Combined inhibition of 20-hydroxyeicosatetraenoic acid formation and of epoxyeicosatrienoic acids degradation attenuates hypertension and hypertension-induced end-organ damage in Ren-2 transgenic rats

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Abstract

Recent studies have shown that the renal CYP450 (cytochrome P450) metabolites of AA (arachidonic acid), the vasoconstrictor 20-HETE (20-hydroxyeicosatetraenoic acid) and the vasodilator EETs (epoxyeicosatrienoic acids), play an important role in the pathophysiology of AngII (angiotensin II)-dependent forms of hypertension and the associated target organ damage. The present studies were performed in Ren-2 renin transgenic rats (TGR) to evaluate the effects of chronic selective inhibition of 20-HETE formation or elevation of the level of EETs, alone or in combination, on the course of hypertension and hypertension-associated end-organ damage. Both young (30 days of age) prehypertensive TGR and adult (190 days of age) TGR with established hypertension were examined. Normotensive HanSD (Hannover Sprague–Dawley) rats served

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as controls. The rats were treated with N-methylsulfonyl-12,12-dibromododec-11-enamide to inhibit 20-HETE formation and/or with N-cyclohexyl-N-dodecyl urea to inhibit soluble epoxide hydrolase and prevent degradation of EETs. Inhibition in TGR of 20-HETE formation combined with enhanced bioavailability of EETs attenuated the development of hypertension, cardiac hypertrophy, proteinuria, glomerular hypertrophy and sclerosis as well as renal tubulointerstitial injury. This was also associated with attenuation of the responsiveness of the systemic and renal vascular beds to AngII without modifying their responses to noradrenaline (norepinephrine). Our findings suggest that altered production and/or action of 20-HETE and EETs plays a permissive role in the development of hypertension and hypertension-associated end-organ damage in this model of AngII-dependent hypertension. This information provides a basis for a search for new therapeutic approaches for the treatment of hypertension.

INTRODUCTION

Chronic kidney disease and end-stage renal disease present severe medical problems; their incidence has been increasing steadily, especially in industrialized countries [1]. For both conditions, hypertension remains one of the most important risk factors even though antihypertensive treatment has significantly advanced over the recent decades [2]. It is generally accepted that inappropriate activation of the RAS (renin–angiotensin system) is a major factor in the development of AngII (angiotensin II)-dependent forms of hypertension [3]. However, the role of the RAS in the pathophysiology of hypertensive target organ damage is still poorly understood.

It is well recognized that CYP450 (cytochrome P450)-dependent metabolites of AA (arachidonic acid), 20-HETE (20-hydroxyeicosatetraenoic acid) and EETs (epoxyeicosatrienoic acids), play an important role in the regulation of renal tubular ion transport and renal and systemic vascular tone [4,5]. Moreover, recent studies strongly suggest that altered production and/or action of CYP450-dependent metabolites contribute to the development of AngII-dependent forms of hypertension [6–11]. The hypertensive rat transgenic for the mouse Ren-2 renin gene [TGR; strain name TGR(mRen2)27] represents a unique AngII-dependent animal model in which the development of hypertension is attributable to a single gene alteration [12]. We have found recently that TGR exhibit increased intrarenal levels of 20-HETE and, simultaneously, an intrarenal deficiency of EETs [13]. 20-HETE is a vasoconstrictor and is commonly regarded as a natriuretic agent, a combination of properties forming the background for both its pro- and anti-hypertensive potential [4,6,7]. On the other hand, we have recently provided evidence that 20-HETE may induce antinatriuresis in TGR [13]. EETs exhibit antihypertensive properties related to their vasodilator and natriuretic potency [4–6].

In the present study, we tested the hypothesis that in TGR, a monogenic model of AngII-dependent hypertension, increased intrarenal 20-HETE combined with intrarenal deficiency of EETs may at least partially account for the enhanced renal and systemic vascular responsiveness to AngII and thereby contribute to the development and/or maintenance of hypertension. To examine this possibility, we evaluated the possible antihypertensive effects of chronic selective inhibition of 20-HETE formation and of an elevation of EET levels in young prehypertensive heterozygous TGR rats; the effect of the treatment on the associated hypertension-induced end-organ damage was also examined. In addition, to make the study more relevant to the clinical condition of hypertensive patients, we assessed the effects of chronic selective inhibition of 20-HETE production and of enhanced bioavailability of EETs on blood pressure and target organs in adult TGR with established hypertension.

Finally, to gain a more detailed insight into the mechanism(s) underlying the potential beneficial (antihypertensive) effects of selective pharmacological interventions into the CYP450-dependent metabolism of AA, experiments were performed in which systemic and renal vascular responses to AngII and NE (norepinephrine) [norpinephrine]] were evaluated in TGR and HanSD (Hannover Sprague–Dawley) rats. TGR and HanSD rats were either untreated or exposed to chronic reduction of 20-HETE production, or to an increase in the level of EETs, or they were subjected to both (combined) treatments at the same time.

MATERIALS AND METHODS

Ethical approval and animals

The studies were performed in accordance with guidelines and practices established by the Animal Care and Use Committee of the Institute for Clinical and Experimental Medicine. All animals used in the present study were bred at the Department of Experimental Medicine of the Institute for Clinical and Experimental Medicine, Prague, from stock animals supplied by the Max Delbrück Center for Molecular Medicine, Berlin, Germany, which
is accredited by the Czech Association for Accreditation of Laboratory Animal Care. The animals were kept on a 12-h/12-h light/dark cycle. Throughout the experiments, rats were fed a normal salt, normal protein diet (0.45 % NaCl, 19–21 % protein) produced by SEMED and had free access to tap water.

Chemicals
DDMS [(N-methylsulfonyl-12,12-dibromododec-11-enamide), a selective inhibitor of 20-HETE formation [14], was administered using osmotic minipumps implanted at the dorsal neck; the concentration used allowed the delivery of 2 mg/day. As tested in our preliminary experiments, this dose blocks 20-HETE formation selectively and persistently [13].

NCND (N-cyclohexyl-N-dodecyl urea) selectively inhibits sEH (soluble epoxide hydrolase), the enzyme responsible for the conversion of EETs into DHETEs (dihydroxyicosatetraenoic acids), the biologically inactive metabolites of EETs. NCND was administered i.p. (intraperitoneally) at a dose of 10 mg · kg⁻¹ of body weight · day⁻¹, as described previously [15]. It has been demonstrated that this dose selectively blocks sEH activity and increases the bioavailability of EETs [16].

Experimental protocols
Series 1: inhibition of 20-HETE formation and of sEH activity starting in young prehypertensive rats (early treatment protocol)
Male heterozygous TGR rats, aged 28 days, and age-matched male HanSD rats from several litters were randomly assigned to experimental groups, to make sure that the animals from a single litter did not prevail in any of the groups. Beginning from 30 days of age, SBP (systolic blood pressure) was measured every second day in appropriately trained conscious animals by tail-plethysmography, using a tail-cuff apparatus (MC 4000; Hatteras Instruments); in all cases, a mean SBP of four measurements was taken. This approach (MC 4000; Hatteras Instruments) is regularly employed in our laboratory, and a close correlation between tail-plethysmography and direct blood pressure measurements with an indwelling catheter was established [11,17,18]. At 80 and 140 days of age, the animals were placed in individual metabolic cages, and after appropriate habituation training, their 24-h urine was collected for sodium and water excretion and protein determination. At the end of the experiment (150 days of age), animals were again placed in metabolic cages, food was withheld to prevent contamination of urine samples and 12-h urine samples were collected on dry ice. The samples were stored at −80 °C until assayed. The urinary 20-HETE, EETs and DHETEs concentrations were measured by ELISA using commercially available kits, according to the manufacturer’s instructions (Detroit R&D Inc.). Thereafter, rats were killed by decapitation, and AngII levels in plasma, whole right kidney and left ventricular heart tissue were measured by RIA as described in detail in our previous studies [19–21]. We used the LVW/TL (ratio of left ventricular weight to tibial length) to evaluate the degree of cardiac hypertrophy, since we have demonstrated previously that this is the most suitable index to assess cardiac hypertrophy [21].

To assess renal glomerular damage, the left kidney was quickly removed, fixed in 4 % formaldehyde, dehydrated and embedded in paraffin. The sections stained with haematoxylin–eosin and PAS (periodic acid–Schiff reaction) were examined and evaluated in a blind-test fashion. Fifty glomeruli in each kidney were examined on a semiquantitative scale as described previously [22]: grade 0, entire glomerulus normal; grade 1, sclerotic area up to 25 % (minimal sclerosis); grade 2, sclerotic area 25–50 % (moderate sclerosis); grade 3, sclerotic area 50–75 % (moderate-to-severe sclerosis); grade 4, sclerotic area 75–100 % (severe sclerosis). The GSI (glomerulosclerosis index) was calculated using the following formula: GSI = [((1 × n₁) + (2 × n₂) + (3 × n₃) + (4 × n₄))/ (n₂ + n₁ + n₂ + n₃ + n₄)], where n₄ is the number of glomeruli in each grade of glomerulosclerosis.

Cortical tubulointerstitial injury was evaluated as described by Nakano et al. [23] for inflammatory cell infiltration, tubular dilatation and/or atrophy, or interstitial fibrosis and was graded semiquantitatively using the following scale: grade 0, no abnormal findings; 1, mild (< 25 % of the cortex); 2, moderate (25–50 % of the cortex); 3, severe (> 50 % of the cortex). Lesions were assessed for at least 30 random and non-overlapping fields in the renal cortex.

Morphometric evaluation of the glomerular volume was made in the same kidney sections that were examined for morphological changes, using the method developed by Weibel [24] and validated by Lane et al. [25] using the Nikon NIS-Elements AR 3.1 morphometric program. Briefly, this method consists of determination of mean glomerular profile area and calculation of mean glomerular volume from the following formula: glomerular volume = area³ × 1.38/1.01, where 1.38 is β, the shape coefficient for a sphere, and 1.01 is the size distribution coefficient assuming a 10 % coefficient of variation.

Since osmotic minipumps (M 2006; Alzet) have an operating time of 42 days, they were replaced by new minipumps containing DDMS on days 68 and 108 of the experiment. Control rats received osmotic minipumps containing saline vehicle and i.p. saline vehicle at the same time as treated rats. The following experimental groups were investigated: (i) HanSD rats + vehicle (n = 15); (ii) TGR + vehicle (n = 16); (iii) TGR + DDMS (n = 18); (iv) TGR + NCND (n = 16); and (v) TGR + DDMS + NCND (n = 18).
Series 2: inhibition of 20-HETE formation and of sEH activity starting in adult hypertensive rats (late treatment protocol)

In this series, animals were randomly assigned to experimental groups and remained untreated from weaning until 188 days of age. At the age of 189 days, the appropriate treatment(s) was started as described above. At 185, 260 and 300 days of age, the animals were placed in individual metabolic cages, and their 24-h urine was collected for protein determination. New minipumps containing DDMS were implanted on days 228 and 268 of the experiment. At the end (310 days of age), the animals were again placed individually in metabolic cages, and their 12-h urine was collected for determination of 20-HETE, EETs and DHETEs as described above. The following experimental groups were investigated: (i) HanSD rats + vehicle (n = 12); (ii) TGR + vehicle (n = 15); (iii) TGR + DDMS (n = 15); (iv) TGR + NCND (n = 15); and (v) TGR + DDMS + NCND (n = 15).

Series 3: MAP (mean arterial pressure) and RBF (renal blood flow) responses to AngII and NE during inhibition of 20-HETE formation and/or of sEH in the early treatment protocol

In this series, TGR and HanSD rats were subjected to the same experimental protocols as described above (series 1). At the end of the experiments (150 days of age), the animals were fasted overnight and anaesthetized with sodium thiopental (50 mg/kg of body weight). They were placed on a thermoregulated table to maintain body temperature at 37–37.5°C. A tracheotomy was performed to maintain a patent airway, and the exterior way ANOVA was employed when appropriate. The differences within each experimental group. One-way repeated-measures ANOVA was used to detect

Statistical analysis and calculations

Statistical analysis of the data was performed using Graph-Pad Prism software (GraphPad Software). All values are expressed as means ± S.E.M. Two-way repeated-measures ANOVA was used to detect differences within each experimental group. One-way ANOVA was employed when appropriate. The RVR (renal vascular resistance) was calculated as the MAP/RBF ratio during the 30-min equilibration period, using a computerized data-acquisition system (PowerLab/4SP; ADInstruments). Statistical significance was defined as P < 0.05.
RESULTS

Series 1: effects of ‘early’ inhibition of 20-HETE formation or/and of sEH activity

As shown in Figure 1(A), DDMS treatment did not alter the development or the final level of hypertension in TGR, whereas NCND did retard its development significantly. The increase in the pressure was less steep in NCND-treated rats than in untreated TGR, and at the age of 79 days, the SBP levels were 181 ± 4 and 196 ± 4 mmHg, respectively (P < 0.05). Thereafter, the progress of hypertension was similar, and the final SBP did not significantly differ between NCND-treated and untreated TGR (202 ± 6 compared with 199 ± 5 mmHg, respectively). In contrast, the combined treatment with DDMS and NCND both attenuated the progression of hypertension and lowered the final SBP to a level that was significantly different from that in untreated TGR (178 ± 3 compared with 199 ± 5 mmHg, P < 0.05).

Nevertheless, the level was still distinctly higher than that in HanSD rats, which remained normotensive throughout the experiment.

Figure 1(B) shows that HanSD rats exhibited only minor proteinuria over the entire duration of the experiment. In TGR, a pronounced proteinuria was observed already during the early phase of hypertension, at the age of 80 days [26.4 ± 2.8 mg · (24 h)−1 compared with 6.5 ± 1.4 mg · (24 h)−1 seen in HanSD rats]. The proteinuria further increased to 34.8 ± 2.2 mg · (24 h)−1 in the phase of established hypertension (day 140 of age). The treatment with DDMS did not affect the degree of proteinuria in TGR. On the other hand, NCND attenuated proteinuria during the early but not in the established phase of hypertension. In contrast, combined treatment with DDMS and NCND distinctly ameliorated the proteinuria in the early as well as in the established phase of hypertension [8.7 ± 1.5 and 12.2 ± 1.1 mg · (24 h)−1, respectively, compared with
26.4 ± 2.8 and 34.8 ± 2.2 mg·(24 h)−1 observed in untreated TGR (P < 0.05 for both the early and the established phase).

As shown in Figure 1(C), untreated TGR developed distinct cardiac hypertrophy, expressed as the ratio of LVW/TL, which was not attenuated by DDMS or NCND given alone. In contrast, the combined treatment with DDMS and NCND substantially attenuated the degree of cardiac hypertrophy (25.9 ± 0.6 compared with 32.6 ± 0.7 observed in untreated TGR, P < 0.05); however, the levels observed in HanSD rats were not attained.

As shown in Figure 1(D), untreated TGR exhibited a GSI that was substantially higher than in HanSD rats (0.64 ± 0.05 compared with 0.11 ± 0.05, P < 0.05). The treatment with DDMS or NCND given alone did not prevent the development of glomerulosclerosis. In contrast, the combined treatment with DDMS and NCND significantly reduced the GSI, even though it did not decrease to the level observed in HanSD rats.

As shown in Figure 2(A), untreated TGR had markedly higher degree of kidney tubulointerstitial injury, compared with HanSD rats (0.025 ± 0.005 compared with 0.002 ± 0.001, P < 0.05). The treatment with DDMS alone did not attenuate the injury in TGR. In contrast, the treatment of TGR with NCND alone markedly inhibited the development of kidney tubulointerstitial injury, compared with untreated TGR, and the combined treatment with DDMS and NCND completely prevented the development of the injury (Figure 2A).

The glomerular volume in untreated TGR was significantly greater than in HanSD rats (1.43 ± 0.065 compared with 1.08 ± 0.025 × 106 μm3, P < 0.05) (Figure 2B). The treatment with DDMS alone did not change the volume in TGR compared with untreated TGR. In contrast, the treatment of TGR with NCND alone distinctly reduced the volume, and the combined treatment with DDMS and NCND normalized it, bringing the values down to the levels observed in HanSD rats.

As shown in Figure 3(A), the urinary excretion of 20-HETE in untreated TGR was significantly higher than in HanSD rats [1480 ± 165 compared with 460 ± 90 ng (12 h)−1, P < 0.05]. The treatment with NCND did not alter the urinary excretion of 20-HETE. However, as expected, the treatment with DDMS alone, or the combined treatment with NCND and DDMS significantly reduced 20-HETE excretion down to the levels observed in HanSD rats.

In contrast, urinary excretion of EETs was significantly lower in TGR than in HanSD rats [640 ± 120 compared with 1295 ± 110 ng (12 h)−1, P < 0.05]. Treatment with DDMS did not alter EETs excretion, whereas the treatment with NCND, alone or in combination with DDMS, significantly increased it to the levels observed in HanSD rats (Figure 3B).

Figure 3(C) shows that the urinary excretion of DHETEs, the biologically inactive metabolites of EETs, was significantly higher in untreated TGR than in HanSD rats [920 ± 60 compared with 490 ± 60 ng (12 h)−1, P < 0.05]. The treatment with DDMS did not significantly change DHETEs excretion, but treatment with NCND alone or in combination with DDMS significantly decreased it to the levels observed in HanSD rats.

Figure 3(D) summarizes the results on the intrarenal availability of biologically active epoxygenase metabolites when expressed as the EETs/DHETEs ratio. This ratio was significantly lower in untreated TGR than in HanSD rats (0.7 ± 0.07 compared with 2.64 ± 0.76, P < 0.05). It was not significantly changed by DDMS treatment. However, treatment with NCND, alone or
in combination with DDMS, significantly increased this ratio to the levels observed in HanSD rats.

 Plasma, kidney and left heart ventricle AngII levels were significantly higher in untreated TGR than in HanSD rats; in the former, the levels were not altered by any of the three treatment modalities. In addition, urinary output and urinary sodium excretion were not significantly different among experimental groups at any time of the experiment (see Supplementary Table S1 available at http://www.ClinSci.org/cs/118/cs1180617add.htm).

**Series 2: effects of ‘late’ inhibition of 20-HETE formation and/or sEH activity**

As shown in Figure 4(A), untreated TGR remained distinctly hypertensive throughout the experiment, with a final SBP of 204 ± 5 mmHg, whereas HanSD rats were invariably normotensive, with a final SBP of 138 ± 3 mmHg. In TGR, the treatment with DDMS did not significantly change SBP at any time point. Within the first 8 days of treatment, NCND significantly decreased SBP from 204 ± 6 to 181 ± 3 mmHg ($P < 0.05$), but during the further 10 days, it returned to the levels observed in untreated TGR, reaching a final value of 202 ± 7 mmHg. In contrast, the combined treatment with DDMS and NCND resulted in a distinct decrease in SBP from 205 ± 4 to 179 ± 2 mmHg ($P < 0.05$) within 8 days. Thereafter, despite a gradual return of the pressure towards the original hypertensive levels, SBP remained significantly lower than in untreated TGR (191 ± 2 compared with 204 ± 5 mmHg, $P < 0.05$).

Figure 4(B) shows that on day 185, the basal values of proteinuria were markedly higher in TGR than in HanSD rats. This pattern was not reduced by chronic treatment with either DDMS or NCND alone. In contrast, the combined treatment with DDMS and NCND decreased proteinuria from 47.4 ± 4.8 to 34.9 ± 1.1 mg·(24 h)$^{-1}$ ($P < 0.05$). This was still substantially higher than the low
Figure 4 Changes in SBP (A), proteinuria (B), heart left ventricle weight to tibia length ratio (C) and GSI (D) in untreated TGR (heterozygous Ren-2 renin transgenic rats) and HanSD (transgene-negative) rats, and in TGR treated either with DDMS or NCND, or their combination.

Results are taken from the ‘late’ treatment protocol. *P < 0.05 compared with pretreatment values. #P < 0.05 compared with untreated TGR; @P < 0.05 compared with the values in each of TGR groups.

Levels of proteinuria observed in HanSD rats throughout the entire experiment.

As shown in Figure 4(C), untreated TGR had a markedly higher LVW/TL index than observed in HanSD rats (35.8 ± 0.9 compared with 22.4 ± 0.6, P < 0.05), which was not altered by DDMS or NCND treatment given alone, but was reduced by the combined treatment with DDMS and NCND to 32.1 ± 0.4 (P < 0.05). Nevertheless, the LVW/TL index remained significantly higher than in HanSD rats.

As shown in Figure 4(D), untreated TGR exhibited a significantly higher GSI than did HanSD rats (0.98 ± 0.07 compared with 0.19 ± 0.04, P < 0.05); the index was not reduced by any of the three treatment modalities.

As shown in Figure 5(A), untreated TGR showed a markedly higher degree of kidney tubulointerstitial injury compared with HanSD rats (0.19 ± 0.04 compared with 0.02 ± 0.01, P < 0.05). The treatment with DDMS or NCND given alone did not reduce the kidney tubulointerstitial injury score in TGR. In contrast, the combined treatment with DDMS and NCND substantially attenuated the injury but did not bring it down to the levels observed in HanSD rats (Figure 5A).

The glomerular volume in untreated TGR was significantly greater in than in HanSD rats (1.65 ± 0.095 compared with 1.22 ± 0.084 × 10⁶ μm³, P < 0.05) (Figure 5B). The treatment with DDMS or NCND alone did not change the glomerular volume in TGR compared with untreated TGR. In contrast, the combined treatment with DDMS and NCND normalized it, bringing the values down to the levels observed in HanSD rats.

Figure 6(A) shows that the urinary excretion of 20-HETE was markedly higher in untreated TGR than...
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Figure 5
Kidney tubulointerstitial injury score (A) and glomerular volume (B) in untreated TGR (heterozygous Ren-2 renin transgenic rats) and HanSD (transgene-negative) rats and in TGR treated with DDMS or NCND or their combination

Results are taken from the ‘late’ treatment protocol. \#P < 0.05 compared with untreated TGR; \@P < 0.05 compared with the values for the remaining four groups.

Figure 6
Series 3: MAP and RBF responses to AngII and NE in the ‘early’ treatment protocol
Basal values for MAP, RBF and RVR (average values from the equilibration period) are summarized in Supplementary Table S3 (available at http://www.ClinSci.org/cs/118/cs1180617add.htm). As shown in Figure 7(A), the responses of MAP to intravenous bolus administration of AngII, 50 ng, were significantly greater in untreated TGR than in HanSD rats (+32 ± 3 compared with +14 ± 2 mmHg, P < 0.05). Treatment with either DDMS or NCND alone did not modify the responses, whereas the combined treatment with DDMS and NCND did attenuate them significantly. However, the responses of MAP remained significantly greater than in HanSD rats.

Similarly, as shown in Figure 7(B), the intrarenal administration of AngII, 10 ng, decreased RBF significantly more in untreated TGR than in HanSD rats (−31 ± 3 compared with −14 ± 2%, P < 0.05). The treatment with either DDMS or NCND alone did not attenuate the RBF decreases, whereas in contrast, a distinct attenuation was seen with the combined treatment (−22 ± 2 compared with −31 ± 3% in untreated TGR, P < 0.05). As shown in Figures 7(A) and 7(B), an intravenous or intrarenal administration of NE elicited similar increases in MAP and decreases in RBF in TGR and HanSD rats; in the former, the responses were not altered by any of the three treatment modalities.

Series 4: MAP and RBF responses to AngII and NE in the ‘late’ treatment protocol
Basal values for MAP, RBF and RVR (average values from the equilibration period) are summarized in Supplementary Table S3. As shown in Figure 8(A), an intravenous bolus of 50 ng of AngII increased MAP...
Figure 6 Urinary excretion of 20-HETE (A), EETs (B), DHETEs (C) and the urinary EETs to DHETEs excretion ratio (D) in untreated TGR (heterozygous Ren-2 renin transgenic rats) and HanSD (transgene-negative) rats, and in TGR treated with DDMS or NCND, alone or combined

Results are taken from the 'late' treatment protocol. *$P < 0.05$ compared with untreated TGR.

significantly more in untreated TGR than in HanSD rats ($+35 \pm 3$ compared with $+16 \pm 3$ mmHg, $P < 0.05$). The treatment with either DDMS or NCND alone did not alter the responses, whereas the combined treatment did attenuate them significantly. Similarly, as shown in Figure 8(B), the intrarenal bolus administration of AngII, 10 ng, elicited significantly larger decreases in RBF in untreated TGR than in HanSD rats ($−35 \pm 4$ compared with $−16 \pm 2\%$, $p < 0.05$). The responses were not modified by treatment with either DDMS or NCND alone but were significantly reduced by the combined treatment with DDMS and NCND.

As shown in Figures 8(A) and 8(B), an intravenous or intrarenal administration of NE elicited similar increases in MAP and decreases in RBF in TGR and HanSD rats; in the former, the responses were not altered by any of the three treatment modalities.

**DISCUSSION**

After initial controversies regarding the role of the RAS in the pathophysiology of hypertension in TGR [12,28,29], more recent studies have clearly demonstrated that enhanced formation of AngII leading to elevation of its plasma and kidney tissue levels, and an exaggeration of peripheral and renal vascular responsiveness to AngII, are the main pathogenetic factors in the development and maintenance of hypertension in TGR [19,20,26,27]. Notwithstanding this conclusion, the important role of CYP450-derived eicosanoids in the regulation of renal function and blood pressure as well as in the pathophysiology of hypertension and the associated hypertension-induced end-organ damage has recently been documented for AngII-dependent forms of hypertension [6–11,16,30–32]. Moreover, disturbed intrarenal production and diminished action of
20-HETE, combined with intrarenal deficiency of EETs (a consequence of increased sEH-mediated conversion into biologically inactive DHETEs), have been implicated in the pathophysiology of hypertension in TGR [11,13]. Evaluation of the proposed in vivo functional role of 20-HETE and EETs is difficult. One potential approach is to apply pharmacological interventions aimed at an abolishment or substantial reduction of the agents’ activity. This requires their chronic selective blockade and prolonged follow-up studies of transgenic rats subjected to the treatment and parallel studies of appropriate control animals. The present study evaluates the effect of such blockade on the development of hypertension and of the end-organ damage in TGR.

20-HETE has been shown to promote vasoconstriction via mechanisms involving inhibition of large-conductance, calcium-activated potassium channels [33], activation of calcium channel conductance leading to depolarization of the VSMC (vascular smooth muscle cell) [34] and activation of Rho-kinase, which causes sensitization of the contractile apparatus to calcium [35]. 20-HETE is also known to augment vascular reactivity to vasoconstrictor agents [36,37] and to play a critical role as a second messenger for AngII-mediated vasoconstriction [7,8]. Overall, it has been claimed that enhanced formation of 20-HETE in the vasculature is the basis of its prohypertensive action and contributes to the development and maintenance of hypertension in some experimental models [7–9,30,38].
In addition to its action on the vasculature, 20-HETE inhibits sodium reabsorption in the proximal tubule [39] and in the thick ascending limb of the loop of Henle [40]. Accordingly, it has been postulated that increased 20-HETE levels along the renal tubule should promote natriuresis and thereby oppose its constrictor action on the vasculature and the development of hypertension [4,6,30]. The notion that intrarenal 20-HETE also exerts an antihypertensive action is strengthened by the demonstration that chronic blockade of 20-HETE formation promotes the development of salt-sensitive hypertension in otherwise salt-resistant rats [41]. In a clear contrast with the transport inhibitory effect of 20-HETE observed in normotensive rats and in a number of experimental or genetic hypertensive rat models, in a recent study, we found that in hypertensive TGR, increased intrarenal 20-HETE concentrations cause sodium retention without decreasing renal haemodynamics, which suggests an increase in the tubular sodium transport [13]. Thus, in hypertensive TGR, 20-HETE could be prohypertensive both owing to its vasoconstrictor and to the sodium-retaining activity.

EETs have been consistently shown to cause vasodilation through stimulation of the large-conductance, calcium-activated potassium channels (K_{Ca}) [5]. They have also been identified as an EDHF (endothelium-derived hyperpolarizing factor) that mediates nitric oxide- and prostaglandin-independent vasodilatation, and have been found to oppose the vasoconstrictor actions of AngII [42–45]. Moreover, EETs inhibit tubular sodium reabsorption in the renal proximal tubule [46] and in the cortical collecting duct [47]. Lastly, an increasing body of evidence suggests that EETs exhibit cardiovascular and renal protective actions [48].

A major finding of the present study is that combined chronic inhibition of 20-HETE synthesis (DDMS) and of sEH activity (NCND), when applied in young prehypertensive TGR, significantly attenuated the development of hypertension and had considerable nephroprotective and cardioprotective effects, including reduction of proteinuria, glomerulosclerosis, kidney tubulointerstitial injury and cardiac hypertrophy. This was associated with reduction of urinary excretion of 20-HETE and DHETEs, and elevation of urinary excretion of EETs to the levels observed in normotensive HanSD rats. Of special interest is our finding that nephroprotective effects of the combined treatment with DDMS and NCND were associated with restoration of glomerular volume to values observed in normotensive HanSD rats. Given a strong correlation between the glomerular size and the degree of glomerulosclerosis, these findings are in good agreement with the demonstration that the nephroprotective effect of antihypertensive therapy is also associated with the reduction of the size of the glomerulus [49,50].

Remarkably, isolated chronic 20-HETE inhibition did not significantly affect the development of hypertension or of the end-organ damage, even though the urinary excretion of 20-HETE was reduced to levels close to those in the animals treated with the combination of DDMS and NCND. Similarly, chronic isolated inhibition of sEH increased the intrarenal availability of biologically active epoxygenase metabolites 3-fold, but elicited only transitory antihypertensive effects and did not alter the extent of the final end-organ damage. Nevertheless, it is important to emphasize that in TGR, the treatment with NCND alone exhibited some minor nephroprotective actions, e.g. the degree of tubulointerstitial injury was reduced, and glomerular hypertrophy was prevented. However, these nephroprotective effects were minimal compared with those of the combined treatment with DDMS and NCND. On the whole, the present findings agree well with those of our previous studies with TGR, which showed that altered production and/or action of CYP450-derived metabolites contributes to the development of hypertension and of the associated end-organ damage [11,13]. However, this is the first study clearly showing that only the combination of the blockade of 20-HETE formation and of sEH inhibition exerts long-term antihypertensive and renal and cardiovascular protective actions in the monogenic model of AngII-dependent hypertension.

It is difficult to explain why the beneficial effects were achieved exclusively with the combined DDMS and NCND treatment. Since in most situations 20-HETE has both pro- and anti-hypertensive properties, one cannot easily predict the consequence of its inhibition on blood pressure [4,6]. However, we have shown earlier that in TGR rats, 20-HETE causes sodium retention rather than natriuresis [13], which means that both components, its vascular and tubular actions, should promote the development of hypertension. Therefore, we assumed that in this AngII-dependent model, a chronic and selective inhibition of 20-HETE formation should be more effective in attenuating blood pressure elevation, and possibly also the associated organ damage, than could be expected in other models of hypertension. However, we found that although DDMS treatment lowered the elevated urinary excretion of 20-HETE to the levels observed in normotensive HanSD rats, it did not exhibit any antihypertensive and organ-protective action. Moreover, chronic treatment with DDMS did not alter plasma and kidney AngII levels and did not attenuate the enhanced systemic and renal vascular responsiveness to AngII.

Similar to the effects of specific 20-HETE inhibition, our TGR chronic inhibition of sEH activity alone by NCND did not exhibit long-term antihypertensive and organ-protective actions, even though the availability of biologically active epoxygenase products was substantially increased. Most probably, it is not tissue EETs or
tissue 20-HETE but the elevated AngII concentrations and the augmented vascular responsiveness to AngII, which are the dominant factors in the development of hypertension and in the end-organ damage in this transgenic AngII-dependent model. This is in contrast to the results obtained in rats rendered hypertensive by chronic infusion of AngII in whom the endogenous RAS activity is substantially suppressed [3,16]. In these animals, isolated pharmacological manipulations of the CYP450 pathway of AA metabolism clearly reduced blood pressure and the end-organ damage [6,32,51]. Overall, the results strengthen our earlier conclusions that the substantial chronic elevation of RAS activity is the crucial step in the pathophysiology of hypertension in the TGR model [19,20,27].

We found that the combination of suppression of 20-HETE production with an elevation of EETs can partially counteract the vasoconstrictor actions of chronically elevated circulating and tissue AngII levels in TGR and can partly attenuate the development of hypertension and hypertension-induced end-organ damage. The reasons why the combined treatment was generally effective while the isolated treatment, e.g. with NCND, was not, may be complex. It should be kept in mind that in TGR with established hypertension, the isolated inhibition of sEH did not lower blood pressure or attenuate proteinuria, whereas the combined treatment had appreciable effects. This could be related to the very high basal sEH activity (in TGR the DHETE excretion increased with age), and also to a high rate of 20-HETE synthesis; the urinary excretion of 20-HETE was almost twice that seen in young TGR and 7-fold higher than in old HanSD. One might speculate that the extremely high 20-HETE synthesis diminished the amount of the substrate AA available for the synthesis of EETs and decreased their synthesis rate, in accordance with the repeatedly proposed hypothesis assuming ‘substrate shifts’ between the individual pathways of AA metabolism [4,52–55]. At a reduced EETs synthesis rate, an inhibition of their degradation with NCND alone would not increase available EETs very effectively. On the other hand, a simultaneous suppression of 20-HETE synthesis (DDMS treatment) would, in addition to the specific direct effect, make the AA substrate available for the epoxygenase subpathway of CYP450-dependent AA metabolism, and at a high EETs synthesis rate, their action would increase more than with NCND treatment alone; the final result would be an appreciable reduction of blood pressure and proteinuria. It is commonly recognized that antihypertensive therapy is more effective when applied early during the development rather than during established hypertension [56]. Therefore, in a separate series of experiments, we evaluated the effects of chronic blockade of 20-HETE formation and sEH inhibition on blood pressure and on proteinuria, an index of kidney damage, in the TGR with sustained elevation of blood pressure, i.e. a condition that more closely resembles clinical hypertension. We found that in this setting – similar to the first series of experiments – a reduction in blood pressure, proteinuria and cardiac hypertrophy was seen only with the combined treatment with DDMS and NCND. The improvement was associated with a reduction in the urinary excretion of 20-HETE and DHETEs and an increase in the urinary excretion of EETs to levels observed in normotensive HanSD rats. Thus, the findings strengthen our conclusion based on the results of ‘early’ treatment experiments. It should be noticed, however, that the amelioration of end-organ damage was significantly smaller than that observed when the same treatment was started immediately after weaning.

After the late-onset combined therapy, proteinuria and cardiac hypertrophy were only modestly attenuated, whereas tubulointerstitial injury was markedly attenuated, and the glomerular hypertrophy was prevented. There was no difference between untreated TGR and TGR treated with the combination of DDMS and NCND with regard to the index of glomerulosclerosis – this was so, despite a similar blood pressure-lowering effect observed with the early and late-treatment protocols. The failure of the combined treatment to inhibit the progress of glomerulosclerosis could be related to the fact that renal tissue AngII and 20-HETE progressively increased in TGR and were much higher in the phase of established hypertension than in the young prehypertensive rats. It should be noticed that AngII, among other actions, activates Ras/mitogen-activated protein kinase by increasing the synthesis of CYP4A-derived 20-HETE; this stimulates proliferation of the VSMCs [57–60]. One might speculate that prolonged exposure of the glomeruli to increasing concentrations of AngII resulted in proliferation-based changes that could not be reversed by our combined therapy. Interestingly, cardiac hypertrophy could still be attenuated by the combined treatment, possibly because the plasma concentration, unlike kidney tissue AngII, did not increase with the age of the TGR. Overall, in the TGR with established hypertension, alterations of 20-HETE and EETs activities have only limited effects on end-organ damage. This suggests that the CYP450-dependent active agents play a ‘permissive’ rather than causal role in the pathophysiology of hypertension and the associated end-organ damage in TGR.

We found that only the combined blockade of 20-HETE formation and of sEH activity significantly attenuated MAP and RBF responses to bolus injections of AngII; this was similar when examined in young prehypertensive TGR and in the rats with established hypertension. The responses to NE were not altered. These findings are in accordance with the previous studies showing that TGR display an exaggerated systemic and renal vascular reactivity to AngII [26,27].
However, our present findings are the first to clearly show that, in contrast to rats rendered hypertensive by chronic subpressor AngII infusion [7,8,16,28,32,51], in TGR, an augmented vascular responsiveness to AngII is not attenuated by an isolated blockade of 20-HETE formation or an isolated enhancement of EETs bioavailability. The fact that an alteration of the activities of the two types of CYP450 metabolites does result in an appreciable attenuation of the vasoconstrictor indication that an augmentation of the vascular responsiveness to AngII is one of the mechanism(s) underlying the beneficial antihypertensive actions of combined DDMS and NCND treatment in TGR.

In summary, the results show that selective blockade of 20-HETE formation and of sEH activity, when applied chronically and in combination in young prehypertensive TGR, suppresses the development of hypertension and provides a substantial protection from the associated cardiac hypertrophy, proteinuria and glomerulosclerosis. Also, in adult TGR with established hypertension, the combined treatment with DDMS and NCND reduced blood pressure and slightly decreased proteinuria and cardiac hypertrophy. We found also that a reduction in 20-HETE combined with an increase in EETs levels attenuated the enhanced vascular responsiveness to AngII in TGR. Taken together, the findings strongly suggest that alteration in the production and/or action of 20-HETE and EETs plays a permissive role in the development of hypertension and hypertension-associated end-organ damage in this AngII-dependent model. This information derived from our present study should be considered in attempts at the development of new therapeutic approaches or tools for the treatment of early stages of human hypertension.

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Combined inhibition of 20-hydroxyeicosatetraenoic acid formation and of epoxyeicosatrienoic acids degradation attenuates hypertension and hypertension-induced end-organ damage in Ren-2 transgenic rats

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1 These authors contributed equally to the study and should be considered as joint first authors.

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Table S1  Kidney and left heart ventricle AngII levels and urinary sodium and water excretion in individual experimental groups

Results are taken from the ‘early’ treatment protocol. Values are means ± S.E.M. HanSD, transgene-negative normotensive rats; TGR, heterozygous Ren-2 renin transgenic hypertensive rats. *P < 0.05 compared with the corresponding values in the other four groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameter</th>
<th>Age (days) . .</th>
<th>80</th>
<th>140</th>
<th>150</th>
<th>80</th>
<th>140</th>
<th>150</th>
<th>80</th>
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<th>150</th>
<th>80</th>
<th>140</th>
<th>150</th>
</tr>
</thead>
<tbody>
<tr>
<td>HanSD+vehicle</td>
<td>Plasma AngII (fmol/ml)</td>
<td></td>
<td>19 ± 3*</td>
<td>53 ± 4</td>
<td>56 ± 4</td>
<td>51 ± 4</td>
<td>56 ± 3</td>
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<tr>
<td>TGR+vehicle</td>
<td></td>
<td></td>
<td>42 ± 2*</td>
<td>110 ± 10</td>
<td>97 ± 5</td>
<td>95 ± 4</td>
<td>96 ± 4</td>
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<tr>
<td>TGR+DDMS</td>
<td>Heart AngII (fmol/g)</td>
<td></td>
<td>24 ± 2*</td>
<td>32 ± 2</td>
<td>33 ± 2</td>
<td>33 ± 3</td>
<td>34 ± 3</td>
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<tr>
<td>TGR+NCND</td>
<td>Kidney AngII (fmol/g)</td>
<td></td>
<td>654 ± 48</td>
<td>672 ± 51</td>
<td>694 ± 65</td>
<td>637 ± 41</td>
<td>688 ± 56</td>
<td>684 ± 61</td>
<td>634 ± 55</td>
<td>652 ± 48</td>
<td>614 ± 73</td>
<td>664 ± 56</td>
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<tr>
<td>TGR+DDMS+NCND</td>
<td>Na excretion (μmol/24 h)</td>
<td>8.8 ± 0.7</td>
<td>9.2 ± 0.6</td>
<td>8.9 ± 0.9</td>
<td>9.4 ± 1.1</td>
<td>8.7 ± 0.8</td>
<td>8.9 ± 0.9</td>
<td>9.6 ± 0.8</td>
<td>8.5 ± 0.9</td>
<td>9.1 ± 0.5</td>
<td>9.2 ± 0.8</td>
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<tr>
<td></td>
<td>Water excretion (ml/24 h)</td>
<td>8.8 ± 0.6</td>
<td>9.9 ± 0.8</td>
<td>9.7 ± 0.9</td>
<td>9.6 ± 0.6</td>
<td>9.7 ± 0.7</td>
<td>9.1 ± 0.7</td>
<td>9.2 ± 0.9</td>
<td>9.9 ± 0.5</td>
<td>9.2 ± 0.5</td>
<td>9.1 ± 0.4</td>
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</table>

Table S2  Kidney and left heart ventricle AngII levels and urinary sodium and water excretion in individual experimental groups

Results are taken from the ‘late’ treatment protocol. Values are means ± S.E.M. HanSD, transgene-negative normotensive rats; TGR, heterozygous Ren-2 renin transgenic hypertensive rats. *P < 0.05 compared with the corresponding values in the other four groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameter</th>
<th>Age (days) . .</th>
<th>80</th>
<th>140</th>
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<th>80</th>
<th>140</th>
<th>150</th>
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<th>150</th>
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<tbody>
<tr>
<td>HanSD+vehicle</td>
<td>Plasma AngII (fmol/ml)</td>
<td></td>
<td>16 ± 3*</td>
<td>46 ± 4</td>
<td>47 ± 5</td>
<td>45 ± 5</td>
<td>44 ± 5</td>
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</tr>
<tr>
<td>TGR+vehicle</td>
<td></td>
<td></td>
<td>38 ± 4*</td>
<td>129 ± 22</td>
<td>114 ± 15</td>
<td>126 ± 14</td>
<td>124 ± 11</td>
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</tr>
<tr>
<td>TGR+DDMS</td>
<td>Heart AngII (fmol/g)</td>
<td></td>
<td>20 ± 2*</td>
<td>30 ± 2</td>
<td>29 ± 2</td>
<td>30 ± 3</td>
<td>31 ± 3</td>
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</tr>
<tr>
<td>TGR+NCND</td>
<td>Kidney AngII (fmol/g)</td>
<td></td>
<td>724 ± 59</td>
<td>772 ± 82</td>
<td>738 ± 59</td>
<td>737 ± 61</td>
<td>757 ± 54</td>
<td>744 ± 69</td>
<td>755 ± 69</td>
<td>782 ± 78</td>
<td>759 ± 52</td>
<td>709 ± 79</td>
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<tr>
<td>TGR+DDMS+NCND</td>
<td>Na excretion (μmol/24 h)</td>
<td>9.8 ± 0.6</td>
<td>9.9 ± 0.8</td>
<td>9.7 ± 0.9</td>
<td>9.6 ± 0.6</td>
<td>9.7 ± 0.7</td>
<td>9.1 ± 0.7</td>
<td>9.2 ± 0.9</td>
<td>9.9 ± 0.5</td>
<td>9.2 ± 0.5</td>
<td>9.1 ± 0.4</td>
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</table>
Table S3  Baseline values of arterial blood pressure (MAP), RBF and RVR in individual experimental groups

Results are from series evaluating MAP and RBF responses to bolus injections of AngII and NE. Values are means ± S.E.M. Abbreviation are as given in Tables S1 and S2. *P < 0.05 compared with the corresponding values in TGR+vehicle, TGR+DDMS and TGR+DDMS groups; # P < 0.05 compared with the values in the four remaining groups.

<table>
<thead>
<tr>
<th>Series</th>
<th>Group</th>
<th>MAP (mmHg)</th>
<th>RBF (ml·min⁻¹·g⁻¹)</th>
<th>RVR (mmHg·ml⁻¹·min⁻¹·g⁻¹)</th>
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<tr>
<td>‘Early’ treatment protocol</td>
<td>HanSD+vehicle</td>
<td>121 ± 4#</td>
<td>6.58 ± 0.22</td>
<td>18.39 ± 0.61*</td>
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<td>TGR+vehicle</td>
<td>189 ± 4</td>
<td>6.41 ± 0.21</td>
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<td>TGR+DDMS</td>
<td>191 ± 5</td>
<td>6.52 ± 0.28</td>
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<tr>
<td></td>
<td>TGR+NCND</td>
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<td>28.79 ± 0.56</td>
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<tr>
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<td>TGR+DDMS+NCND</td>
<td>166 ± 4*</td>
<td>6.61 ± 0.24</td>
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<tr>
<td>‘Late’ treatment protocol</td>
<td>HanSD+vehicle</td>
<td>132 ± 4#</td>
<td>6.22 ± 0.31</td>
<td>21.22 ± 0.29*</td>
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<tr>
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<td>TGR+vehicle</td>
<td>191 ± 4</td>
<td>6.12 ± 0.29</td>
<td>31.21 ± 0.37</td>
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<tr>
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<td>TGR+DDMS</td>
<td>192 ± 3</td>
<td>6.09 ± 0.31</td>
<td>31.53 ± 0.41</td>
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<tr>
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<td>TGR+NCND</td>
<td>189 ± 4</td>
<td>6.11 ± 0.31</td>
<td>30.93 ± 0.42</td>
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<tr>
<td></td>
<td>TGR+DDMS+NCND</td>
<td>161 ± 4*</td>
<td>6.36 ± 0.44</td>
<td>25.31 ± 0.52*</td>
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