Whole body norepinephrine kinetics in ANG II-salt hypertension in the rat

Andrew J. King,1 Martin Novotny,2 Greg M. Swain,2 and Gregory D. Fink1
Departments of 1Pharmacology and Toxicology and 2Chemistry, Michigan State University, East Lansing, Michigan
Submitted 12 November 2007; accepted in final form 31 January 2008

King AJ, Novotny M, Swain GM, Fink GD. Whole body norepinephrine kinetics in ANG II-salt hypertension in the rat. Am J Physiol Regul Integr Comp Physiol 294: R1262–R1267, 2008. First published February 6, 2007; doi:10.1152/ajpregu.00819.2007.—The purpose of this study was to investigate total body norepinephrine (NE) kinetics as an index of global sympathetic nervous system (SNS) outflow in a rat model of chronic ANG II-salt hypertension. Male Sprague-Dawley rats fed a 0.4% (normal salt, NS) or 2% (HS) NaCl diet were instrumented with arterial and venous catheters. After 5 days of recovery and a 3-day control period, ANG II (150 ng·kg−1·min−1) was given subcutaneously by minipump for 14 days. Plasma NE levels and total body NE spillover and clearance were determined on control day 3 and ANG II infusion days 7 and 14 using radioisotope dilution principles. To perform this analysis, 4H-NE and NE were measured in arterial plasma after a 90-min infusion of tracer amounts of 4H-NE. Mean arterial pressure (MAP) was similar during the control period in NS and HS rats; however, MAP increased to a higher level in HS rats. During the control period, plasma NE tended to be lower in rats on HS, whereas NE clearance tended to be higher in HS rats. As a result NE spillover was similar in NS and HS rats during the control period. In NS rats, plasma NE, NE spillover, and NE clearance were unchanged by ANG II. In contrast, in rats on the HS diet, plasma NE and NE spillover increased during ANG II infusion, whereas NE clearance was unchanged. In conclusion, a HS diet alone or ANG II infusion in animals fed NS do not affect global sympathetic outflow. However, the additional hypertensive response to ANG II in animals fed HS is accompanied by SNS activation.

norepinephrine spillover; norepinephrine clearance; sympathetic nervous system; salt-sensitive hypertension

SYMPATHETIC NERVOUS SYSTEM (SNS) activity is commonly increased in human essential hypertension (29). The most compelling evidence for SNS activation in hypertensive humans comes from direct nerve recordings of muscle sympathetic nerve activity (MSNA) (1, 27, 52) and measurements of whole body and regional norepinephrine (NE) spillover (15, 17, 18, 20, 21). Although SNS activation is also thought to be a common feature of many experimental animal models of hypertension, direct measures of SNS activity are relatively lacking. Indirect evidence for SNS activation includes enhanced depressor responses to ganglion or adrenergic receptor blockade and regional denervation techniques to laboratory animals have been described (31). Angiotensin type 1 (AT1) receptor antagonists have been shown to significantly reduce MSNA in lean and obese hypertensive humans (3, 28), implicating ANG II in the pathogenesis of SNS activation in human hypertension. We have used depressor responses to acute ganglionic blockade with hexamethonium (32, 33) and regional denervation techniques (33), as indirect evidence for sympathetic activation in a rat model of chronic ANG II hypertension. That evidence suggested, however, that sympathoexcitation to ANG II only occurs in rats fed a high-salt diet. Therefore, in the current experiments, we specifically tested the hypothesis that global SNS activity is increased in chronic ANG II-salt hypertension only in rats ingesting a high-salt diet.

METHODS

Animals

All protocols were approved by the Michigan State University All University Committee on Animal Use and Care. Male Sprague-Dawley rats (Charles River Laboratories, Portage, MI), weighing 225 to 250 g at the beginning of the study, were allowed free access to either a 0.4% (n = 8) or 2% NaCl (n = 9) diet (Research Diets, New Brunswick, NJ) and distilled water for 7 days prior to surgery. During this time, rats were housed 3 per cage in a temperature- and humidity-controlled room with a 12:12-h light-dark cycle.

Catheterization

Under isoflurane anesthesia, rats were instrumented with arterial and venous catheters, which were exteriorized into a stainless steel spring attached to the rat by a loosely fitted rubber jacket (Instech, Plymouth Meeting, PA), as described previously (39). The arterial catheter was advanced into the abdominal aorta from the left femoral artery and the venous catheter positioned in the abdominal vena cava via the left femoral vein. Antimicrobial prophylaxis was achieved by...
administration of ticarcillin-clavulanate (200 mg/kg iv) and enrofloxacin (5 mg/kg iv). Carprofen (5 mg/kg sc) was administered for postoperative analgesia. The rats were then loosely tethered in individual plastic cages to allow continuous access to all catheters without handling or disturbing the rat. Ticarcillin-clavulanate (200 mg/kg iv) and enrofloxacin (5 mg/kg iv) were administered daily for the duration of the experiment, and vascular catheters were flushed with heparin saline each day.

**Hemodynamic Measurements**

Cardiovascular measurements were made using our previously published methods (32). Briefly, briefly, systolic, diastolic, and mean arterial pressure (MAP) and heart rate (HR) were recorded at the same time each morning for 30 min by connecting the exteriorized arterial catheter to a pressure transducer that was connected to a digital pressure monitor (Digi-Med BPA-400; Micro-Med, Louisville, KY). The pressure monitoring was linked to a computerized data acquisition program (DMSI-400; Micro-Med). The pressure transducers were calibrated using a sphygmomanometer and zeroed daily using a column of water placed at the level of the rat’s heart.

**NE Kinetics**

Total body NE clearance and spillover were determined by applying radioisotope dilution principles using established methods in the rat (31). To perform this analysis, concentrations of 3H-NE and NE were measured in arterial plasma after a 90-min infusion of tracer amounts of 3H-NE.

**3H-NE infusion.** Levo-[ring-2,5,6-3H]-NE (PerkinElmer, Wellesley, MA) was infused intravenously at 0.13 Ci/kg/hr262 infusion protocol has been shown to produce steady-state plasma amounts of 3H-NE.

**NE concentrations.** After infusion of 3H-NE for 90 min, 1 ml of blood was drawn from the arterial catheter into a 1-ml syringe primed with 25 μl of 0.2 mol/l acetic acid, 50 μl sodium sulfate (100 mg/ml), and 350 μl reduced glutathione (6 mg/ml) to a conical polystyrene tube and mixing it. 3H-NE was then added in amounts based on the weight of the rats (~25 μl for 300 g rats), and 0.9% saline was used to bring the final volume of the solution to 10 ml. A syringe infusion pump was connected to the exteriorized venous catheter to deliver the solution to the rat intravenously.

**Blood sampling.** After infusion of 3H-NE for 90 min, 1 ml of blood was drawn from the arterial catheter into a 1-ml syringe primed with 25 μl (pH = 7) of an EGTA (9 mg/ml) and reduced glutathione (6 mg/ml) solution and placed on ice. The blood was centrifuged at 4°C (14,000 rpm for 15 min), and the plasma was stored at -80°C until analysis.

**NE concentrations.** Catecholamines were determined in duplicate in 100 μl of freshly thawed plasma by batch alumina extraction followed by reverse-phase high-performance liquid chromatography separation with coulometric detection (HPLC-CD). The alumina extraction procedure and analyte quantification were performed using a method modified from the one originally reported by Holmes et al. (30).

**NE extraction.** In a 1.5-ml plastic tube, 100 μl of freshly thawed plasma, 10 mg of acid-washed alumina (EcoChrom MP Alumina A; MP Biomedicals, Eschwege, Germany), 15 μl of 3.4-dihydroxybenzylamine internal standard and 400 μl of 2 M Tris/0.5 M EDTA buffer (pH 8.1) were added. After shaking for 25 min on a vortex mixer, the samples were briefly centrifuged, and the supernatant was discarded. The alumina pellet was then washed with dionized water (18 MΩ), mixed for 15 s, and then again centrifuged; this step was repeated twice. Catecholamines and metabolites were then eluted from alumina with 100 μl of 0.04 M phosphoric acid-0.2 M acetic acid (20:80, vol/vol). The eluate was then directly injected onto the HPLC column (10–40 μl injection). The average extraction recovery of NE was 78.2%.

**HPLC-CD.** HPLC-CD was performed using a commercial system (ESA Biosciences, Chelmsford, MA) consisting of a solvent delivery module (model 584), an autosampler (model 542) cooled to 4°C, and a Coulochem III detector, which was equipped with a 5021A conditioning cell (electrode I) and a 5011A high-sensitivity analytical cell (electrode II and III). Both cells use flow-through porous graphite electrodes. The high surface area of the detection electrodes results in an almost 100% reaction of the electroactive compound. Hydrodynamic voltammograms were obtained to determine the optimum potential for detection. The highest signal-to-noise results were obtained when electrode I was set at +200 mV, electrode II at +100 mV, and electrode III at -280 mV. Chromatograms were obtained by monitoring the reduction current for working electrode III. The catecholamines and metabolites were separated on an HR-80 (3,3-μm particle size, 80-μm length × 4.6 mm ID) reversed-phase column (ESA Biosciences). The mobile phase was a commercial Cat-A-Phase II (ESA Biosciences) that consisted of a proprietary mixture of acetomitrile, methanol, phosphate buffer, and an ion-pairing agent (approximately pH 3.2). The optimum flow rate for the separation was 1.1 ml/min. The separation column was maintained at 35°C. The limit of detection of NE was 19.1 pg/ml.

**3H-NE concentrations.** After HPLC analysis, the NE fraction was collected using a Gilson model 203 fraction collector (Gilson Medical Electronics), and the 3H-NE was quantified using a Packard model TRI-CARB-2100TR liquid scintillation analyzer (Packard Instruments, Downer’s Grove, IL).

**NE clearance and spillover calculations.** Total body NE clearance and spillover were calculated using established methods (13, 31, 41). NE clearance was calculated as NE clearance (ml/min) = 3H-NE infusion rate (dpm/min)/steady state plasma 3H-NE concentration (dpm/ml). NE spillover was calculated as NE spillover (ng/min) = NE clearance (ml/min) × plasma NE concentration (ng/ml). Both NE clearance and NE spillover were normalized to body weight and expressed as milliliters per minute per kilogram and nanogram per minute per kilogram, respectively.

**Experimental Protocol**

Five days of recovery were allowed after catheterization followed by a 3-day control period. ANG II (150 ng·kg⁻¹·min⁻¹) was then delivered subcutaneously by osmotic minipump (2ML2, Alzet) for 14 days. Endogenous plasma NE levels and total body NE spillover and clearance were determined on control days and ANG II infusion days 7 and 14. Rats were allowed free access to either 0.4% NaCl or 2% NaCl diet and distilled water for the duration of the experiment.

**Statistical Analysis**

Within-group changes in MAP, HR, plasma NE, NE clearance, and NE spillover were assessed with a one-way repeated-measures ANOVA and post hoc multiple comparisons with the Dunnett test. The effect of salt intake on hemodynamic and NE kinetic responses to ANG II between groups was assessed using a two-way mixed-design (one repeated factor) ANOVA. Post hoc multiple comparisons were corrected using Bonferroni’s procedure. A P value of <0.05 was considered statistically significant. All results are presented as means ± SE.

**RESULTS**

The MAP and HR response to chronic infusions of ANG II in animals fed 0.4% NaCl or 2% NaCl is shown in Fig. 1. Both MAP (0.4% NaCl: 106 ± 3, 2% NaCl: 103 ± 2 mmHg) and HR (0.4% NaCl 395 ± 5, 2% NaCl 372 ± 4 beats/min) were unaffected by a high-salt diet during the control period. ANG II caused a significant (P < 0.01) increase in MAP for the entire duration of infusion, in rats fed both 0.4% NaCl and 2% NaCl. However, the effect of ANG II to increase MAP was...
dependent on salt intake, as indicated by a statistically significant \((P < 0.01)\) interaction (two-way mixed-design ANOVA) between the two factors. MAP increased to a greater level in animals fed 2\% NaCl. HR responses to ANG II infusion were similar in rats fed both salt diets.

Infusion of 0.13 \(\mu\)Ci·min\(^{-1}\)·kg\(^{-1}\) of \(^{3}\)H-NE for 90 min resulted in an average steady-state plasma concentration of \(^{3}\)H-NE of 1,908 dpm/ml. The specific activity of the infused \(^{3}\)H-NE was 525 dpm/pg. Therefore, our infusion protocol increased total plasma NE concentration by an average of 3.6 pg/ml. The average plasma NE concentration measured in the study was 215.25 pg/ml. Therefore, \(^{3}\)H-NE contributed on average only 1.6\% of the total NE measured. We also determined that the infusion of \(^{3}\)H-NE at this rate did not affect MAP or HR. Figure 2 shows plasma NE and total body NE clearance and spillover during the control period and on ANG II infusion days 7 and 14 in rats fed 0.4\% or 2\% NaCl. *Interaction \(P < 0.05\) two-way ANOVA.

A high-salt diet alone or ANG II infusion in rats fed a normal salt diet did not increase global sympathetic outflow in this study. However, when administered in combination, high salt and ANG II caused global SNS activation associated with a further increase in arterial pressure. The finding of sympathoactivation in chronic ANG II hypertension only in the

**DISCUSSION**

A high-salt diet alone or ANG II infusion in rats fed a normal salt diet did not increase global sympathetic outflow in this study. However, when administered in combination, high salt and ANG II caused global SNS activation associated with a further increase in arterial pressure. The finding of sympathoactivation in chronic ANG II hypertension only in the

**Fig. 1.** Mean arterial pressure (A) and heart rate (B) response to chronic subcutaneous infusion of ANG II (150 ng·kg\(^{-1}\)·min\(^{-1}\)) in rats fed 0.4\% or 2\% NaCl. #Interaction \(P < 0.05\) two-way ANOVA.

**Fig. 2.** Plasma norepinephrine (A), whole body norepinephrine clearance (B), and whole norepinephrine spillover (C) during control and on ANG II infusion days 7 and 14 in rats fed 0.4\% or 2\% NaCl. *\(P < 0.05\) vs. control period. #Interaction \(P < 0.05\) two-way ANOVA.
setting of a high-salt diet is consistent with our previous work showing enhanced depressor responses to ganglionic blockade during chronic ANG II infusion only in rats eating a high-salt diet (32, 33). In addition, we have recently shown that surgical removal of the celiac plexus to selectively disrupt splanchnic sympathetic innervation significantly attenuated chronic ANG II hypertension, but again only in rats fed a high-salt diet (33). Therefore, the SNS activating actions of ANG II in this model appear critically dependent on the presence of a high-salt diet.

In this study, we documented global SNS activation in rats fed 2% NaCl but not rats fed 0.4% NaCl on day 7 of ANG II infusion, despite similar increases in MAP at this time point. Therefore, it appears that while the magnitude of ANG II-induced hypertension is similar by day 7, the underlying mechanism must be different depending on salt intake. Under conditions of a normal salt diet, ANG II seems to be acting to increase MAP by non-neural mechanisms. While the exact nature of these mechanisms is unclear, it may involve the direct vasoconstrictor or renal actions of ANG II. In contrast, in rats fed a high-salt diet, neurogenic mechanisms of hypertension appear to predominate. Studies have demonstrated ANG II can increase sympathetic outflow by a central action but also has stimulatory effects on sympathetic ganglia, including the adrenal medulla and can facilitate neurotransmission at the neuromuscular junction via both presynaptic and postsynaptic actions (49). The results of this study are consistent with the actions of ANG II at any level of the SNS.

Reports on the ability of ANG II to activate the SNS in humans are conflicting. Studies show that AT1 receptor antagonists significantly reduce MSNA in lean (3) and obese (28) hypertensive humans and that AT1 receptor blockade or angiotensin-converting enzyme inhibition reduces MSNA in hypertensive patients with chronic kidney disease (44). However, Esler’s group combined microneurography and radioisotope dilution methodology in a randomized, placebo-controlled crossover study to demonstrate that MSNA and whole body NE spillover were unchanged by AT1 receptor antagonism in human essential hypertension and concluded that the blood pressure-lowering actions of AT1 blockade are not related to sympathoinhibition (38). The evidence for ANG II-mediated sympathoactivation in experimental animals is also controversial. For example, Luft and colleagues (40) attenuated chronic ANG II hypertension in the rat by adrenergic blockade and showed significant increases in splanchnic nerve activity in conscious ANG II-infused rats instrumented with splanchnic nerve electrodes. However, Kline et al. (34) showed no significant differences in NE turnover in the heart, kidney, skeletal muscle, or intestine in ANG II-hypertensive rats, indicating that SNS activity was not increased, although depressor responses to ganglionic blockade were significantly larger in the ANG II-infused rats (34). Therefore, it appears as though the effect of ANG II on SNS activity depends on the specific human population or the experimental conditions under which it is studied. It is the hypothesis of our group that one of the conditions promoting ANG II-mediated sympathoactivation is a high-salt diet, and potential mechanisms mediating this interaction have been reviewed recently (48).

Other studies, using measurements of plasma catecholamines as an index of sympathetic activity, have also suggested that salt enhances ANG II-mediated SNS activation in the rat (51). Recently, Malpas and colleagues (42) showed in rabbits that the effects of ANG II on SNS activity were dependent on the dose of ANG II, as well as on dietary salt intake. The ability of salt to enhance SNS activation in experimental animal models of hypertension is not unique to ANG II. O’Donough and Brooks (46) have elegantly demonstrated using direct recordings of lumbar sympathetic nerve activity that the elevated blood pressure and sympathoactivation seen in DOCA-salt hypertension in the rat is driven by increased NaCl levels (46). Elevated osmolality appears to be acting centrally in the brain to support increased blood pressure and SNS activation in the DOCA-salt model, as bilateral intracarotid infusion, but not intravenous administration, of hypotonic fluid rapidly decreased blood pressure in DOCA-salt rats, and this fall in blood pressure was partially prevented by ganglionic blockade (47). A high-salt diet also stimulates SNS activity in SHR (11, 35) and Dahl salt-sensitive rats (24, 37).

More importantly, there is considerable evidence that neurogenic mechanisms may play a role in the pathophysiology of salt sensitivity in human essential hypertension. The phenomenon of salt sensitivity, an increase in blood pressure with increasing sodium intake, is common in human essential hypertension, and the risk of cardiovascular events is more than three times higher in salt-sensitive patients (43, 59). Measurements of spontaneous arterial baroreflex sensitivity demonstrate abnormalities in the autonomic control of the cardiovascular system in association with salt sensitivity, supporting the hypothesis that salt sensitivity is at least in part neurogenically driven (12). In addition, salt-sensitive hypertensive subjects show an abnormal relationship between plasma catecholamines and salt intake, as they fail to suppress plasma NE levels during high sodium intake, whereas plasma NE concentrations fall significantly in salt-resistant individuals on a high-salt intake (9, 25). Salt-sensitive patients also exhibit exaggerated pressor responses to infused NE (53).

Although abundant evidence supports SNS activation as a possible cause of salt-sensitive hypertension, the exact mechanism by which salt increases SNS activity is unknown. It has been proposed that a high-salt diet causes salt retention, which modestly increases plasma NaCl concentrations, which activate brain osmoreceptors to ultimately increase SNS activity (4). Lesions of key brain areas, including the osmosensitive anteroventral third ventricle (AV3V) region, prevent a number of models of salt-sensitive hypertension (10, 50). These osmosensitive neurons in the AV3V region, including the subfornical organ and organum vasculosum of the lamina terminalis, have projections to brain regions, such as the paraventricular nucleus, that can modulate SNS activity directly or via the rostral ventrolateral medulla (48, 56). ANG II and other humoral factors appear to amplify the SNS, activating actions of increased osmolality, perhaps by directly activating or sensitizing the osmosensitive neurons in the forebrain circumventricular structures (4, 48, 58). However, the exact mechanism remains to be elucidated.

Measurements of whole body NE spillover, as an index of global sympathetic outflow, have been widely applied in the investigation of SNS activity in human cardiovascular diseases (13, 14). In this study, we successfully employed these radioisotope dilution principles to demonstrate SNS activation in chronic ANG II-salt hypertension in the rat. The advantage of this method to distinguish the dynamic processes of NE spillover and clearance is evident on analyzing the measurements...
obtained in the control period. Interpreting plasma NE concentrations alone during the control period may suggest a tendency toward global SNS inhibition in rats fed a high-salt diet, although this was not statistically significant (P = 0.09). However, rats fed a high-salt diet during the control period also tended (P = 0.06) to have increased NE clearance. As a result, NE spillover was similar, independent of salt diet, indicating similar levels of global SNS overflow and the possible fallibility of interpreting plasma NE levels in isolation.

Perspectives

A high-salt diet alone or ANG II infusion in animals fed a normal salt diet does not increase global sympathetic outflow. However, when administered in combination, the additional hypertensive response to ANG II in rats fed a high-salt diet is accompanied by SNS activation. Measurements of regional NE spillover and direct sympathetic nerve recordings are required to identify the regional pattern of ANG II–salt-mediated sympatheoactivation; however, regional denervation studies indicate the splanchnic bed is the critical peripheral neural target. The phenomenon of salt sensitivity is common in human essential hypertension; however, the exact mechanism remains to be elucidated. This study indicates that one possible mechanism of salt sensitivity is SNS activation. Therefore, sympathoinhibition may be a successful therapeutic strategy for salt-sensitive hypertension in humans.

ACKNOWLEDGMENTS

Plasma NE and [3H]-NE were measured by Robert Burnett. Hannah Garver provided technical assistance in the animal studies.

GRANTS

This study was supported by National Institutes of Health Grant HL-076312 to the Neurogenic Cardiovascular Diseases Consortium (NCDC). A. J. King is supported by a Pfizer Safety Sciences Training Fellowship.

REFERENCES


