SEM. A significant P value was < 0.05.

Results

Hemodynamic data for the Ang II group are presented in Table I. LBNP produced no significant change in mean arterial pressure, and it did not change during the Ang II infusions. LBNP produced no change in heart rate; however, a very small but statistically significant increase occurred during the two Ang II infusions. LBNP reduced central venous pressure (CVP) by 1.9±0.4 mmHg from control ($P < 0.05$). CVP was similarly reduced from control during both Ang II infusions by 2.4±0.5 and 2.6±0.5 mmHg; the reduction in CVP was similar during all three LBNP periods, $P = 0.3$. No significant hemodynamic changes occurred in the saline group. For the four periods mean arterial pressure was 89.8±1.7, 84.5±3.9, 87.8±2.3, and 88.8±3.0 mmHg ($P = 0.58$), and heart rate was 56.2±3.2, 57.5±5.4, 63.5±4.4, and 60.6±2.4 min^{-1} ($P = 0.11$).

The goal of regulating forearm blood flow at 10 ml/min · 100 ml (approximately three to four times normal) by the intraarterial infusion of nitroprusside was successfully ac-

Table I. Hemodynamics and NE Data (Mean±SEM) under Control Conditions before LBNP, during LBNP Alone at −15 mmHg, and during LBNP Plus Ang II Infusion into the Brachial Artery at Low and High Concentrations (n = 8)

	Control	LBNP alone	Ang II (4 ng/min)	Ang II (16 ng/min)
MAP (*mmHg*)	78±2	79±2	80±3	83±3
Heart rate	53±1	54±1*	56±2‡	57±2‡
FBF	10.0±0.7	10.2±0.8	10.2±1.0	9.9±1.2
Arterial NE	123±17	163±20§	163±20§	170±23§
Venous NE	138±21	173±23§‖	189±26§‖	224±33§

FBF, milliliters per minute · 100 ml forearm tissue; NE, in picograms per milliliter. ‡ $P < 0.05$ and § $P < 0.01$ vs control; * $P < 0.05$ and ‖ $P < 0.01$ vs Ang II (16 ng/min).

complished (Table I). When the FBF data were evaluated by two-way ANOVA, there was no significant difference between any of the four periods (time effect: $P = 0.37$), or between the Ang II and saline infusion groups (group effect: $P = 0.67$).

When the dose of nitroprusside was analyzed similarly, there was a significant time effect ($P = 0.003$) over the four periods. Post hoc analysis revealed that the dose of nitroprusside had to be increased over time to keep forearm blood flow constant in the Ang II group ($P < 0.001$; nitroprusside doses: 3.3, 4.8, 6.6, and 10.4 ng/min), but not in the saline group ($P = 0.47$; nitroprusside doses: 3.3, 5.0, 5.5, and 6.7 ng/min).

Arterial and venous plasma NE data for the Ang II group are illustrated in Table I and Fig. 1. All subjects had normal control arterial (123±17 pg/ml) and venous (138±21 pg/ml) plasma NE levels. There was a significant increase in arterial plasma NE (163±20 pg/ml; $P < 0.01$) with LBNP alone, suggesting systemic stimulation of the SNS. There was no further increase in arterial plasma NE with either the low (4 ng/min) or high (16 ng/min) dose of intraarterial Ang II. The venous plasma NE also increased significantly (173±23 pg/ml; $P < 0.01$) with LBNP. With low dose Ang II, venous NE was significantly higher than control (189±26 pg/ml; $P < 0.01$) but not LBNP. With high dose Ang II, venous NE was significantly higher than each of the three previous values (224±33 pg/ml; $P < 0.01$). This suggested that there was a local effect of the intraarterial Ang II on either forearm NE release or forearm NE clearance.

In the saline group LBNP increased arterial and venous NE, but these values did not change further during the three subsequent 20-min periods. (arterial: 134±18, 175±12, 173±13, and 184±29 pg/ml. venous: 136±40, 178±23, 180±19, and 180±21 pg/ml).

Systemic NE kinetics were calculated from both arterial and venous plasma. The [^{3}H]NE samples for the control period were lost for one subject in the Ang II group. In the remaining seven, control systemic NE spillover (SO) was 0.68±0.11 and 1.44±0.22 nmol/min · m^2, respectively, for arterial and venous samples. Comparable systemic clearance data was 0.89±0.04 and 1.63±0.09 liters/min · m^2. Since the principal question to be answered was whether Ang II altered NE SO or NE clearance (CL), the ANOVA statistical analysis was performed on the data from the three time periods (LBNP alone, LBNP plus low dose Ang II, LBNP plus high dose Ang II) for the entire group of eight subjects. These data are presented in Fig. 2 and Table II. The systemic arterial NE SO and CL during LBNP were unchanged by intraarterial Ang II. The venous systemic NE SO progressively rose with intraarterial Ang II during LBNP, and was significantly greater during high dose Ang II. Ang II produced no significant change in venous systemic NE CL. The data for systemic NE SO are consistent with the changes in arterial and brachial venous plasma NE concen-

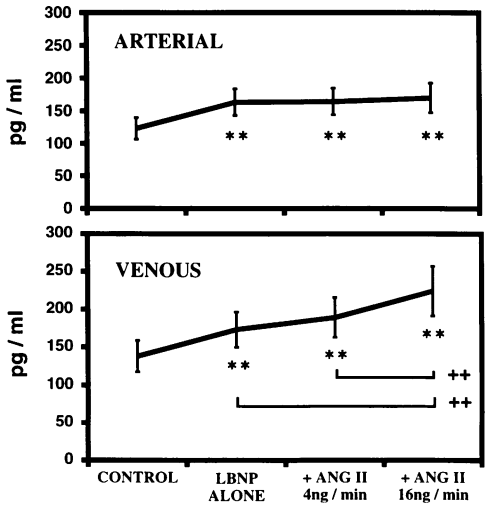

Figure 1. Arterial plasma norepinephrine (*top*) (mean±SEM) and brachial venous plasma norepinephrine (*bottom*) under control conditions, during LBNP at −15 mmHg alone, LBNP plus low dose Ang II (4 ng/min), and LBNP plus high dose (16 ng/min) Ang II infusion into the ipsilateral brachial artery. ** $P < 0.01$ vs control; ++ $P < 0.01$ between bracketed values.

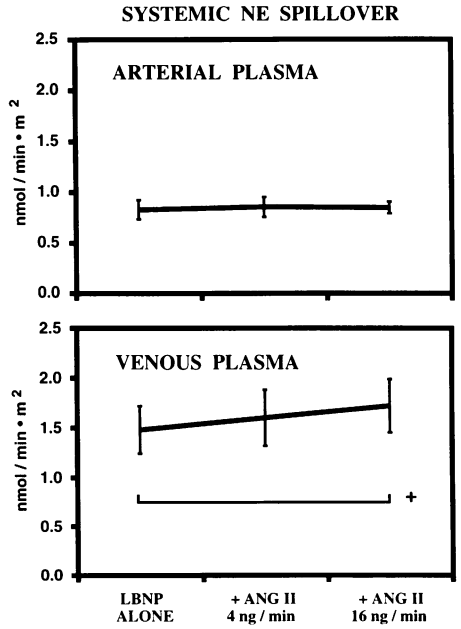

Figure 2. Systemic NE spillover calculated from arterial plasma, representing the systemic circulation (*top*), and brachial venous plasma (*bottom*) which also reflects NE dynamics in the experimental forearm. + $P < 0.05$.

Table II. Systemic and Forearm NE Kinetic Data during LBNP Alone at −15 mmHg, and during LBNP Plus the Brachial Artery at Low and High Concentrations

	LBNP alone	Ang II (4 ng/min)	Ang II (16 ng/min)
Arterial NE CL (*liters/min · m²*)	0.88±0.07	0.90±0.06	0.85±0.06
Arterial NE SO (*nmol/min · m²*)	0.83±0.10	0.86±0.10	0.84±0.12
Venous NE CL (*nmol/min · m²*)	1.43±0.09	1.41±0.07	1.29±0.05
Venous NE SO (*nmol/min · m²*)	1.48±0.24*	1.59±0.28	1.72±0.27
Forearm NE CL (*ml/min · 100 ml tissue*)	2.06±0.20	1.90±0.15	1.76±0.19
Forearm NE SO (*pg/min · 100 ml tissue*)	384±65‖	439±67*	560±85
Fex	0.38±0.04	0.35±0.04	0.34±0.04

Systemic NE SO and CL are calculated from arterial and brachial venous plasma samples. Fex is the fractional extraction of [³H]NE by the forearm ($n = 8$). * $P < 0.05$ and ‖ $P < 0.01$ vs Ang II (16 ng/min).

trations, and suggest that intraarterial infusion of Ang II enhances neuronal NE release in the forearm. In the four subjects in whom intraarterial saline was infused, there was no significant changes in any variable during the three 20-min periods of continuous LBNP (Table III).

Regional (forearm) NE kinetic data for the Ang II group are presented in Table II and Fig. 3. There was a progressive rise in forearm NE SO from LBNP alone to LBNP plus low dose Ang II infusion and LBNP plus high dose Ang II infusion. These data parallel the rise in venous plasma NE. The forearm NE SO

Table III. Systemic and Forearm NE Kinetic Data during Three Consecutive 20-min Periods after Instituting LBNP at −15 mmHg

	LBNP plus intraarterial saline		
	Period 1	Period 2	Period 3
Arterial NE CL (*liters/min · m²*)	0.88±0.03	0.87±0.02	0.88±0.06
Arterial NE SO (*nmol/min · m²*)	0.91±0.08	0.90±0.08	0.97±0.19
Venous NE CL (*nmol/min · m²*)	1.40±0.09	1.42±0.08	1.32±0.04
Venous NE SO (*nmol/min · m²*)	1.35±0.38	1.50±0.23	1.40±0.20
Forearm NE CL (*ml/min · 100 ml tissue*)	1.92±0.24	2.05±0.37	1.70±0.32
Forearm NE SO (*pg/min · 100 ml tissue*)	357±133	375±80	286±37
Fex	0.36±0.04	0.38±0.04	0.33±0.04

During each period saline (0.9%) was infused into the brachial artery. Systemic NE SO and CL are calculated from arterial and brachial venous plasma samples. Fex is the fractional extraction of [³H]NE by the forearm. There were no significant changes. ($n = 4$).

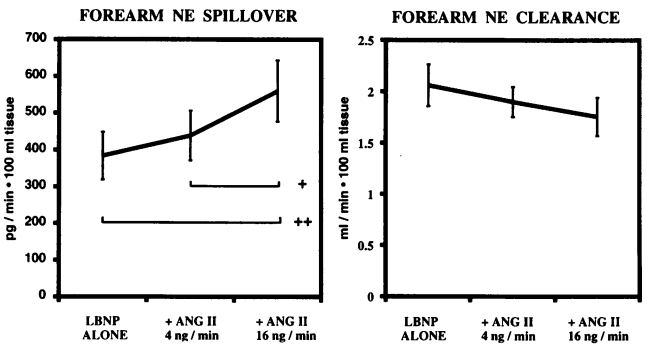

Figure 3. Forearm NE spillover and clearance. $^{+}P < 0.05$ and $^{++}P < 0.01$.

with high dose Ang II infusion was significantly greater than with LBNP plus low dose Ang II infusion and LBNP alone. Although there was a downward trend in forearm NE CL, these changes were not statistically significant. In the group in whom saline was infused instead of Ang II, no significant differences were seen in forearm NE kinetic variables (Table III).

When the data for arterial and venous plasma Ang II concentrations under basal condition (artery: 2.11±0.32; vein: 2.44±0.26 pM) and during LBNP (artery: 3.08±0.42; vein: 1.32±0.47) were evaluated by two-way ANOVA, there was a significant interaction ($P = 0.016$). Post hoc analysis revealed that LBNP increased systemic arterial Ang II ($P = 0.07$), and resulted in a significant arterial venous Ang II difference ($P = 0.017$). This suggests that there was significant consumption of Ang II across the forearm circulation during activation of the SNS by cardiopulmonary baroreceptor unloading. During intraarterial infusion of Ang II at 4 and 16 ng/min, venous plasma Ang II increased to 25.3±3.7 and 96.6±14.2 pM, respectively.

Discussion

This study was performed to test the hypothesis that prejunctional Ang II receptors exist in humans and function to facilitate the neuronal release of NE. To enhance the ability to demonstrate this effect, the study was performed in a state of heightened sympathetic tone induced by cardiopulmonary baroreceptor unloading using LBNP at −15 mmHg. Forearm blood flow was also maintained at approximately three to four times normal by the simultaneous intraarterial infusion of nitroprusside. This was done to facilitate NE washout into the circulation and to minimize masking of an Ang II effect, which might not be seen if the higher interstitial NE concentration was subjected to enhanced neuronal uptake, or if high local NE inhibited further NE release by activation of prejunctional alpha$_2$ receptors. The major findings from our data are the following: (*a*) both arterial and venous plasma NE concentration increased with LBNP, however, arterial NE remained constant while venous NE continued to increase with incremental intraarterial Ang II infusions; (*b*) during LBNP systemic NE spillover calculated from arterial blood samples was unchanged by regional Ang II infusion, whereas NE spillover calculated from venous plasma increased significantly with the high dose intraarterial infusion of Ang II; and (*c*) forearm NE spillover was increased significantly by high dose intraarterial Ang II infusion during steady state cardiopulmo-

nary baroreceptor unloading. To our knowledge these are the first data to demonstrate directly that prejunctional Ang II receptors regulating NE release are both present and functional in humans.

The increase in arterial NE concentration with LBNP provides evidence of a systemic increase in sympathetic tone resulting from cardiopulmonary baroreceptor disengagement. We have shown previously that the increase in arterial NE with LBNP is primarily because of a significant decrease in arterial NE clearance, with an increase in arterial NE spillover that was found not to be significant (41). Since the decreased clearance is related to a vasoconstriction that reduced cardiac output (42), we have suggested that this increase in arterial NE is an indirect reflection of SNS activation. Thus, we achieved the desired amplification of sympathetic activity with LBNP.

To accept the conclusion that locally administered Ang II facilitated local NE release, it is important to demonstrate that it was the local Ang II that caused the local changes we observed and not a changing level of systemic sympathetic activation. The significant reduction in central venous pressure caused by LBNP alone tended to be greater during Ang II but this was not significant. Mean arterial pressure tended to rise but this too was insignificant. Heart rate was slightly but significantly higher during the high Ang II infusion. However arterial NE, NE clearance, and NE spillover were all unchanged during the three LBNP periods. This was also true for the control subjects who only received intraarterial saline. Moreover, all indices of local forearm NE release increased significantly with local Ang II infusion (Table II) and did not change with local saline infusion (Table III). The progressive rise in venous NE concentration with the addition of intraarterial Ang II suggests that a local effect was being produced in the regional forearm circulation. NE spillover calculated from venous plasma, reflecting a regional effect, increased with high dose intraarterial Ang II. Forearm NE spillover increased in a similar fashion, suggesting that regional infusion of Ang II at 16 ng/min facilitated the neuronal release of NE.

Although our data imply that intraarterial Ang II facilitated neuronal NE release, they have to be interpreted with caution. One of the inherent limitations of NE kinetic methodology is that NE spillover only reflects that fraction of released NE that escapes neuronal reuptake and spills over into the circulation (34). It is possible that Ang II could have altered the reuptake/spillover fraction. It has been recently noted that regional blood flow is an important regulator of spillover (43). As forearm blood flow was increased with intraarterial nitroprusside, Grossman et al. (43) found that forearm NE fractional extraction declined, and calculated NE spillover increased. This was one reason we held forearm flow constant during LBNP and Ang II infusions by adjusting the rate of nitroprusside infusion. As expected, significantly more nitroprusside was required to hold flow constant in the Ang II group than in the control group receiving a continuous saline infusion. Although blood flow was not a factor in our study, we cannot exclude an Ang II or nitroprusside effect on spillover independent of flow. A second related limitation for kinetic methodology to be valid is that a steady state must be present. Using a bolus of tracer before constant infusion allowed us to reach a steady state more quickly than an infusion alone. Although we determined that the plasma radioactivity in two plasma samples had achieved a stable plateau during each experimental period, it is likely that the tracer specific activity in the interstitial space was lower than the circulation. It is impossible to exclude a drug effect that might change this relationship independent of an effect on NE spillover. However, it is likely that the most important factor that would cause this to happen is a changing blood flow, which we held constant. A third limitation of this technique is that it assumes unidirectional transport of tracer into the nerves from the circulation. To test the possibility that there is a rerelease of [^{3}H]NE, we infused [^{3}H]NE for 30 min, allowed it to clear from the circulation for 30 min, and attempted to release [^{3}H]NE from the nerves into forearm and systemic circulations by a mild, then moderate stress. Although LBNP at -15 and -40 mmHg increased forearm venous and systemic arterial NE twofold, no increase in plasma radioactivity was detected. Thus despite the limitations of [^{3}H]NE kinetic methodology in humans, we have attempted to control the major factors that could have spuriously affected our data.

The existence of prejunctional Ang II receptors on peripheral sympathetic neurons has been well documented experimentally (21–27). These receptors facilitate the neuronal release of NE when stimulated. In an isolated perfused rabbit heart model Starke et al. (21) reported that 1,300 pM Ang II produced maximal facilitation of NE release, a concentration that was 10 times threshold. Starke's group (22) later noted that angiotensin concentrations of 2.0–20 nM produced approximately a twofold increase in NE spillover. These concentrations are much higher than true Ang II found in human plasma under basal conditions (2–5 pM), with nonhypotensive LBNP ($\sim$ 8 pM), with exercise (20–22 pM), or in patients with CHF ($\sim$ 25 pM) (5, 12, 14, 43, 44). In one subject who experienced vasovagal syncope during LBNP, Ang II rose to $\sim$ 45 pM (45).

Randall and Zimmerman (27) studied the effects of acute and chronic ACE inhibition on the blood pressure and vascular conductance response to sympathetic nerve stimulation in anesthetized rabbits, a subgroup of which underwent bilateral nephrectomy. They demonstrated that chronic ACE inhibition attenuated the vasoconstrictor response, which could be restored with a 24-h infusion of Ang II (1 μg/kg·h). This suggested that the renin-angiotensin system potentiated the effect of sympathetic nerve stimulation by Ang II acting at a prejunctional receptor. This study, however, used an indirect index of sympathetic activity. Using similar techniques Hilgers et al. (46) demonstrated these receptors in a rat skeletal muscle preparation. Schwieler et al. (47) failed to confirm their existence in a canine gracilis muscle preparation, however, they did not employ NE kinetic techniques. Finally, Majewski (26) used a radiotracer infusion of [^{3}H]NE to calculate the NE release rate in response to spinal sympathetic electrical stimulation in a pithed rat model. Infusion of Ang II at a rate of 0.1 μg/kg·min had no effect on NE release rate because of the very high endogenous plasma renin activity, however, a higher dose (1.0 μg/kg·min) produced a marked increase in the NE release rate, presumably from a direct effect on chromaffin tissue independent of the prejunctional Ang II receptor. The administration of captopril and the Ang II blocking agent saralasin, however, decreased the NE release rate. After nephrectomy, low dose Ang II infusion (0.1 μg/kg·min) did appear to activate facilitatory prejunctional Ang II receptors.

It has been difficult to demonstrate the existence of these prejunctional Ang II receptors in humans. Most attempts have used indirect techniques. Webb et al. (28) infused Ang II in the brachial artery during LBNP and concomitantly with NE. Ang

II augmented the vasoconstriction induced by LBNP, but did not alter the vasoconstriction due to simultaneously infused intraarterial NE. They concluded that Ang II produced a prejunctional interaction causing an increase in neuronal NE release. Seidelin et al. (48) found no effect of intravenous Ang II on plasma NE levels in humans at rest or during exercise.

The only studies in humans using a direct technique to assess NE release is from Goldsmith's laboratory (29, 49). They used the radiotracer NE kinetic technique similar to ours, however, they evaluated systemic rather than regional kinetics. They found that intravenous Ang II infusion in subpressor doses 2 ng/kg · min did not alter NE spillover under basal conditions or during baroreceptor-stimulated sympathetic nerve activity in normals (29). A pressor dose (5 ng/kg · min) was without effect on NE spillover in patients with heart failure (49). It is unlikely that the plasma concentration of Ang II was too low to demonstrate an increase in systemic NE spillover (50). Therefore it is more likely that the use of systemic (intravenous) infusion of Ang II and measurement of systemic NE kinetics may not have had the power to demonstrate an Ang II effect on NE release when compared with vehicle infusion on a different day.

In our study, we used a regional infusion of Ang II into the brachial artery. This allowed us to increase Ang II locally to clinically significant levels without causing a general pressor response or producing a central nervous system effect. We saw a regional effect of intrabrachial arterial Ang II infusion in the forearm with each of the three indices of forearm NE release we used: there was an increase in forearm venous NE concentration, an increase in systemic NE spillover calculated from forearm venous plasma samples, and lastly, an increase in forearm NE spillover. The key to our success in demonstrating this effect was to markedly increase forearm blood flow by concomitantly infusing intraarterial nitroprusside. This served to eliminate the following three factors which might mask the Ang II facilitation of NE release: (*a*) decreased NE release via stimulation of prejunctional alpha$_2$ adrenergic receptors by higher concentrations of NE in the synaptic cleft; (*b*) neuronal reuptake of the excess released NE; and (*c*) an Ang II-mediated decrease in regional blood flow which retards NE washout into the circulation (35).

The potential implications of our findings depend upon whether this represents a physiologic or pharmacologic effect of Ang II. To determine the pathophysiologic significance of these results, we determined plasma-true Ang II concentration using HPLC methodology to separate the angiotensins and radioimmunoassay to quantify Ang II. We found that LBNP increased arterial Ang II significantly and caused a significant extraction of Ang II by the forearm. The venous plasma concentrations during low dose Ang II (4 ng/min) and high dose Ang II (16 ng/min) intraarterial infusions were 25 and 97 pM, respectively. Normal subjects performing strenuous exercise have plasma Ang II concentrations of ~ 20–22 pM (44), a value often exceeded by patients with severe CHF under basal conditions. Since syncope increased plasma Ang II to ~ 45 pM in a normal volunteer, it is likely that Ang II in ill CHF patients under conditions of stress exceeds the concentration calculated for our high dose Ang II infusion (44). In addition, we have recently measured a true Ang II concentration of 94 pM in a CHF patient at rest. Thus, we suggest that prejunctional Ang II receptors may facilitate NE release at pathophysiologically relevant Ang II concentrations. It is tempting to speculate that one of the mechanisms by which ACE inhibitor therapy produces its beneficial effects in CHF is on blocking Ang II–facilitated NE release under conditions of stress, thereby lessening the toxic effects of NE on the heart and circulation. However, further studies would be required to prove this conclusively.

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