Blood Pressure–Independent Effects in Rats With Human Renin and Angiotensinogen Genes

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Abstract—The blood pressure–independent effects of angiotensin II (Ang II) were examined in double transgenic rats (dTGR) harboring human renin and human angiotensinogen genes, in which the end-organ damage is due to the human components of the renin angiotensin system. Triple-drug therapy (hydralazine 80 mg/L, reserpine 5 mg/L, and hydrochlorothiazide 25 mg/L in drinking water) was started immediately after weaning. Triple-drug therapy normalized blood pressure and coronary resistance, only partially prevented cardiac hypertrophy, and had no effect on ratio of renal weight to body weight. Although triple-drug therapy delayed the onset of renal damage, severe albuminuria nevertheless occurred. Semiquantitative scoring of ED-1–positive and MIB-5–positive (nuclear cell proliferation–associated antigen Ki-67) cells showed profound perivascular monocyte/macrophage infiltration and cell proliferation in kidneys and hearts of untreated dTGR. Triple-drug therapy had only a minimal effect on local inflammatory response or vascular cell proliferation. In contrast, a novel orally active human renin inhibitor (HRI), 30 mg/kg by gavage for 4 weeks, normalized blood pressure and coronary resistance and also prevented cardiac hypertrophy and albuminuria. ED-1–positive cells and MIB-5–positive cells were decreased by HRI in hearts and kidneys almost to levels observed in normotensive Sprague-Dawley rats. The renoprotective effects of HRI were at least in part due to improved renal hemodynamics and distal tubular function, since HRI shifted renal pressure-diuresis/natriuresis curves leftward by ≈35 mm Hg, increased glomerular filtration rate and renal blood flow, and shifted the fractional water and sodium excretion curves leftward. In untreated dTGR, plasma Ang II was increased by 400% and renal Ang II level was increased by 300% compared with Sprague-Dawley rats. HRI decreased plasma human renin activity by 95% and normalized Ang II levels in both plasma and kidney compared with triple-drug therapy. Our findings indicate that in dTGR harboring human renin and angiotensinogen genes, Ang II causes end-organ damage and promotes inflammatory response and cellular growth largely independent of blood pressure. (Hypertension. 2000;35:587-594.)

Key Words: renin n angiotensinogen n angiotensin II n albuminuria n cell proliferation n natriuresis

A nsscher and Anson1 demonstrated a vascular permeability factor causing arterial necrosis in malignant hypertension. Subsequently, other investigators reported similar findings and presented evidence that angiotensin II (Ang II) was responsible.2–4 More recent studies indicated that Ang II can be synthesized not only in the blood compartment but also locally in the tissues.5 Furthermore, alternative pathways to the angiotensin-converting enzyme may exist for local Ang II generation.6,7 Ang II acts as a direct trophic factor for smooth muscle cells and cardiac myocytes in vitro.8,9 The effects are mediated through the AT1 receptor and involve activation of signal transduction pathways associated with cell growth.10 Upregulation of the local renin-angiotensin system (RAS) is involved in hypertension-related injury.11 Ang II promotes mesangial cell hypertrophy and extracellular matrix production in vitro via synthesis of superoxide anion12 and growth factors such as platelet-derived growth factor13 and transforming growth factor-β.14 Several studies suggest that Ang II may control vascular wall cell proliferation in vivo.15,16 Whether induction of cellular growth by Ang II in vivo is due to hemodynamic effects or to direct cellular actions is not invariably clear. We showed recently that Ang II–induced cardiac hypertrophy and renal damage in the double transgenic rats harboring human renin and angiotensinogen genes (dTGR) is mainly dependent on the local tissue RAS.17 In dTGR, RAS blockade by a human renin inhibitor (HRI) at nonantihypertensive doses protected against Ang II–induced end-organ damage.18 We also reported that nuclear transcription factor–κB (NF-κB)–mediated adhesion molecule induction plays a central role in the pathogenesis of end-organ damage.19

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damage. We now tested the hypothesis that Ang II can cause end-organ damage, inflammation, and cell proliferation independent of blood pressure in this model. We normalized blood pressure in dTGR with non-RAS-dependent triple-drug therapy (hydralazine, reserpine, hydrochlorothiazide). We compared this treatment to the actions of a novel, longer-acting HRI.

Methods
Experiments were conducted in 45 four-week-old male dTGR (body weight, 51±2 g) and in 15 normotensive Sprague-Dawley rats (53±2 g). The dTGR line and characteristics are described elsewhere. The rats were purchased from Biological Research Laboratories Ltd (Füllinsdorf, Switzerland) and were allowed free access to standard 0.3% sodium rat chow (SSNIF Spezialitäten GmbH) and drinking water. The study was approved by the local Council on Animal Care (permit G408/97), whose standards correspond to those of the American Physiological Society. dTGR were divided into 3 groups: (1) control group (n=15), (2) HRI group (n=15), and (3) triple-drug therapy group (n=15). HRI was given by gavage once a day (30 mg/kg) for 4 weeks. This novel HRI (RO 66-1132) has improved bioavailability compared with remikiren. In a pilot study, this dose produced maximal human renin inhibition in dTGR. Triple-drug therapy was given in drinking water (hydrochlorothiazide 25 mg/L, reserpine 5 mg/L, hydralazine 80 mg/L) as described. Control dTGR and Sprague-Dawley rats received vehicle by gavage. Systolic blood pressure was measured weekly by the tail-clamp method under light ether anesthesia ~24 hours after the last drug dose, starting at age 4 weeks. Urine samples were collected over a 24-hour period in metabolic cages at 6, 7, and 8 weeks. Rats were killed under thiopental (150 mg/kg IP) at age 8 weeks. Blood for hormone analysis was drawn by aortic puncture into prechilled tubes containing EDTA (6.25 mmol/L) and phenanthroline (26 mmol/L) as anticoagulant and inhibitor of Ang II breakdown in vitro, respectively. Remikiren (1 μmol/L) was added to plasma samples for Ang II measurement to prevent Ang II formation in the tissue. The heart and kidneys were washed with ice-cold saline, blotted dry, and weighed. To examine the effects of chronic drug treatments on coronary resistance, rats (n=5 to 6 in each group) were heparinized and retrograde perfused in a Langendorff apparatus under constant flow anesthetized with thiopental (150 mg/kg IP). The hearts were then excised and the isolated left ventricle was mounted in a fundus perfusion cuvette, which was maintained at 37°C. Pressure was continuously measured throughout the experiment and recorded on a computer system (TSE GmbH).

Human plasma renin activity (PRA), rat PRA, human angiotensinogen, and rat angiotensinogen were measured as described previously. For PRA (50 μL) and angiotensinogen (25 μL) measurements, plasma was incubated for 2 hours at 37°C in the presence and absence of the human specific renin inhibitor remikiren (2 μmol/L). Generated Ang I was measured by direct radioimmunoassay. Human PRA and human angiotensinogen were calculated by subtraction of Ang I generation in the presence of remikiren from total Ang I generation (in the absence of remikiren). Ang II was measured from plasma and kidney tissue according to the method described by van Kats et al. with some modifications. The Ang II recovery from renal tissue was 90%. Urinary rat albumin was measured with a commercially available enzyme-linked immunosorbent assay with rat albumin used as a standard (Immun Diagnostik).

Data are presented as mean±SEM. Statistically significant differences in mean values were tested by ANOVA and Tukey’s multiple range test. A value of P<0.05 was considered statistically significant. The data were analyzed with SYSTAT statistical software (SYSTAT Inc.).

Results
Systolic blood pressure and 24-hour albumin excretion increased progressively in untreated dTGR (Figure 1A and 1B). Both HRI and triple-drug therapy normalized blood pressure in dTGR (Figure 1A). HRI prevented the development of albuminuria (Figure 1B). Although triple-drug therapy delayed the onset of renal damage, severe albuminuria eventually developed (Figure 1B). Cardiac hypertrophy, coronary resistance, and ratio of kidney weight to body weight were markedly increased in untreated dTGR (Figure 2). Triple-drug therapy partially prevented the development of cardiac hypertrophy and normalized coronary resistance but had no effect on relative kidney weight. Cardiac hypertrophy, coronary resistance, and relative kidney weight in HRI-treated dTGR were indistinguishable from values observed in Sprague-Dawley rats.

Plasma and kidney Ang II concentrations were 4- to 5-fold higher in untreated dTGR than in Sprague-Dawley rats.
HRI decreased plasma and kidney Ang II levels to levels observed in normotensive Sprague-Dawley rats, whereas triple-drug therapy had no effect on circulating and tissue Ang II concentrations. Ang II release from the isolated perfused heart was 2-fold higher in dTGR than in Sprague-Dawley rats (Ang II content in the coronary effluent collected for 20 minutes after 15-minute washout period, 19.0±3.9 pg Ang II per 20 minutes in dTGR versus 9.0±1.8 pg Ang II per 20 minutes in Sprague-Dawley rats; P<0.05). HRI decreased human PRA by 95% but did not influence rat PRA, human angiotensinogen, or rat angiotensinogen. Triple-drug therapy had no significant effects on plasma RAS parameters (Figure 3C through 3F).

There was a profound perivascular monocyte/macrophage infiltration in the kidney (Figure 4A through 4D) and heart (data not shown) of untreated dTGR. HRI prevented local monocyte/macrophage infiltration, whereas triple-drug therapy had only a modest effect on inflammatory response in the kidney and the heart (Figure 5A). In untreated dTGR, the number of MIB-5–positive cells in the kidney and heart vascular wall was ~4-fold higher than that of Sprague-Dawley rats (Figure 5B). HRI prevented vascular cell proliferation, whereas the triple therapy had only a modest effect on the number of MIB-5–positive cells in the kidney and heart.

Figure 6 (top) shows the effects of chronic HRI treatment on pressure-natriuretic and -diuretic responses in dTGR. In
untreated dTGR, urinary flow and sodium excretion averaged 6.9±0.6 μL/min per gram kidney weight and 0.4±0.2 μmol/min per gram kidney weight, respectively, at the RPP level of 115 mm Hg. Increasing the RPP to 203 mm Hg in these rats was accompanied by an increase in urinary flow and sodium excretion to 121±16 μL/min per gram kidney weight and 32.5±5.5 μmol/min per gram kidney weight, respectively. HRI shifted the pressure-natriuresis and -diuresis curves leftward by ~35 mm Hg. Figure 6 (middle) shows the relationships between RPP, RBF, and GFR. In untreated dTGR, RBF averaged between 2.8±0.4 and 3.9±0.9 mL/min per gram kidney weight, and GFR averaged between 0.6 and 1.0 mL/min per gram kidney weight. HRI increased RBF by 35% and GFR by 65% to 100%. Figure 6 (bottom) shows fractional sodium and fractional water excretion. In untreated dTGR, fractional sodium and fractional water excretion averaged 0.4±0.2% and 1.5±0.4% at the RPP level of 115 mm Hg and increased to 23.4±3.0% and 15.2±2.3% when RPP was increased to 203 mm Hg. HRI treatment shifted the fractional water and sodium excretion curves leftward.

Discussion

Hypertension and end-organ damage in dTGR are due to the human components of the RAS. Cardiac hypertrophy, renal damage, and severe vasculopathy with pronounced perivascular inflammation and fibrinoid necrosis are mainly due to local Ang II formation in the heart, kidney, and vasculature. We tested the hypothesis that Ang II would cause end-organ damage and induce inflammatory responses and cell proliferation locally in the heart and kidney, independent of systemic blood pressure. We therefore normalized blood pressure in dTGR by a previously described, non–RAS-dependent, triple-drug therapy. The most important finding in our study was that Ang II caused severe renal damage in normotensive dTGR. Furthermore, Ang II induced pronounced monocyte infiltration and vascular cell proliferation in both the kidney and the heart. Our results provide direct evidence that Ang II causes end-organ damage and promotes inflammatory response and cellular growth, independent of blood pressure.

Data from several reports demonstrate that Ang II stimulates the growth of vascular smooth muscle cells and cardiac...
myocytes in vitro. In animals, Ang II infusion typically causes marked hemodynamic changes. Therefore, the direct growth-promoting effects of Ang II are more difficult to evaluate in vivo. Nevertheless, Griffin et al. successfully demonstrated that Ang II causes vascular hypertrophy in rats in part by a nonpressor mechanism. More recently, Su et al. showed convincing evidence that Ang II infusion, when given concomitantly with the vasodilator hydralazine, has a direct, blood pressure–independent effect on cell proliferation in rat mesenteric vessels and carotid arteries. Mazzolai et al. demonstrated that local Ang II production induces myocardial hypertrophy independent of blood pressure in transgenic mice overexpressing the rat angiotensinogen gene in the heart. Furthermore, Montgomery et al. showed that inhibition of tissue angiotensin-converting enzyme activity at nonhypotensive doses prevented malignant hypertension in rats bearing the mouse renin (mREN2) gene. These data suggest that Ang II at the tissue level is capable of vasculotoxic effects, independent of blood pressure. We showed here that Ang II induces inflammatory responses and vascular cell proliferation in the kidney and heart through blood pressure–independent mechanisms. We recently reported that monocyte infiltration and overexpression of adhesion molecules in the kidney and heart are mediated, at least in part, via nuclear transcription factor NF-κB. Evidence suggests that Ang II induces superoxide anion production in the kidney via activation of membrane-bound NAD(P)H oxidase. NF-κB activity is regulated by oxygen radicals. Thus, it is tempting to speculate that in dTGR, oxidative stress activates the genes encoding adhesion molecules, chemokines, and cytokines, all of which are dependent on NF-κB activity. This hypothesis must be tested in further detail.

We measured Ang II concentrations in the plasma and renal tissue with radioimmunoassay after sample purification and separation of the angiotensin peptides by high-performance liquid chromatography. Ang II concentrations were consistently 4- to 5-fold higher in dTGR than in Sprague-Dawley rats. HRI decreased human renin activity by 95% and reduced Ang II concentrations in the plasma and kidney almost to levels observed in Sprague-Dawley rats, indicating that Ang II formation in the circulation and locally in tissues can be effectively blocked by human renin inhibition in dTGR. Neither PRA nor Ang II concentrations were markedly affected by triple-drug therapy, supporting the previous notion that the combination of hydralazine, reserpine, and hydrochlorothiazide decreases blood pressure by RAS-independent mechanisms.
We showed previously that both human renin and human angiotensinogen genes are expressed in the kidney, indicating that dTGR are capable of generating Ang II locally. Other studies demonstrated that high circulating Ang II may also augment intrarenal Ang II content in a tissue-specific manner through AT1 receptor activation. In contrast to the kidney, we were unable to detect the expression of the human renin gene in the heart. However, the markedly increased Ang II concentrations in the coronary effluent of the isolated perfused heart strongly support the existence of a cardiac RAS with local Ang II formation in dTGR. We showed recently that circulating renin can be taken up by cardiac tissue. We suggest that the local Ang II formation in the dTGR hearts is linked to plasma-derived renin uptake.

We showed earlier that the rightward shift in renal pressure-natriuresis and -diuresis relationships in dTGR depends on mechanisms inherent to the kidneys themselves and that blockade of RAS by an angiotensin-converting enzyme inhibitor or AT1 receptor antagonist shifts the pressure-natriuresis and -diuresis curves toward normal. In the present study we examined the effects of an orally active HRI on the pressure-natriuresis and -diuresis relationships.

The HRI shifted the curves approximately 35 mm Hg leftward. Improvement in the pressure-natriuresis/diuresis mechanism is likely due to renal vasodilatation since the HRI increased both RBF and GFR. However, the HRI also shifted fractional sodium and water curves leftward, suggesting that changes in distal tubular function may also be responsible. Our findings are in good agreement with recent studies demonstrating that HRI induce renal vasodilatation in healthy volunteers.

In an earlier study, we used a different renin inhibitor that provided good end-organ protection but had only a transient blood pressure–lowering effect. Renin inhibition has particular appeal since renin is the primary rate-limiting step in Ang II production. Renin inhibition may provide advantages over angiotensin-converting enzyme inhibition, since possible conversion of Ang I to Ang II via chymases is not an issue. Furthermore, chronically elevated Ang II concentrations, as observed with AT1 receptor blockade, are also avoided. Ang II is still able to occupy the AT2 receptor with AT1 blockade. The consequences of this occupancy are not fully known. For instance, the generation of RANTES by Ang II may occur via the AT2 receptor.

Interpretation of our data presents some difficulties because of inherent limitations. The model is necessarily artificial and was developed with the testing of HRI in mind. However, there are similarities to the results reported here and earlier data presented from 2-kidney, 1 clip hypertension.
another Ang II–dependent model. Whitworth et al produced malignant hypertension with pathology similar to what we observed here by introducing genetic susceptibility. They performed a cross between hypertensive transgenic mREN-2 rats and normotensive Sprague-Dawley (Edinburgh) rats. They observed that male F1 hybrids developed malignant hypertension with a penetrance of 75%. Fibrinoid necrosis and microangiopathic hemolytic anemia were prominent features in their model. Their results suggest the presence of genetic susceptibility loci. Thus, our results may not only involve the actions of Ang II but also susceptibility in the particular strain of rats used to develop our model. Another limitation stems from the fact that we did not perform telemetry. We cannot claim to have completely normalized arterial pressure without measuring pressure beat-to-beat for the entire study period. Nevertheless, blood pressure was markedly ameliorated by triple-drug therapy, which only delayed but did not prevent vascular damage.

In conclusion, the present study was undertaken to examine the blood pressure–independent effects of Ang II on vascular structure and function. We lowered blood pressure in dTGR by a non–RAS-dependent triple-drug therapy. In normoten- sive dTGR, Ang II nevertheless caused severe renal damage and induced perivascular inflammation, as well as vascular cell proliferation in the kidney and heart. Thus, our results provide direct evidence that Ang II causes end-organ damage and promotes inflammatory response and cellular growth independent of blood pressure.

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