Modulation of Renin-Angiotensin and Kallikrein Gene Expression in Experimental Hypertension

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Abstract
Previous studies have shown that chronic low-dose administration of 40 ng/min angiotensin II by osmotic minipump to uninephrectomized rats mimics the temporal hypertensive response and the circulating angiotensin II levels observed in two-kidney, one clip Goldblatt rats. Furthermore, renal tissue angiotensin II contents were higher than the circulating angiotensin II levels, suggesting that circulating angiotensin II induces endogenous intrarenal angiotensin II production. The present study examined the molecular mechanisms by which intrarenal angiotensin II production is modulated in angiotensin II-induced and two-kidney Goldblatt hypertension. Two weeks after clipping, intrarenal renin mRNA levels were elevated threefold in the clipped kidney of Goldblatt rats but were markedly suppressed in the non-clipped kidneys of Goldblatt rats (28% of control values) and in the remaining kidney of uninephrectomized angiotensin II-infused rats (7% of control values). In contrast, there were sustained levels of angiotensinogen mRNA in the kidneys and livers of Goldblatt and angiotensin II-infused rats, indicating differential regulation of the genes of the renin-angiotensin system. Renal kallikrein gene expression was not altered in either of the hypertensive groups 14 days after the induction of hypertension, suggesting the absence of an enhanced counteracting kinin influence. (Hypertension. 1994;23[suppl I]: I-131-I-136.)

Key Words: • hypertension, Goldblatt • hypertension, angiotensin II-induced • nephrectomy • RNA, messenger • angiotensinogen

It is well established that the onset of two-kidney, one clip (2K1C) hypertension is an angiotensin II (Ang II) –dependent phenomenon.1 Considerable evidence indicates that the contralateral kidney, although not the initiating factor, plays an important role in the development of 2K1C hypertension. The nonclipped kidney contains increased Ang II content despite marked renin depletion2,3 and exhibits an enhanced angiotensin converting enzyme (ACE) activity,4 In addition to increased ACE activation, however, sustained elevations of Ang II production would necessitate a continued supply of substrate. Accordingly, the primary objective of the present study was to delineate further the molecular mechanisms by which augmented intrarenal Ang II content occurs in 2K1C hypertensive rats. In view of the recent evidence implicating Ang II as a positive modulator of its own synthesis via stimulation of angiotensinogen (Ao) gene expression,5 we compared the responses in the 2K1C rats with those obtained in an Ang II–infused hypertensive model. A chronic infusion of Ang II (40 ng/min SC) approximated the levels of circulating Ang II found in the 2K1C rats 14 days after surgical placement of the renal arterial clip and resulted in a similar intrarenal angiotensin hormonal profile.4 By replacing the clipped kidney of the 2K1C model with an osmotic minipump as the source of circulating Ang II, we were able to examine the solitary effects of Ang II on the contralateral kidney, independent of other confounding humoral and neural factors that could mediate alterations in gene expression. These studies demonstrated that the onset and progression of 2K1C hypertension are mimicked by this chronic Ang II infusion.4 Evaluation of the renin-angiotensin hormonal profile indicated that intrarenal Ang II content and ACE activity were increased in the contralateral kidney of the 2K1C rats and the remaining kidney of the Ang II–infused rats.4 The elevated Ang II levels in the kidneys of the Ang II–infused animals were higher than could be explained by the circulating Ang II levels, indicating enhanced intrarenal Ang II generation elicited by the elevated circulating Ang II levels. The present study was designed to investigate possible molecular mechanisms by which enhanced endogenous Ang II production might occur in the 2K1C and Ang II–infused rats. With these two models, we examined the regulation of renin message in the kidney and Ao gene expression in the liver and kidney to determine if renin substrate was altered.

Considering the vasodilator and natriuretic role of intrarenal kinins,6 and thus the potential to serve as a counteracting force against the effects of high Ang II levels in the kidneys of 2K1C rats, we also investigated the response of the renal kallikrein gene in 2K1C and Ang II–induced hypertension.

Methods
Experimental Design
Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) were housed in wire cages and maintained in a temperature- and light-controlled room. Throughout the experiments, animals had free access to tap water and standard rat chow (Ralston-Purina, St Louis, Mo). All experiments were approved by the Tulane University Animal Care and Use Committee. At 180 to 200 g body weight, rats were divided into four groups, and surgery was performed with rats under pentobarbital anesthesia (30 mg/kg IP). A 0.25-mm silver clip was placed around the isolated right renal artery of the 2K1C
rats (n=6). Ang II–induced hypertensive rats (n=6) underwent a right nephrectomy, and osmotic minipumps (Alza Corp, Palo Alto, Calif) were implanted subcutaneously at the dorsum of the neck to chronically deliver Ang II (Sigma Chemical Co, St Louis, Mo) at a rate of 40 ng/min. This Ang II infusion rate has been shown to achieve circulating Ang II levels similar to those observed in 2K1C rats (72±15 versus 60±13 fmol/mL, respectively). Normal two-kidney rats (2K; n=6) were used as the controls for the 2K1C hypertensive animals. A sham operation was performed with the renal artery being isolated but not constricted by a silver clip. One-kidney rats (1K; n=6) were uninephrectomized, received a saline infusion by osmotic minipump, and therefore served as the controls for the Ang II–infused group.

Conscious blood pressures were measured by tail-cuff plethysmography (Harvard Apparatus, South Natick, Mass) to monitor the progression of hypertension. Fourteen days after surgical manipulation, rats were decapitated, and trunk blood was collected in chilled tubes containing EDTA for the analysis of plasma renin activity (PRA). The plasma was separated and stored at —20°C until assayed using a commercially available angiotensin I (Ang I) radioimmunoassay kit (Incstar Corp, Stillwater, Minn) as described previously. Tissues were immediately harvested, snap-frozen by immersion in liquid nitrogen, and then stored at —70°C for subsequent RNA extraction.

RNA Extraction and Hybridization

Total RNA was extracted using the guanidinium isothiocyanate–phenol–chloroform protocol described by Chomczynski and Sacchi. Denatured RNA was electrophoresed on a 1.2% agarose gel containing 2.2 mol/L formaldehyde. RNA was transferred to a positively charged synthetic nylon membrane (Zetabind, AMF Cuno, Inc, Meriden, Conn) using a vacuum apparatus (Vacugene XL, Pharmacia, Uppsala, Sweden) and cross-linked to the membrane with an ultraviolet (UV) source for 1 minute. UV shadowing at 254 nm allowed visualization of both 28S and 18S ribosomal RNA bands, confirming that the RNA was intact and that equal transfer was achieved. Slot blots were prepared by serial twofold dilutions (2.0 to 0.5 μg) of total RNA in sterile 25 mmol/L phosphate buffer (pH 7.2). RNA was applied to a Zetabind membrane with a Minifold II Slot-Blotter (Schleicher & Schuell, Inc, Keene, NH), and equal loading was assessed by UV shadowing at 254 nm.

Northern and slot blots were hybridized with full-length cDNAs for renin, Ao, and tissue kallikrein. Probes were labeled with [α-32P]deoxycytidine triphosphate ([32P]dCTP; Du Pont–NEN, Boston, Mass) by the random primer technique and purified on G-50 Sephadex Nicks columns (Pharmacia). Specific activity of labeled probes ranged from 1.57×106 to 1.05×109 cpm/μg DNA. After a prehybridization period of 1 hour at 65°C in 0.1× SSPE and 1.0% sodium dodecyl sulfate (SDS), the blots were hybridized with the 32P-labeled cDNAs for 20 hours at 65°C in 1% bovine serum albumin (BSA), 0.1% SDS, 1 mmol/L EDTA, and 0.25 mmol/L phosphate buffer with DNA herring sperm as a carrier (100 μg/mL). Blots were washed six times, for 10 minutes each, under high-stringency (65°C) conditions with a solution containing 0.1% SDS, 20 mmol/L phosphate buffer, 1 mmol/L

**Fig 1. Renal renin gene expression.** A and B, Autoradiographs of Northern blots (20 μg total RNA per lane) hybridized with a 32P-labeled renin cDNA, stripped, and then rehybridized with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe. C and D, Densitometric analysis of renal renin mRNA factored for GAPDH and expressed relative to two-kidney (2K) controls, which were given a value of 1.0. n indicates the number of samples per group of kidneys. *P<.05 vs 2K; tP<.05, clip vs nonclip of two-kidney, one clip (2K1C) animals. C, The right, clipped kidney (striped bar) shows a threefold increase in renin mRNA compared with the normal right kidney (open bar). The left kidneys of the 2K1C and angiotensin II (Ang II)–infused groups (filled bars) demonstrate significant suppression in renin gene expression compared with the 2K left counterpart. D, Renin message is significantly reduced in the Ang II–infused group (hatched bar) compared with the 2K (open bar) and one-kidney (1K) (filled bar) control groups.
Fig 2. Renal angiotensinogen (Ao) gene expression. A, A representative autoradiograph of Northern blots (20 μg total RNA per lane) hybridized with angiotensinogen- and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-radiolabeled probes. B, Densitometric analysis of the Northern blot for renal angiotensinogen mRNA presented in A, factored for GAPDH, and expressed relative to the left kidney of two-kidney (2K) controls, which were given a value of 1.0. C, Densitometric analysis of a slot blot hybridized with an angiotensinogen probe. Linear regression lines are presented for each kidney of the experimental groups; their similar slopes indicate no significant differences in gene expression. 1K indicates one-kidney controls.

**Results**

**Arterial Pressure and Plasma Renin Activity**

Systolic blood pressures in the 2K1C (n=6) and Ang II-infused (n=6) rats used in these studies averaged 171±7 and 167±8 mm Hg, respectively, and were significantly elevated compared with their respective controls (2K, 142±5 mm Hg, n=6; 1K, 130±3 mm Hg, n=6; P<.05). 2K1C rats exhibited significantly elevated increases in PRA, which averaged 12.58±2.06 ng Ang I·mL⁻¹·h⁻¹ compared with the 2K control animals (4.97±0.53 ng Ang I·mL⁻¹·h⁻¹; P<.05). PRA in Ang II-infused rats was dramatically suppressed to 0.14±0.10 ng Ang I·mL⁻¹·h⁻¹ compared with the 1K group, which averaged 4.26±0.45 ng Ang I·mL⁻¹·h⁻¹ (P<.05).

**Renal Renin Gene Expression**

Northern blots of total kidney RNA (20 μg per lane) were hybridized with full-length cDNA probes for renin and GAPDH, giving the expected 1.6- and 1.4-kb bands on the autoradiograms, respectively. As shown in Fig 1, 2 weeks after surgical placement of the arterial clip in the 2K1C group, renal renin message was elevated threefold in the right clipped kidneys and suppressed to 28% in the contralateral nonclipped kidneys compared with their respective right and left kidney 2K controls (P<.05). Renin mRNA in the remaining kidney of the Ang II-infused group was

**Data Analysis**

Autoradiographic signals were quantified by laser densitometry (Ultrascan XL, Pharmacia). Densitometric values represent the integration of the area under the curve corrected for the baseline background reading. Data are expressed as mean±SEM. Systolic blood pressure measurements and PRA values were statistically analyzed using one-way analysis of variance (ANOVA) and Fisher's least significant difference (FLSD) post hoc test. For the Northern blots, relative gene expression was factored for GAPDH and then statistically compared using ANOVA and FLSD. Pearson product-moment correlation was used to determine if significant relations were present between systolic blood pressure and hepatic and renal Ao gene expression. Regression lines were calculated for each tissue sample from the densitometric values of the three serial dilutions of the slot blots. Only those samples exhibiting linearity of the hybridization signals with values of r≥.90 were accepted for further consideration. The slopes from the various treatments were statistically compared by one-way ANOVA with repeated measures. Statistical significance was defined at a value of P<.05.
markedly suppressed to 7% to 10% of sham 2K levels \((P<.05, \text{Fig } 1\). The suppression of renin message in the Ang II-infused rats was independent of the contralateral nephrectomy because the 1K controls did not exhibit significant alterations in renin gene expression (86% of 2K controls).

Renal and Hepatic Angiotensinogen Gene Expression

Despite the marked differences in renin gene expression, Ao gene expression in the clipped and nonclipped kidneys of the 2K1C rats and the kidneys of the control 2K rats were not significantly different. Clipped and nonclipped kidney mRNA levels were 95% and 72% of control values, respectively \((P=.34\). As shown in Fig 2A and 2B, there was no significant modulation of Ao message in the contralateral kidney of 2K1C and the remaining kidney of Ang II-infused rats; renal Ao message factored for GAPDH was 70% and 82%, respectively, of normal 2K control values \((P=.20\). The more sensitive technique of slot blot analysis (serial dilutions of 2.0, 1.0, and 0.5 \(\mu\)g total RNA per slot) confirmed that there were no significant alterations in renal Ao gene expression in the kidneys of the various experimental groups. No significant correlations were found between relative Ao expression in the kidney and systolic blood pressure.

Northern blot analysis of hepatic Ao gene expression \((10 \mu\)g total RNA per lane\) revealed the expected 1.8-kb mRNA size, similar to that in the kidney (Fig 3). No significant alterations in the relative abundance of Ao message, corrected for GAPDH, were seen 14 days after surgical manipulation in either of the hypertensive groups. Relative to 2K control, 2K1C and Ang II-infused rats were 82% and 101% of normal values, respectively \((P=.44\). Hepatic Ao mRNA increased 20% in Ang II-infused rats and decreased 25% in 1K rats, but these changes did not reach statistical significance \((P=.20\). When the relation between hepatic expression of Ao and systolic blood pressure was examined, the uninephrectomized control group demonstrated a significant correlation \((P<.05\).

Renal Kallikrein Gene Expression

Northern blots \((20 \mu\)g total RNA per lane\) hybridized with cDNA probes for tissue kallikrein and GAPDH exhibited the anticipated 1.0- and 1.4-kb bands, respectively \((P<.05\). No significant modulation of kallikrein gene expression occurred in the clipped (74%) and contralateral (73%) kidneys of the 2K1C rats compared with normal 2K rats \((P=.29, \text{Fig } 4\). Likewise, kallikrein message was not significantly altered in the Ang II-infused and 1K rats \((P=.18, \text{Fig } 4\).

Discussion

The present data revealed that the renin message is enhanced threefold in the clipped kidney and markedly suppressed in the contralateral kidney 2 weeks after unilateral arterial clipping. The reduction in renin gene expression in the contralateral kidney is largely attributed to elevated levels of circulating Ang II and/or the resultant increases in arterial pressure because the Ang
II–infused animals exhibited a similar suppression of renin mRNA. These results confirm previous findings by other investigators10,11 and indicate that Ang II exerts a negative feedback effect on the renin gene. It also appears that this effect may be mediated via the type-I Ang II receptor (AT₁); a recent preliminary report by Tufro-McReddie and Gomez12 indicated a sevenfold increase in renal renin gene expression in losartan-treated (AT₁ antagonist) versus vehicle-treated rats. However, it should be recognized that Ang II alone may not be a sufficient inhibitory stimulus because the clipped kidney, subjected to the same circulating Ang II levels, exhibited enhanced renin gene expression.

The results of the present study did not demonstrate upregulation of hepatic and/or renal Ao gene expression in 2KIC and Ang II–infused rats. It must be emphasized, however, that the abundance of the Ao message was maintained in the two hypertensive models, thus allowing for the continued production of intrarenal Ang II. The current data agree with our previous studies in which Ao gene expression was examined in 2KIC rats 7 and 25 days after induction of hypertension.13 Renal Ao message was not altered at either stage, and hepatic Ao mRNA, although sustained at 7 days, was reduced 70% at 25 days. Likewise, Morishita et al10 reported no modulation in hepatic Ao gene expression 4 weeks after clipping. These findings differ from those of other investigators demonstrating increases in renal or hepatic Ao gene expression in response to exogenous administration of Ang II.5,14,15 In a recent study, Schunkert et al5 reported dose-dependent increases in both renal and hepatic steady-state Ao mRNA levels. These results were obtained at 3 days of infusion and with circulating levels of Ang II that were greater than values observed in either of our hypertensive groups. These temporal and dosage differences could account for the divergence in these reports. In addition, the lack of change in Ao gene expression in the remaining kidneys of Ang II–infused rats cannot be related to the effects of contralateral nephrectomy because the 1K uninephrectomized saline-infused rats also displayed no significant changes in hepatic or renal Ao gene expression. Although we cannot rule out that Ao gene expression was increased at an earlier stage, it was not apparent at 14 days. Thus, other mechanisms must be evaluated to determine the basis for the augmented intrarenal Ang II levels. The possibility that Ao mRNA levels may not reflect the expression of its protein should also be considered. Cassis16 has recently demonstrated normal circulating Ao levels in the diabetic rat despite a significant reduction in hepatic Ao message. Renal kallikrein gene expression was examined to clarify the role that the kallikrein-kinin system may play in the onset of 2KIC and Ang II–induced hypertension. Previous studies have documented decreases in renal kallikrein content and urinary excretion in the clipped kidney during the sustained phase of hypertension in 2KIC rats.17,18 A recent study documented a 50% downregulation of renal kallikrein mRNA levels at 25
days but not at 7 days after clipping. In the present study, no detectable differences were observed in the expression of kallikrein mRNA between the nonclipped and sham-operated kidney at 2 weeks. The lack of an increase in renal kallikrein gene expression in 2K1C or Ang II–infused rats therefore does not support the concept that kinins are counteracting the effects of Ang II in these hypertensive models. However, rigorous testing of this hypothesis requires further study.

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