Increased Vascular Angiotensin Formation in Female Rats Harboring the Mouse Ren-2 Gene

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Rats harboring the mouse Ren-2 transgene develop hypertension despite low levels of plasma renin activity. We tested the hypothesis that these rats exhibit an increase in vascular angiotensin formation caused by the presence of the transgene. We measured the release of angiotensins I and II from isolated perfused hindquarters by high-performance liquid chromatography and radioimmunoassay. Female rats heterozygous for the transgene had significantly elevated mean arterial pressure compared with control rats (189.3±9.5 versus 110.0±5.4 mm Hg, p<0.05). Plasma angiotensin II was significantly decreased in transgenic rats. Transgenic rat hindquarters released more angiotensin I (121±37 versus 39±12 fmol/30 min, n=7 each) and more angiotensin II (210±21 versus 62±12 fmol/30 min, p<0.05, n=7 each) than control rat hindquarters. Captopril increased angiotensin I release and decreased angiotensin II values in both transgenic and control rat hindquarters. Bilateral nephrectomy 24 hours before hindquarter perfusion greatly reduced angiotensin release from control rat hindquarters but not from transgenic rat hind limbs. We also tested for the presence of Ren-2 messenger RNA in mesenteric and aortic tissue by RNase protection assay and Northern blot analysis. We found that Ren-2 messenger RNA was present in mesenteric and aortic tissue of transgenic but not of control rats. We conclude that the Ren-2 transgene is expressed in vascular tissue of transgenic rats and may be responsible for substantial increases in vascular angiotensin formation. (Hypertension 1992; 19:687-691)

KEY WORDS • transgenic animals • renin • hind limb • converting enzyme inhibition

Recently, Mullins et al. 1 were successful in producing rats transgenic for the mouse Ren-2 gene. These rats develop severe hypertension; however, they exhibit low, rather than elevated, plasma levels of renin and circulating angiotensin II (Ang II) despite the expression of the Ren-2 gene in several tissues. 1 Nevertheless, the hypertension in these rats is readily responsive to low doses of angiotensin converting enzyme inhibitors. 1 However, no evidence has been published that Ang II production is increased in these animals.

During the past decade, attention has been drawn to local renin and angiotensin formation. Such systems have been described in the brain, heart, adrenal gland, and walls of blood vessels. 2-5 However, the role of local Ang II production in the regulation of vascular tone remains unclear. We and others have used the isolated perfused rat hindquarter to demonstrate the activity of the vascular renin system by measuring local formation of angiotensin peptides. 5-7

In the current study, we tested the hypothesis that rats transgenic for the mouse Ren-2 gene exhibit an increased formation of angiotensin peptides within the walls of blood vessels. We examined angiotensin release from isolated perfused rat hindquarters and demonstrated the expression of the transgene within the vessels of these rats. Our results provide direct evidence for increased blood vessel angiotensin formation related to the presence of the transgene.

Methods

Animals

We used 12–18-week-old female rats heterozygous for the mouse Ren-2 gene. 1 These rats were derived from cross-breeding homozygous, transgenic rats (strain number 27) with outbred Sprague-Dawley rats (ZI, Hannover, FRG). We compared these transgenic animals (TGpos) with female Sprague-Dawley outbred rats (TGneg) of similar age, derived from the same supplier. In addition, four TGneg and four TGpos rats underwent bilateral nephrectomy via flank incisions 24 hours before hindquarter perfusion.

Blood Pressure Measurements

Blood pressure was measured directly in six TGpos and six TGneg rats. Catheters were implanted in the left carotid artery under hexobarbital anesthesia. On the following day, arterial blood pressure was recorded on a polygraph recorder (Hellige, Freiburg, FRG) in conscious rats for 1 hour. Thereafter, blood for measurement of plasma Ang II was obtained from the arterial catheter. One-milliliter aliquots were sampled in syringes containing 50 μl inhibitor cocktail (125 mM EDTA and 26 mM ortho-phenanthroline). 6 These sam-
Hindquarter Perfusion

Preparation and perfusion of isolated rat hindquarters were performed as previously described. Briefly, rats underwent median laparotomy under thiobarbital anesthesia (60 mg/kg body wt i.p.). After evisceration, the abdominal aorta and inferior vena cava were cannulated and the perfusion was begun immediately. The hindquarters were perfused in a nonrecirculating system with modified Tyrode’s solution containing 2 g/l glucose and 20 g/l of the artificial colloid Ficoll 70 (Pharmacia, Freiburg, FRG). The perfusate was gassed with O2–CO2, adjusted to pH 7.4, and maintained at 38°C. The hindquarter perfusion was performed at a constant flow rate (10 ml/min) by using a two-channel peristaltic pump (Harvard Apparatus, South Natick, Mass.). Fractions of the perfusate were collected for biochemical analysis from the venous cannula. The perfusion pressure was monitored via a side arm of the aortic perfusion cannula by means of transducers connected to a polygraph.

Experimental Protocols

After an initial 30-minute period of baseline perfusion, Sep-Pak C18 cartridges (Waters Chromatography Div., Milford, Mass.) were connected on-line to the venous cannula for direct extraction of peptides as previously described. Per fusate was collected without the addition of enzyme inhibitors from seven TGpos and seven TGNeg rats for 30 minutes. Thereafter, the cartridges were changed, and captopril (10 μM) was added to the perfusate. Peptides were thus extracted from perfusate for another 30 minutes. Perfusate from hindquarters of nephrectomized rats was collected for 60 minutes. In addition, perfusate was also collected from perfusion channels (n=5) without hindquarters (blank, control channels) according to the same protocol.

Measurement of Peptides

Peptides were eluted from the Sep-Pak cartridges by using a previously described method. The eluates were lyophilized, and the dry residues were dissolved in 0.1 M Tris acetate buffer (pH 7.4) containing 0.1% bovine serum albumin. All perfusate samples were analyzed by high-performance liquid chromatography (HPLC) and subsequent radiomimmune assay (RIA) for angiotensin I (Ang I) and Ang II determination. For reverse-phase HPLC (gradient pump 2249, Pharmacia LBK, Freiburg, FRG), aliquots (500 μl) were loaded onto a 7-μm Nucleosil C18 column (Macherey Nagel & Co., Dueren, FRG) and eluted using a methanol gradient (35% for 10 minutes, then increasing to 80% over a period of 35 minutes) in a 10 mM ammonium acetate buffer, pH 5.4, at a flow rate of 1 ml/min and a temperature of 40°C. Fractions of 500 μl each were collected, gassed with nitrogen, and lyophilized. These fractions were redissolved in 250 μl of 0.1 M Tris acetate buffer, and peptides were quantified by RIA for Ang I and II. Synthetic angiotensins were used for calibration of the HPLC column. The sensitivity of the Ang I and II RIAs was 1 pg per tube. The cross-reactivity of the Ang I antibody (K18) was 0.05% for Ang II. Ang II in HPLC fractions was measured with an antibody (Celine, Reference 8) that exhibits 1% cross-reactivity with Ang I and 100% with both the Ang(2–8) heptapeptide (angiotensin III) and the Ang(3–8) heptapeptide. Ang II in plasma samples was measured using an antibody that does not cross-react with any angiotensin metabolite. RIAs were performed using a modification of previously described methods; bound and unbound tracers were separated by a second antibody, and polyethylene glycol was used instead of charcoal. All samples were estimated in duplicate.

Demonstration of Mouse Ren-2 Gene Expression in Vascular Tissue

The presence of mouse Ren-2 messenger RNA (mRNA) in mesenteric and aortic tissue was investigated both by mouse Ren-2–specific RNase protection assay and by Northern blot analysis. Endogenous rat renin in vascular tissues was examined by an RNase protection specific for rat renin. These methods have been described in more detail elsewhere. Briefly, tissue samples were taken immediately after rats had been killed by decapitation. Mesenteric tissue was dissected from the gut, and aortic tissue was freed from fat. Total RNA was isolated by homogenization in guanidine isothiocyanate. For the RNase protection assay, phosphorus-32–labeled RNA transcripts were prepared by transcription of a 244-nucleotide antisense RNA from the plasmid pSLM1 by using SP6 RNA polymerase, resulting in a 223-nucleotide mouse-specific protected fragment, or by transcription of a 315-nucleotide antisense RNA from plasmid pREN412. Samples were dissolved in 30 μl of 80% formamide containing 40 mM PIPES, 400 mM NaCl, 1 mM EDTA, and 200,000 cpm of the gel-purified transcript. They were then denatured at 100°C for 1 minute and incubated at 45°C for 20 hours. RNase digestion was performed in 300 μl buffer containing 40 μg/ml RNase A and 2 μg/ml RNase T1 for 45 minutes at 37°C. After digestion with proteinase K, samples were electrophoresed on denaturing 5% polyacrylamide gels. Northern blots were prepared and hybridized as previously described, with a phosphorus-32–labeled renin cDNA probe (pDD1D2), by random priming and were washed with 0.1x SSC and 0.1% sodium dodecyl sulfate at 65°C.

Statistical Analysis

Results are expressed as mean±SEM. The Mann-Whitney test was used to assess significant differences between TGpos and TGNeg rats. The Wilcoxon matched-pairs test was used to compare values before and after captopril treatment. A value of p<0.05 was considered significant.

Results

Mean arterial blood pressure was significantly higher in TGpos than in TGNeg rats (189.3±9.5 versus 110.0±5.4 mm Hg), with a very pronounced increase in diastolic blood pressure (162.2±9.9 mm Hg in TGpos).
Release of Angiotensin I

![Graph showing release of Angiotensin I](image1)

Release of Angiotensin II

![Graph showing release of Angiotensin II](image2)

FIGURE 1. Bar graphs showing spontaneous release of angiotensin from isolated hindquarters from female rats heterozygous for the mouse Ren-2 gene (TGpos) and from outbred female Sprague-Dawley rats (TGneg) during 30 minutes of perfusion, measured by radioimmunoassay (mean±SEM, n=7 each). Upper panel: Angiotensin I (ANG I). Lower panel: Angiotensin II (ANG II). +Significant differences between control period and captopril; *significant differences between TGpos and TGneg.

versus 96.3±2.4 mm Hg in TGneg rats, p<0.05). Plasma Ang II was significantly lower in TGpos animals (32±10 fmol/ml) than in TGneg rats (98±23 fmol/ml). During hindquarter perfusion, baseline perfusion pressure was significantly higher in TGpos than in TGneg rats (29.5±2.3 versus 21.1±2.3 mm Hg).

The release of Ang I and II from perfused hindquarters is shown in Figure 1. Hindquarters of TGpos rats released significantly more Ang II (p<0.05) and Ang I than TGneg hindquarters did. Captopril increased Ang I and decreased Ang II release significantly from both TGpos and TGneg (p<0.05) rat hindquarters (Figure 1). Typical examples of HPLC runs are shown in Figure 2. In the presence of captopril, significantly more Ang I was released from TGpos than from TGneg hindquarters (Figure 1).

After bilateral nephrectomy, TGpos rat hindquarters released 415±63 fmol/30 min Ang I and 398±164 fmol/30 min Ang II, whereas angiotensin release was greatly reduced in TGneg rats (p<0.05). In addition, the RNase protection assay (Figure 3) demonstrated the mRNA for the Ren-2 gene in mesenteric and aortic tissue of TGpos but not of TGneg rats. Expression of the rat renin gene could not be detected under the conditions used here (Figure 3). Northern blot analysis detected renin mRNA only in TGpos but not in TGneg rat vascular tissues (Figure 4).

Discussion

Rats heterozygous for the mouse Ren-2 gene exhibit severe hypertension and low plasma Ang II values. We demonstrated the expression of the transgene in vascular tissue of these rats and found substantially increased local angiotensin formation in isolated perfused rat hindquarters. Vascular angiotensin formation in TGpos rat hindquarters was still present after bilateral nephrectomy. These data provide direct evidence for increased local tissue levels of Ang II in TGpos rats. The local Ang II production may have contributed to the severe hypertension in these rats.

Rats harboring the Ren-2 gene of DBA/2 mice develop severe hypertension that is fatal at an early age in homozygous animals. We used heterozygous female rats...
in this study. These animals exhibit less severe hypertension than homozygous males do, and they live longer. The transgene is expressed in a number of tissues. For instance, very high levels of transgene mRNA are detected in the adrenal glands. The reasons for the fulminating hypertension are only beginning to be understood. Renal renin, plasma renin, and plasma Ang II values are actually lower in TGpos rats than in TGneg control rats. However, the striking efficacy of converting enzyme inhibitors on blood pressure in TGpos rats supports the notion that Ang II is nonetheless involved. Our data support that notion and indicate that angiotensin production in the vasculature may be in part responsible.

Our data are the first to show increased local Ang II production at the tissue level in TGpos rats. The susceptibility of vascular angiotensin formation to captopril demonstrates that the peptide formation is due to renin, since no other Ang I–producing proteases are active at pH 7.4,11,12 Our data did not allow us to determine whether the peptide was formed by the original rat renin or by a product of the transgene. However, vascular angiotensin production in TGpos rats was independent of renal renin, as shown by its persistence after nephrectomy. Further, the transgene was highly expressed in vascular tissue, while expression of the original rat renin gene was not detectable when an equal amount of RNA was used. These observations support the notion that the expression of the transgene in the vessel wall is by far the most likely reason for the stimulated vascular angiotensin production in TGpos rats.

Vascular renin may cleave Ang I, either from locally synthesized or from plasma-derived angiotensinogen. Hellmann et al13 showed that angiotensinogen is expressed within the vessel wall. Naftilan and colleagues14
extended these observations by means of in situ hybridization. They localized angiotensinogen production to the smooth muscle cells. We previously found increased circulating angiotensinogen levels in rats with low plasma renin values.\textsuperscript{15} These rats nevertheless had local Ang II production no different from control rats. However, the elevated angiotensinogen levels did not stimulate vascular Ang II production in that model, despite a decrease in angiotensin degradation.\textsuperscript{15} We have no data on vascular angiotensinogen synthesis in TGpos rats; however, plasma angiotensinogen values were not increased. Thus, it appears unlikely that changes in plasma angiotensinogen levels were responsible for increased vascular angiotensin production in TGpos rats.

There is convincing indirect evidence that substantial vascular angiotensin generation may occur in vivo.\textsuperscript{3,16} The effective formation of Ang I from angiotensinogen and of Ang II from Ang I can be reproduced in vitro.\textsuperscript{5,6} Whether the renin, which is active in the vessel wall, is derived from local synthesis or from uptake of plasma renin is still a matter of debate. Attempts to demonstrate renin gene expression in vascular tissue have thus far yielded controversial results.\textsuperscript{17,18} In normal rats, the prevailing evidence suggests that vascular renin is taken up from the circulation.\textsuperscript{6,19} Renin activity in the vessel wall decreases sharply to levels below the detection limit after bilateral nephrectomy.\textsuperscript{19,20} Similarly, angiotensin release from the rat hind limb is not detectable in animals that underwent prior bilateral nephrectomy.\textsuperscript{5} Furthermore, the addition of exogenous renin stimulates both vascular renin activity\textsuperscript{20} and angiotensin release from perfused rat hind limbs.\textsuperscript{5} Our inability to measure expression of the original renin gene in normal rats in this study does not necessarily imply the absence of gene expression. We did not design our blots for that purpose. For instance, we did not use poly(A)-purified RNA or the polymerase chain reaction, which may improve the detection threshold.\textsuperscript{17,18} Instead, our data demonstrate that expression of the original renin gene in vascular tissue is not detectable under conditions that readily allow detection of the transgene's expression.

Our data show that rats harboring the mouse Ren-2 transgene provide a model of hypertension with increased vascular angiotensin production. Our data do not allow us to determine the quantitative importance of the increased vascular angiotensin formation. However, several observations suggest that vascular angiotensin formation may contribute to the high blood pressure in Ren-2 transgenic rats. The exquisite responsiveness of TGpos rats to converting enzyme inhibition may be due to inhibition of vascular angiotensin formation. The exceedingly high diastolic blood pressure may indicate a vascular mechanism in TGpos hypertension. The elevated baseline perfusion pressure in TGpos rats suggests that changes in the vessel wall had already occurred before perfusion.\textsuperscript{21} Finally, in addition to the effects of hypertension per se, trophic actions of locally produced Ang II may have contributed to vessel wall hypertrophy in TGpos rats.\textsuperscript{22,23}

In summary, the present data show that vascular angiotensin production is enhanced in rats harboring the DBA/2 mouse Ren-2 transgene. The increased local angiotensin production in these rats is most likely caused by the transgene, which is expressed in vascular tissue. Vascular angiotensin generation in transgenic rats may contribute to high blood pressure and blood vessel hypertrophy. Further studies are required to elucidate the importance of vascular angiotensin formation relative to other tissue renin systems in Ren-2 transgenic rats.

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References

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