The Subtype 2 (AT_2) Angiotensin Receptor Mediates Renal Production of Nitric Oxide in Conscious Rats

Helmy M. Siragy and Robert M. Carey
Department of Medicine, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

Abstract

The angiotensin AT_2 receptor modulates renal production of cyclic guanosine 3′, 5′-monophosphate (cGMP; *J. Clin. Invest.* 1996. 97:1978–1982). In the present study, we hypothesized that angiotensin II (Ang II) acts at the AT_2 receptor to stimulate renal production of nitric oxide leading to the previously observed increase in cGMP. Using a microdialysis technique, we monitored changes in renal interstitial fluid (RIF) cGMP in response to intravenous infusion of the AT_2 receptor antagonist PD 123319 (PD), the AT_1 receptor antagonist Losartan, the nitric oxide synthase (NOS) inhibitor nitro-L-arginine-methyl-ester (L-NAME), the specific neural NOS inhibitor 7-nitroindazole (7-NI), or Ang II individually or combined in conscious rats during low or normal sodium balance.

Sodium depletion significantly increased RIF cGMP. During sodium depletion, both PD and L-NAME caused a similar decrease in RIF cGMP. Combined administration of PD and L-NAME decreased RIF cGMP to levels observed with PD or L-NAME alone or during normal sodium intake.

During normal sodium intake, Ang II caused a twofold increase in RIF cGMP. Neither PD nor L-NAME, individually or combined, changed RIF cGMP. Combined administration of Ang II and either PD or L-NAME produced a significant decrease in RIF cGMP compared with that induced by Ang II alone. Combined administration of Ang II, PD, and L-NAME blocked the increase in RIF cGMP produced by Ang II alone.

During sodium depletion, 7-NI decreased RIF cGMP, but the reduction of cGMP in response to PD alone or PD combined with 7-NI was greater than with 7-NI alone. During normal sodium intake, 7-NI blocked the Ang II–induced increase in RIF cGMP. PD alone or combined with 7-NI produced a greater inhibition of cGMP than did 7-NI alone. During sodium depletion, 7-NI (partially) and L-NAME (completely) inhibited RIF cGMP responses to L-arginine.

These data demonstrate that activation of the renin–angiotensin system during sodium depletion increases renal nitric oxide production through stimulation by Ang II at the angiotensin AT_2 receptor. This response is partially mediated by neural NOS, but other NOS isoforms also contribute to nitric oxide production by this pathway. (*J. Clin. Invest.* 1997. 100:264–269.) Key words: AT_2 receptor • nitric oxide • kidney • L-NAME • 7-nitroindazole

Introduction

The majority of studies suggest that the renal actions of angiotensin II (Ang II)[1] (1) are mediated via subtype 1 angiotensin (AT_1) receptors (2). Subtype 2 angiotensin (AT_2) receptors, however, are also present in the kidney (3), and have been reported to regulate pressure natriuresis in rats (4). Recently, we demonstrated that activation of the renin–angiotensin system during sodium depletion increases renal interstitial fluid (RIF) cyclic guanosine 3′, 5′ monophosphate (cGMP), an effect mediated via the AT_2 receptor (5).

In this study, we used a novel microdialysis technique (5–8) to investigate whether changes in RIF cGMP observed during AT_2 receptor blockade are related to changes in renal nitric oxide (NO) production. We studied conscious rats during sodium depletion, a condition known to activate the renin–angiotensin system. Our microdialysis technique has several advantages over the traditional measurements conducted in blood or urine. First, repeated blood sampling in small animals may cause undesirable hemodynamic changes. Second, RIF sampling provides the ability to monitor autocoids in vivo at almost any site in an organ or tissue. Measurement of circulating hormones/autocoids may not reflect local changes within that organ. Third, the concentration of hormones/autocoids in the circulation may differ from that in the interstitial space, which is closer to target receptors. Fourth, autocoids (e.g., kinins) can be formed and degraded in urine, measurement of which does not reflect their concentrations within the target organ. Fifth, the molecular weight cutoff of the microdialysis membrane can function as a barrier separating small and large molecules, and can help to exclude undesirable substances (degrading enzymes and carrier proteins). The isolation of free (unbound) materials can facilitate their bioanalytical measurement in a

Address correspondence to Helmy M. Siragy, M.D., Department of Medicine, Box 482, University of Virginia Health Sciences Center, Charlottesville, VA 22908. Phone: 804-924-5510; FAX: 804-982-3626; E-mail: hms7a@virginia.edu

Received for publication 9 December 1996 and accepted in revised form 17 April 1997.

J. Clin. Invest.

0021-9738/97/07/0264/06 $2.00
Volume 100, Number 2, July 1997, 264–269

1. *Abbreviations used in this paper:* 7-NI, 7-nitroindazole; Ang II, angiotensin II; AT_1, subtype 1 angiotensin receptor; AT_2, subtype 2 angiotensin receptor; D-Arg, D-arginine; D_5W, 5% dextrose in water; L-Arg, L-arginine; LNa, low sodium intake; L-NAME or NAM, nitro-L-arginine methyl ester; MAP, mean arterial pressure; NNa, normal sodium intake; NO, nitric oxide; NOS, nitric oxide synthase; PD, PD-123319; RIF, renal interstitial fluid; $U_{NA}V$, urinary sodium excretion.

small volume without a need for complicated extraction procedures.

In this study, we hypothesized that Ang II acts at the AT_2 receptor to stimulate renal production of NO leading to an increase in RIF levels of cGMP.

Methods

In vivo renal microdialysis technique. For the determination of RIF cGMP, we constructed a microdialysis probe as previously described (5–8). Each end of single 0.5-cm-long hollow fiber dialysis tubing (0.1-mm inner diameter; molecular mass cutoff, 5,000 D; Hospal, Meyzieu, France) was inserted into a manually dilated end of two 30-cm-long (inflow and outflow) hollow polyethylene tubes (0.12-mm inner diameter, 0.65-mm outer diameter; Bioanalytical Systems, Indianapolis, IN). The distance between the ends of the polyethylene tubes was 3 mm (dialysis area), and the dialysis fiber was sealed in place within the polyethylene tubes with cyanoacrylic glue. The dead volume of the dialysis tubing and outflow tube was 3.6 μl. The microdialysis probe was sterilized by a gas sterilization method.

In vitro microdialysis. In vitro best recoveries for cGMP were observed with a perfusion rate of 3 μl/min, and were ~ 70% for cGMP (5). A negligible amount of cGMP sticks to the polyethylene tubes of the dialysis probes, as demonstrated by the in vitro recovery of [^{3}H]cGMP at 99.8% (5).

Animal preparation. Experiments were conducted in 4-wk-old female Sprague-Dawley rats (n = 8–10 in each group) purchased from Harlan Sprague-Dawley Inc. (Harlan Teklad, Madison, WI). With the rats under general anesthesia with 80 mg/kg ketamine intramuscularly (IM) (Fort Dodge Laboratories, Fort Dodge, IA) and 8 mg/kg xylazine intramuscularly (IM) (Bayer, Animal Health Div., Shawnee, KS), the right and left kidneys were exposed via a midline abdominal incision. The renal capsule of each kidney was penetrated with a 31-gauge needle that was tunneled in the outer renal cortex ~ 1 mm from the outer renal surface for 0.5 cm before it exited by penetrating the capsule again. The tip of the needle was inserted into one end of the dialysis probe, and the needle was pulled together with the dialysis tube until the dialysis fiber was situated in the renal cortex. The inflow and outflow tubes of dialysis probes were tunneled subcutaneously through a bevel-tipped stainless steel tube, and were exteriorized near the interscapular region. To obtain vascular access, a heparinized polyethylene tube (PE 50; Becton Dickinson, Sparks, MD) was inserted into the right carotid artery. This tube was flushed daily with 10% heparin in 5% dextrose in water (D_5W), and was capped with a small piece of copper wire. Exterior ends of these tubes were secured in place by suturing them to skin at the exit site. The exteriorized portions of the tubes were placed in a stainless steel spring to prevent the rats from damaging them. Rats were allowed 7 d to recover and to acclimatize to the laboratory. Rats were housed under controlled conditions (temperature: 21±1°C; humidity: 60±10%; lighting: 8–20 h). Experiments were started at the same time (8 a.m.) each day to avoid any diurnal variation of the measured substances. For collection of RIF, the inflow tube was connected to a gas-tight syringe filled with lactated Ringer's solution and perfused at 3 μl/min. The effluent was collected from the outflow tube for 30-min sample periods in nonheparinized plastic tubes, and was stored at −80°C until assayed for cGMP.

Analytical methods. Urinary sodium levels were measured by a NOVA analyzer (NOVA Biomedical, Waltham, MA). RIF cGMP levels in dialysate samples were measured by enzyme immunoassay kit (Cayman Chemical Co. Inc., Ann Arbor, MI). The sensitivity and specificity of this method for cGMP were 0.11 pmol/ml and 100%, respectively. The intra- and interassay coefficients of variation were < 10%. Cross-reactivity of the cGMP assay was < 0.01% with other cyclic nucleotides.

Effects of sodium depletion, angiotensin AT_2 receptor blockade, and nitric oxide synthase inhibition (NOS) on RIF cGMP. In this study, rats (n = 8) were placed in metabolic cages. A baseline 24-h urine collection was obtained for calculation of urinary sodium excretion ($U_{Na}V$), and RIF samples were obtained for cGMP (experimental day 1) while rats were consuming a normal sodium diet (0.28% NaCl; BioServe Biotechnologies, Frenchtown, NJ). Then the rats were placed on a low sodium diet (0.05% NaCl) for 10 d. We continued to monitor 24-h $U_{Na}V$ and RIF cGMP daily for 4 d (experimental days 2–6). While the rats continued to consume the low sodium diet (days 7–10), RIF cGMP was monitored during the right intracarotid administration (20 μl/min for 30 min) in random order of (*a*) D_5W at 20 μl/min, (*b*) PD123319 (PD; Parke-Davis, Warner Lambert Co., Ann Arbor, MI), a specific AT_2 receptor antagonist (half-maximal inhibitory concentration [IC_{50}]2 × 10^{-8} M for AT_2 receptors and > 10^{-4} M for AT_1 receptors) (4, 5), or (3) nitro-L-arginine methyl ester (L- NAME), an NOS inhibitor (Sigma Chemical Company, St. Louis, MO). PD was infused at 50 μg/kg/min, which yields plasma concentrations near 3 × 10^{-6} M (a value that remains highly specific for AT_2 receptors) (2, 4, 5), and L-NAME was infused at 1 μg/kg/min. To determine the L-NAME dose, L-NAME was infused intravenously at 0.1, 1, 10, and 100 μg/kg/min in conscious rats on normal sodium intake, and BP and RIF cGMP were monitored. There were no significant changes in BP or RIF cGMP associated with L-NAME at 0.1 and 1 μg/kg/min. L-NAME at 10 and 100 μg/kg/min caused a decrease in RIF cGMP, but did not change BP. We chose the L-NAME dose at 1 μg/kg/min since this dose did not elicit any changes in BP or cGMP while animals were on normal sodium intake. Mean arterial pressure (MAP) was measured every 10 min in the rat tail (Rat Tail Manometer-Tachometer System, Natsume model KN-210; Peninsula Laboratories Inc., Belmont, CA) and the recorded values were averaged for each study period. The study was repeated during concomitant administration of PD (50 μg/kg/min) and L-NAME (1 μg/kg/min).

Effects of sodium depletion, angiotensin AT_1 receptor blockade, and inhibition on RIF cGMP. To evaluate the relationship between AT_1 receptor and NO during sodium depletion, the above study was repeated in another group of rats (n = 8) except that PD replaced by Losartan (DuPont Merck Pharmaceutical Co., Wilmington, DE), a long-acting nonpeptide Ang II antagonist at AT_1 receptors (IC_{50} 3 × 10^{-4} and 7 × 10^{-9} M for AT_2 and AT_1 receptors, respectively) (2, 4, 5).

Effects of angiotensin AT_2 receptor blockade and neural NOS inhibition on RIF cGMP during sodium depletion. To differentiate the effects of blockade of neurally derived NOS, the above protocol was repeated in a separate group of rats (n = 10), except that a selective inhibitor of neural NOS, 7-nitroindazole (7-NI, 50 mg/kg; BIOMOL Research Labs, Inc., Plymouth Meeting, PA) was given by intraperitoneal injection instead of L-NAME. This compound, 7-NI, has an IC_{50} of 0.47 μM (9) and selectively blocks the synthesis of NO by neuronal NOS (9, 10). In vitro, 7-NI completely blocks cerebellar conversion of L-arginine to citrulline. In vivo, a dose of 20–50 mg/kg of 7-NI blocks nociceptive responses mediated by noncholinergic, nonadrenergic neurons.

Effects of angiotensin AT_2 receptor blockade and L-NAME on RIF cGMP during normal sodium intake. To evaluate whether the observed changes in RIF cGMP during AT_2 receptor blockade and NOS inhibition were related to changes in renal Ang II during sodium depletion, we repeated the above study (days 6–10) after rats (n = 8) were placed on a normal sodium diet for 5 d. RIF cGMP was monitored during a control period (D_5W was infused into right carotid artery at 20 μl/min for 30 min), and during an experimental period (30 min) during which (*a*) D_5W (20 μl/min), (*b*) Ang II (30 ng/kg/min), (*c*) PD (50 μg/kg/min), or (*d*) L-NAME (1 μg/kg/min) individually or combined were administered intravenously. The dose of Ang II was determined from a dose-pressor response curve for Ang II (5). We chose the largest dose of Ang II that did not elicit any rise in blood pressure.

Effects of angiotensin AT_1 receptor blockade and L-NAME on RIF cGMP during normal sodium intake. To evaluate the relationship between the AT_1 receptor and NO during normal sodium intake,

we repeated the above study after rats ($n = 8$) were placed on normal sodium diet, except that PD was replaced by Losartan.

Effects of L-arginine, D-arginine, L-NAME, and 7-NI on RIF cGMP during sodium depletion. To evaluate if the observed changes in RIF cGMP during PD or L-NAME administration were specifically related to changes in NO generation, RIF cGMP levels were monitored in conscious rats ($n = 10$) while they were on low sodium balance, and during intravenous administration of the NO precursor L-arginine (2.5 mg/kg/min), its inactive isoform D-arginine (2.5 ng/kg/min), L-NAME (1 μg/kg/min), or PD (50 μg/kg/min) individually or combined.

This study was repeated in a separate group of rats ($n = 10$) with replacement of L-NAME with 7-NI (50 mg/kg by intraperitoneal injection).

Statistical analysis of data. Comparisons among pharmacologic agents and controls were examined by ANOVA including a repeated measures term, using the General Linear Models procedure of the Statistical Analysis System. Multiple comparisons of individual pairs of effect means were conducted by use of values of least-square means pooled variance. Data are expressed as mean±1 SE. Statistical significance was identified at $P < 0.05$.

Results

MAP responses to PD, L-NAME or Ang II individually or combined. When rats ($n = 8$) were in metabolic balance at low sodium intake, MAP was 115±3 mmHg. There were no significant changes in MAP during administration of PD, Losartan, L-NAME, or 7-NI. During normal sodium intake, MAP was 114±4 mmHg, and did not change significantly during infusion of Ang II, PD, Losartan, L-NAME, or 7-NI.

RIF cGMP responses to sodium depletion, PD, and/or L-NAME during low sodium intake. A progressive reduction in 24 h $U_{Na}V$ was observed during a low sodium intake. 24-h urine sodium excretion decreased progressively from 600±30 μmol/d during a normal sodium diet to 30±12 μmol/d on day 5 of low sodium intake (Fig. 1). RIF cGMP levels (Fig. 1) increased significantly and progressively during dietary sodium depletion. RIF cGMP increased from 1.0±0.1 to 1.9±0.1 pmol/min ($P < 0.0001$) by the fifth day of sodium depletion. At the end of the fifth day of sodium depletion (Fig. 2), RIF cGMP decreased significantly in response to administration of PD from 1.9±0.1 to 1.0±0.2 pmol/min ($P < 0.0001$). Similarly, RIF cGMP significantly decreased from 2.1±0.1 to 0.7±0.1 pmol/min ($P < 0.0001$) in response to L-NAME. Combined infusion of PD and L-NAME significantly decreased RIF cGMP to the levels observed during normal sodium diet ($P < 0.0001$). There were no significant differences between RIF cGMP levels during sodium depletion and administration of PD or L-NAME alone or combined.

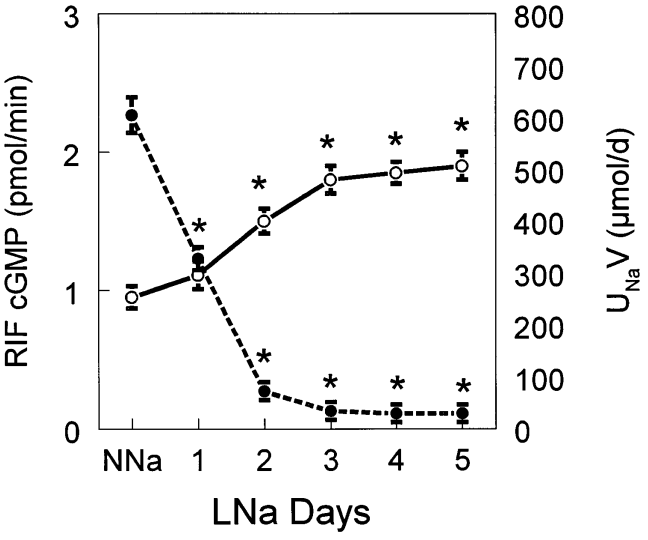

Figure 1. 24-h urinary sodium excretion ($U_{Na}V$; *solid dots and dashed lines*) and RIF cGMP levels (*open dots and solid lines*) in conscious rats ($n = 8$) on normal sodium intake (*NNa*) or on each of 5 d dietary sodium depletion (*LNa*). Data represent mean±SE; *$P < 0.0001$ from values during NNa.

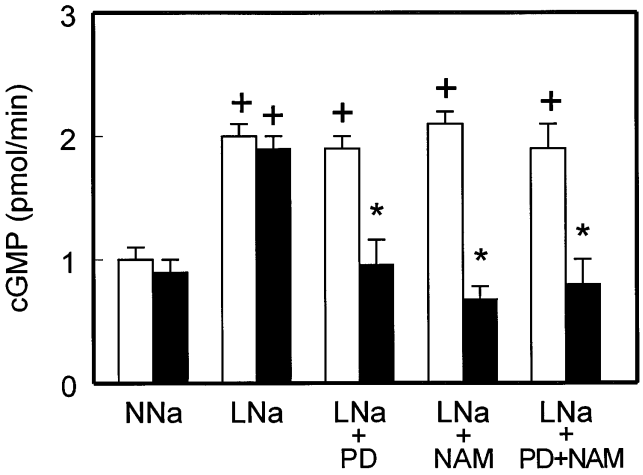

Figure 2. RIF cGMP responses to NNa, LNa, PD, or L-NAME (*NAM*) alone or combined in conscious rats ($n = 8$). Open bars represent time control values in which vehicle was administered. Closed bars represent experimental values in which vehicle, PD, NAM, or the combination was administered. Data represent mean±SE. $^{+}P <$ 0.0001 vs. NNa; *$P < 0.0001$ from respective time control values.

RIF cGMP responses to Ang II, PD, and/or L-NAME during normal sodium intake. RIF cGMP (Fig. 3) increased during Ang II infusion from 0.9±0.1 pmol/min to 1.8±0.1 pmol/min ($P < 0.0001$). PD or L-NAME individually did not change RIF cGMP. Combined administration of Ang II and PD or L-NAME blocked the cGMP response to Ang II (0.8±0.09 and 0.7±0.1 pmol/min, respectively) ($P < 0.0001$). Simultaneous administration of Ang II, PD, and L-NAME blunted the RIF cGMP response to Ang II ($P < 0.0001$). There were no significant differences among RIF cGMP levels during combined administration of Ang II and PD, Ang II and L-NAME, and Ang II, PD, and L-NAME.

RIF cGMP responses to sodium depletion, Losartan, and/or L-NAME during low sodium intake. RIF cGMP levels (Fig. 4) increased significantly during dietary sodium depletion. RIF cGMP increased from 1.0±0.1 to 2.0±0.2 pmol/min ($P < 0.0001$) by the fifth day of sodium depletion. RIF cGMP during sodium depletion (Fig. 4) did not change in response to Losartan, but decreased to levels observed during normal sodium intake in response to L-NAME ($P < 0.0001$). Combined infusion of Losartan and L-NAME significantly decreased RIF cGMP to the levels observed during normal sodium diet and during combined treatment with low sodium intake and L-NAME.

RIF cGMP responses to Ang II, Losartan, and/or L-NAME during normal sodium intake. RIF cGMP (Fig. 5) increased

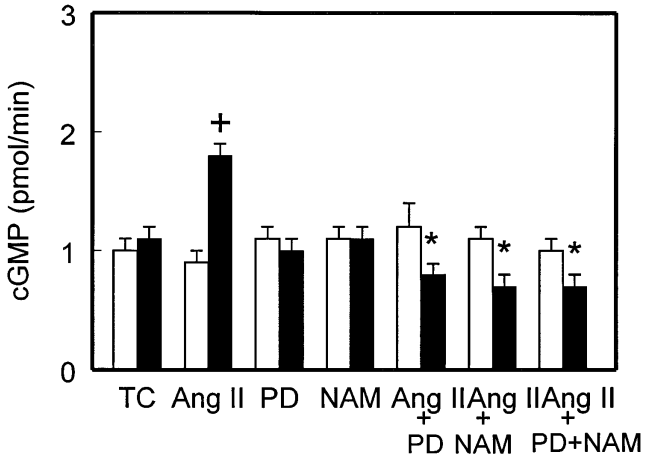

Figure 3. RIF cGMP responses to Ang II, PD, or L-NAME (*NAM*) alone or combined in conscious rats ($n = 8$) during normal sodium intake. TC represents time control values in which vehicle was administered. Open bars represent control values before administration of pharmacologic agent. Closed bars represent experimental values in which vehicle, Ang II, PD, or NAM were administered alone or combined. Data represent mean±SE. $^{+}P < 0.0001$ vs. TC; $^{*}P < 0.0001$ vs. respective control values or Ang II.

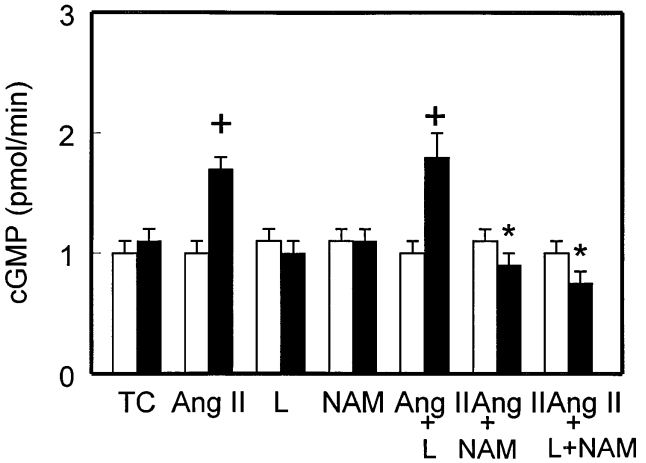

Figure 5. RIF cGMP responses to Ang II, Losartan (*L*), or L-NAME (*NAM*) alone or combined in conscious rats ($n = 8$) during normal sodium intake. TC represents time control values in which vehicle was administered. Open bars represent control values before administration of pharmacologic agent. Closed bars represent experimental values in which vehicle, Ang II, PD, or NAM were administered alone or combined. Data represent mean±SE. $^{+}P < 0.0001$ vs. TC; $^{*}P < 0.0001$ vs. respective control values or Ang II.

during Ang II infusion from 1.0±0.1 to 1.7±0.1 pmol/min ($P < 0.0001$). Losartan or L-NAME individually in the absence of Ang II did not change RIF cGMP. Combined administration of Ang II and Losartan did not change the cGMP response to Ang II. Combined Ang II and L-NAME (Fig. 5) blocked the cGMP response to Ang II (0.9±0.1 vs. 1.8±0.1) ($P < 0.0001$). There were no significant differences among RIF cGMP levels during combined administration of Ang II and L-NAME, or Ang II, Losartan, and L-NAME.

RIF cGMP responses to L-arginine, D-arginine, or combined L- and D-arginine during sodium depletion. RIF cGMP (Fig. 6) increased during L-arginine infusion from 1.5±0.1 pmol/min to 2.5±0.18 pmol/min ($P < 0.001$). D-arginine did not cause any significant change in RIF cGMP. Combined administration of L-arginine and L-NAME blocked the increase in RIF cGMP observed with L-arginine alone ($P < 0.001$).

RIF cGMP responses to PD and/or 7-NI during sodium depletion. Fig. 7 depicts RIF cGMP levels in rats on the fifth day of dietary sodium depletion. PD administration decreased RIF cGMP ($P < 0.001$). Administration of 7-NI decreased cGMP

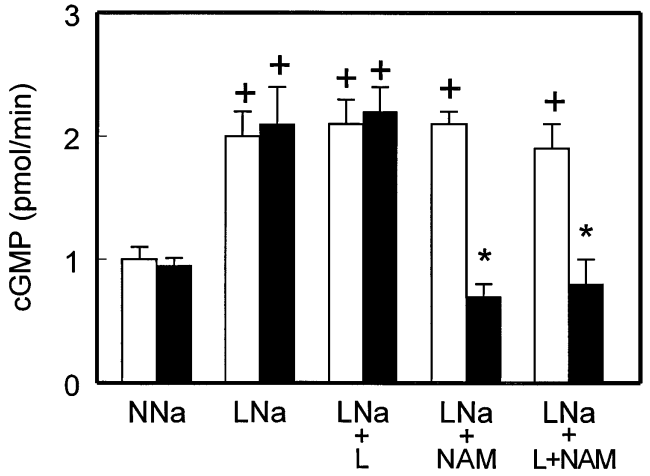

Figure 4. RIF cGMP responses to NNa, LNa, Losartan (*L*), or L-NAME (*NAM*) alone or combined in conscious rats ($n = 8$). Open bars represent time control values in which vehicle was administered. Closed bars represent experimental values in which vehicle, L, NAM, or the combination were administered. Data represent mean±SE. $^{+}P < 0.0001$ vs. NNa; $^{*}P < 0.0001$ from respective time control values.

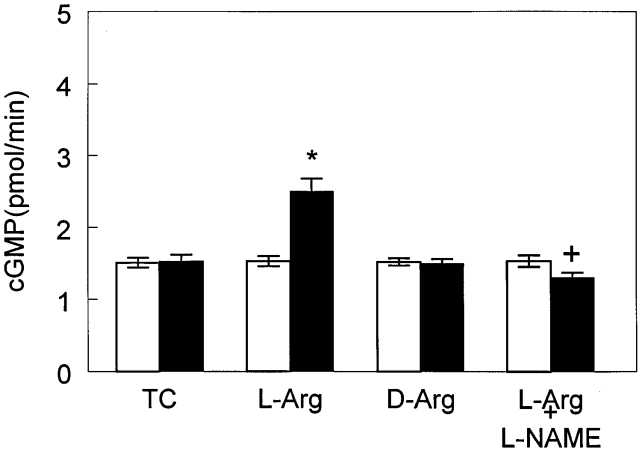

Figure 6. RIF cGMP in sodium-depleted conscious rats ($n = 10$) in response to L-arginine (*L-Arg*), D-arginine (*D-Arg*) or L-NAME combined with L-Arg. TC represents time control values in which vehicle was administered. Open bars represent control values before administration of pharmacologic agent. Closed bars represent experimental values in which vehicle, L-Arg, D-Arg, or L-Arg combined with L-NAME were administered. Data represent mean±SE. $^{*}P < 0.001$ from TC and respective control values; $^{+}P < 0.001$ from L-Arg administration alone and respective control.

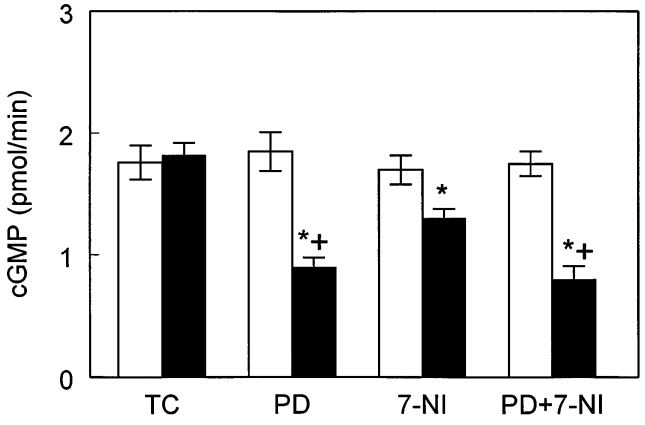

Figure 7. RIF cGMP in sodium-depleted conscious rats ($n = 10$) in response to vehicle, PD, or 7-NI alone or combined. TC represents time control values in which vehicle was administered. Open bars represent control values before administration of pharmacologic agent. Closed bars represent experimental values in which PD or 7-NI alone or combined were administered. Data represent mean±SE. *$P < 0.001$ from respective control and time control; $^{+}P < 0.001$ from 7-NI alone.

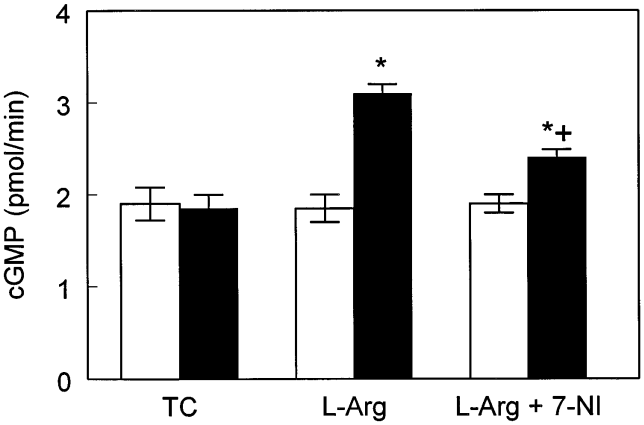

Figure 9. RIF cGMP in sodium-depleted rats ($n = 10$) in response to vehicle, L-arginine (*L-Arg*), or L-Arg combined with 7-NI. TC represents time control values in which vehicle was administered. Open bars represent control values before administration of pharmacologic agent. Closed bars represent experimental values in which vehicle, L-Arg or L-Arg, and 7-NI were administered. *$P < 0.001$ vs. respective control and time control values. $^{+}P < 0.001$ from L-Arg.

($P < 0.001$), but the reduction in cGMP due to PD was greater than that due to 7-NI ($P < 0.001$). Combined administration of PD and 7-NI resulted in a similar decrease in RIF cGMP as observed with PD alone.

RIF cGMP responses to Ang II, PD, and/or 7-NI during normal sodium intake. Ang II increased RIF cGMP from 1.1±0.1 to 2.3±0.1 pmol/min ($P < 0.0001$) (Fig. 8). Neither PD nor 7-NI alone influenced RIF cGMP. The increase in RIF cGMP resulting from Ang II administration was blocked completely by PD, 7-NI, or the combination ($P < 0.0001$). The degree of inhibition of cGMP, however, was greater for PD alone and for PD combined with 7-NI than for 7-NI alone ($P < 0.05$).

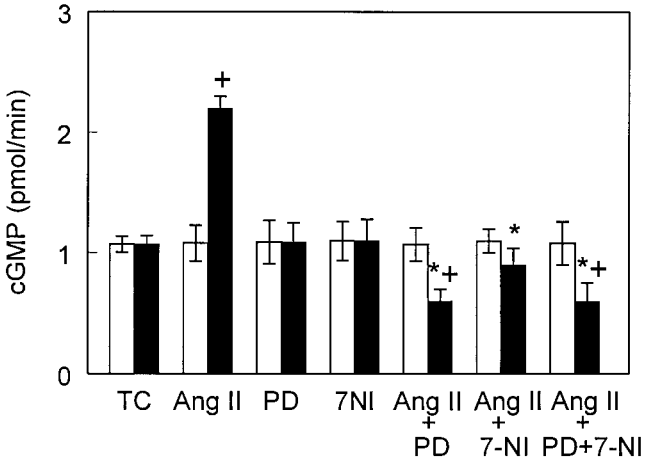

Figure 8. RIF cGMP in conscious rats ($n = 8$) on normal sodium intake in response to vehicle, Ang II, PD, or 7-NI alone or combined. TC represents time control values in which vehicle was administered. Open bars represent control values before administration of pharmacologic agent. Closed bars represent experimental values in which vehicle, Ang II, PD, or 7-NI alone or combined were administered. Data represent mean±SE. $^{+}P < 0.0001$ from TC; *$P < 0.0001$ from Ang II.

RIF cGMP responses to L-arginine and 7-NI during sodium depletion. L-arginine infusion increased RIF cGMP ($P < 0.001$) (Fig. 9). 7-NI partially blocked the increase of RIF cGMP engendered by L-arginine.

Discussion

This study demonstrates to our knowledge for the first time the interaction between the renal subtype-2 angiotensin (AT_2) receptor and NO in the rat. RIF cGMP increased by approximately twofold in response to dietary sodium depletion or Ang II administration. This response was blocked by administration of the AT_2 receptor antagonist PD. These data confirmed our previous observations that the renal AT_2 receptor stimulates the release of cGMP (5). The modulation of renal cGMP by the AT_2 receptor is active only during states of increased activity of the renal renin–angiotensin system as demonstrated by absence of PD effects on cGMP during normal sodium intake.

In this study, we hypothesized that Ang II acting via the AT_2 receptor stimulates renal production of NO leading to the previously described increase in RIF levels of cGMP. We observed that the NOS inhibitor L-NAME inhibited cGMP release in response to endogenous Ang II stimulated by sodium depletion or exogenous administration of Ang II. Administration of PD or L-NAME inhibited to the same degree the cGMP response to sodium depletion or exogenous administration of Ang II. In the presence of PD or L-NAME, cGMP levels were not significantly different from control values before sodium depletion or exogenous ANG II, respectively. In this study, the lack of L-NAME effect on cGMP during normal sodium intake was dose dependent since larger doses caused a decrease in RIF cGMP. These results suggest that NO is produced tonically under normal conditions, leading to production of cGMP, and are in agreement with results of previous studies demonstrating an important physiological role for endogenous renal NO (11).

We confirmed the specificity of L-NAME effects on NO

production by demonstrating that L-NAME blocked the increase in RIF cGMP levels engendered by L-arginine, a substrate for NO. We also validated the specificity of L-arginine to produce a rise in RIF cGMP by demonstrating the absence of a similar effect by the inactive isomer, D-arginine. The absence of additive inhibition of RIF cGMP when PD and L-NAME were coadministered strongly suggests that each of these inhibitors decreases cGMP by inhibition of NO production.

Previously published data demonstrated interactions between the AT_1 receptor and NO in the kidney (12). In the present study we failed to demonstrate that the AT_1 receptor modulates NO production in the kidney based on our observations that Ang II– or sodium depletion–stimulated increases in RIF cGMP were not inhibited by Losartan, a specific AT_1 receptor antagonist. In conscious rats, pretreatment with Losartan did not modify the renal effects of the NOS inhibitor, L-NAME (13), a finding consistent with the observations of the present study. Recent studies also have shown that sodium restriction stimulated the expression of NOS isoforms in the kidney that were not influenced by the AT_1 receptor antagonist, Losartan (14). Ang II, however, can increase the production of NO in some tissues through an action at the AT_1 receptor. For example, in the rat carotid artery, contractions to Ang II are decreased by AT_1 receptor–stimulated release of endothelium-derived NO (15).

All types of NOS genes are present in the kidney (16–19). The effects of Ang II on NOS expression in the kidney to our knowledge have not been studied previously. A recent study (20) demonstrated that short-term Ang II infusion in anesthetized rats increased the excretion of the NO stable metabolites, nitrate and nitrite. In a preliminary communication (21), Ang II was shown to increase type III NOS (endothelial NOS) gene expression in bovine pulmonary endothelial cells. Endothelial (constitutive) NOS is expressed in the tubular epithelium and vascular endothelium. Neuronal NOS is localized to the macula densa and epithelium of the Bowman's capsule. Inducible NOS is expressed in the proximal tubule, glomerulus, medullary thick ascending limb, and inner medullary collecting duct (16–19). In the present study, we investigated the possible role of neuronal NOS in mediating the Ang II stimulatory effect on NO release through the AT_2 receptor. We demonstrated that neuronal NOS is partially involved, but that it is likely that one or both of the other NOS enzymes also are involved. While 7-NI inhibited cGMP responses to sodium depletion and Ang II administration, the 7-NI–induced reduction in cGMP was always significantly less than that due to PD. Since 7-NI also only partially abrogated the increase in cGMP due to L-arginine administration, but L-NAME completely inhibited this increase, it is highly likely that several NOS enzymes (including neuronal NOS) are involved in the generation of NO in renal interstitial fluid.

In conclusion, we have shown that the renal AT_2 receptor is stimulated tonically to enhance the production of NO in response to a physiologic stimulus, sodium depletion. The AT_2 receptor antagonist PD and the NOS inhibitor L-NAME blocked the increase in renal interstitial fluid cGMP engendered by dietary sodium depletion or by exogenous Ang II during normal sodium intake. There was no additive inhibition of renal interstitial fluid cGMP when PD and L-NAME were coadministered, suggesting that the increase in renal NO production associated with sodium depletion or Ang II administration is highly likely to be mediated by an action at the angiotensin AT_2 receptor.

Acknowledgments

This work was supported by grants HL-47669 and HL-57503 (H.M. Siragy) and HL-49575 (R.M. Carey) from the National Institutes of Health. Dr. Siragy is the recipient of Research Career Development Award K04-HL-03006 from the National Institutes of Health.

References

1. Navar, L.G., and L. Rosival. 1984. Contribution of the renin-angiotensin system to the control of intrarenal hemodynamics. *Kidney Int.* 25:857–868.

2. Macari, D., S. Bottani, S. Whitebread, M. De Gasparo, and N. Levens. 1993. Renal actions of the selective angiotensin AT_2 receptor ligands CGP 42112 B and PD 123319 in the sodium-depleted rat. *Eur. J. Pharmacol.* 249:85–93.

3. Ernsberger, P., J. Zhou, T. Damon, and J.G. Douglas. 1992. Angiotensin II receptor subtypes in cultured rat renal mesangial cells. *Am. J. Physiol.* 263: F411–F416.

4. Lo, M., K. Liu, P. Lantelme, and J. Sassard. 1995. Subtype 2 of angiotensin II receptors controls pressure-natriuresis in rats. *J. Clin. Invest.* 95:1394–1397.

5. Siragy, H.M., and R.M. Carey. 1996. The subtype-2 (AT_2) angiotensin receptor regulates renal cyclic guanosine 3′,5′-monophosphate and AT_1 receptor-mediated prostaglandin E_2 production in conscious rats. *J. Clin Invest.* 97:1978–1982.

6. Siragy, H.M., N.L. Howell, N.V. Ragsdale, and R.M. Carey. 1995. Renal interstitial fluid angiotensin modulation by anesthesia, epinephrine, sodium depletion, and renin inhibitor. *Hypertension (Dallas).* 25:1021–1024.

7. Siragy, H.M., M.M. Ibrahim, A.A. Jaffa, R. Mayfield, and H.S. Margolius. 1994. Rat renal interstitial bradykinin, prostaglandin E_2, and cyclic guanosine 3′, 5′-monophosphate. Effects of altered sodium intake. *Hypertension (Dallas).* 23:1068–1070.

8. Siragy, H.M., A.A. Jaffa, and H.S. Margolius. 1993. Stimulation of renal interstitial bradykinin by sodium depletion. *Am. J. Hypertens.* 6:863–866.

9. Moore, P.K., R.C. Babbedge, P. Wallace, Z.A. Gaffen, and S.L. Hart. 1993. 7-Nitroindazole, an inhibitor of nitric oxide synthase, exhibits anti-nociceptive activity in the mouse without increasing blood pressure. *J. Pharmacol.* 108: 296–297.

10. Mayer, B., P. Klatt, E.R. Werner, and K. Schmidt. 1994. Identification of imidazole as L-arginine-competitive inhibitor of porcine brain nitric oxide synthase. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 350:199–202.

11. Baylis, C., and C. Qui. 1996. Importance of nitric oxide in the control of renal hemodynamics. *Kidney Intl.* 49:1727–1731.

12. DeNicola, L., R.C. Blantz, and F.B. Gabbai. 1992. Nitric oxide and angiotensin II. Glomerular and tubular interaction in the rat. *J. Clin. Invest.* 89: 1248–1256.

13. Sigmon, D.H., J.M. Newman, and W.H. Beierwaltes. 1994. Angiotensin II: endothelium-derived nitric oxide interaction in conscious rats. *J. Am. Soc. Nephrol.* 4:1675–1682.

14. Tojo, A., K.M. Madsen, and C.S. Wilcox. 1995. Expression of immunoreactive nitric oxide synthase as it forms in rat kidney. Effects of dietary salt and losartan. *Jpn. Heart J.* 36:389–398.

15. Boulanger, C.M., L. Caputo, and B.I. Levy. 1995. Endothelial AT_1-mediated release of nitric oxide decreases angiotensin II contractions in rat carotid artery. *Hypertension (Dallas).* 26:752–757.

16. Mohaupt, M.G., J.L. Elzie, K.Y. Ahn, W.L. Clapp, C.S. Wilcox, and B.C. Kane. 1994. Differential expression and induction of mRNAs encoding two inducible nitric oxide synthases in rat kidney. *Kidney Int.* 46:653–665.

17. Lau, K.S., O. Nakashima, G. Aalund, L. Hogarth, K. Ujue, J. Yuen, and R.A. Star. 1995. TNF-α and IFN-γ induce expressions of nitric oxide synthase in cultured rat medullary interstitial cells. *Am. J. Physiol.* 269:F212–F217.

18. Conger, J., J. Robinette, A. Villar, L. Raij, and P. Shultz. 1995. Increased nitric oxide synthase activity despite lack of response to endothelium-dependent vasodilators in postischemic acute renal failure in rats. *J. Clin. Invest.* 96:631–638.

19. Bachmann, S., H. Bosse, and P. Mundel. 1995. Topography of nitric oxide synthesis by localizing constitutive NO synthases in mammalian kidney. *Am. J. Physiol.* 268:F885–F898.

20. Deng, X., W.J. Welch, and C.S. Wilcox. 1996. Role of nitric oxide in short-term and prolonged effects of angiotensin II on renal hemodynamics. *Hypertension.* 27:1173–1179.

21. Millat, L.J., and R.A. Johns. 1996. Angiotensin II increases type III nitric oxide synthase (eNOS) gene expression in bovine pulmonary endothelial cells. *J. Vascular Res.* 33(Suppl. 1): 67(Abstr.).