

E S D.

- Como bien es sabido por ese Despacho, las patentes pueden concederse sobre productos y procedimientos más no sobre usos y métodos de tratamiento. Al estudiar de la reivindicación 1 a la 7 es claro que se pretende obtener exclusividad sobre el USO de composiciones farmacéuticas y principios activos, lo que como es sabido no es permitido de acuerdo al artículo 14 de la Decisión 486 de 2000.

De igual manera hago notar que el USO que se pretende tiene el agravante de ser parte de METODOS DE TRATAMIENTO para toda clase de patologías de diversa índole, lo cual igualmente se encuentra prohibido por la Decisión 486

- En cuanto a la inespecificidad y amplitud de la Reivindicación 1 me permito reiterar que la presente solicitud no menciona, especifica o reivindica los principios activos preferidos dentro de la composición, por tanto, no puede ser aceptada por el Despacho

Ahora bien, si se tiene en cuenta lo revelado en las demás reivindicaciones en donde el principio activo es seleccionado de un amplio grupo de sustancias activas, dichos compuestos son conocidos *per se*, están comprendidos en el arte anterior y son ampliamente conocidos por ese Despacho, tal es el caso de antagonistas de receptores AT1-Valsartan, losartan, candesartan, eprosartan, irbesartan y sapnsartan, y de inhibidores de la HMG-CoA reductasa, Atorvastatina, cerivastatina, fluvastatina, lovastatina, entre otros

Por tanto, ni el uso separado, ni el empleo combinado, simultáneo o secuencial de esta clase de principios activos, en presencia de los auxiliares que se mencionan en la solicitud, suponen un "salto técnico" que confiera a la solicitud el indispensable requisito de Nivel Inventivo

- En cuanto a la composición farmacéutica revelada en la reivindicación 9, ésta carece de novedad y nivel inventivo si se tiene en cuenta que la publicación de patente europea EP 0930 076, "Medicinal compositions", se refiere a una composición farmacéutica que comprende como ingrediente activo una o más drogas seleccionadas del grupo que consiste de antagonistas del receptor de angiotensina II, entre los que se encuentra Valsartan e inhibidores de la enzima convertidora de angiotensina como Temocapril, y uno o más agentes que mejoran la resistencia a la insulina, útiles en el tratamiento de la aterosclerosis, principios activos y uso divulgados en la presente solicitud de patente

De igual manera, la publicación PCT/WO97/36874, "Eprosartan dihydrate and a process for its production and formulation" afecta las Rvs 1 a 3, 6 y 7, y especialmente la No 9, puesto que se revela el uso de Eprosartan, solo o en combinación con un segundo principio activo seleccionado del grupo que

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consiste de un diurético, un bloqueador de los canales de calcio, un inhibidor de la renina y un inhibidor de la enzima convertidora de Angiotensina (ACE) (captopril, enalapril), en el tratamiento de la hipertensión, falla congestiva cardíaca y falla renal

Por tanto, si una persona versada en la materia tiene a su alcance de manera individual o combinada estas y otras revelaciones, podría derivar de manera evidente el objeto reivindicado, razón por la cual el mismo, carece por completo de los requisitos de novedad y nivel inventivo y no puede en consecuencia ser objeto de privilegio de patente

II COMPLEMENTACIÓN DE LA INFORMACIÓN

Ruego al Despacho se sirva tener en cuenta el Informe Internacional de Búsqueda practicado por la Oficina Europea de Patentes a la aplicación internacional PCT/EP01/04115, publicada en 10-03-2001, y equivalente a la solicitud en trámite, en tanto invocan la misma prioridad, y donde se señala la existencia de siete (7) anterioridades de categoría "X", que afectan la novedad y el nivel inventivo de dicha solicitud y por ende, los de la solicitud colombiana. Algunos de estas anterioridades fueron mencionadas con detenimiento en el escrito de oposición presentado en noviembre 26 de 2003 y otras de ellas se mencionarán a continuación

- El artículo "Angiotensin-Converting Enzyme Inhibition and AT1 Receptor blockade modify the pressure-natriuresis relationship by additive mechanisms in rats with human renin and angiotensinogen genes"¹ afecta las reivindicaciones 1 a 3, 6, 7 y 9 de la solicitud colombiana 02-091-883, ya que revela la administración de Cíazapril (inhibidor de la ACE) o Losartan (antagonista del receptor AT1) o una combinación de los mismos para normalizar la presión sanguínea, incrementar el flujo sanguíneo renal (RBF) y la tasa de filtración glomerular (GFR)

¹ Mervaala, Eero Et Al "Angiotensin-Converting Enzyme Inhibition and AT1 Receptor blockade modify the pressure-natriuresis relationship by additive mechanisms in rats with human renin and angiotensinogen genes" Journal of the American Society of Nephrology, (Aug. 1999) Vol 10 N°8, pp 1669-1680

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- El artículo "Safety of the combination of valsartan and benazepril in patients with chronic renal disease"² también afecta las reivindicaciones 1 a 3, 6, 7 y 9 de la solicitud colombiana 02-091-883, ya que como su mismo título lo menciona, revela la combinación de Benazepril (inhibidor de la ACE) y Valsartan (antagonista del receptor AT1) que proporciona un aumento gradual del bloqueo del sistema renina-angiotensina que el que podría proporcionar cada agente por separado y que beneficia a los pacientes que exhiben declinación de la función renal debido a la enfermedad renal crónica

Queda claro una vez más, que la pretensión que patentabilidad de una combinación de al menos dos compuestos seleccionados de los grupos de un antagonista del receptor AT-1 o un inhibidor ACE o un inhibidor de la HMG-CoA reductasa carece de nivel inventivo si se tiene en cuenta lo revelado en el arte anterior, puesto que en los documentos expuestos tanto en este escrito como en el de oposición ilustran la existencia de combinaciones de fármacos inhibidores de la ACE y antagonistas del receptor AT-1

EN CONCLUSIÓN

Es claro que la presente solicitud de patente CO 02-091-883 se encuentra afectada tanto en novedad como en nivel inventivo, teniendo en cuenta los argumentos expuestos en el escrito de oposición presentada ante ese Despacho el 26 de mayo de 2003 y en lo establecido en el Informe Internacional de Búsqueda practicado por la Oficina Europea de Patentes a la aplicación internacional PCT/EP01/04115 equivalente

III PRUEBAS

Además de las solicitudes en el escrito de oposición, y de aquellas que oficiosamente considere el despacho, ruego que se tengan como tales las siguientes

- 3 1 Copia del artículo "Angiotensin-Converting Enzyme Inhibition and AT1 Receptor blockade modify the pressure-natriuresis relationship by additive

² Ruilope L M et al "Safety of the combination of valsartan and benazepril in patients with chronic renal disease" Journal of hypertension, current science Philadelphia, PA, US, Vol 18, N° 1 January 2000, pp 89-95

mechanisms in rats with human renin and angiotensinogen genes" Mervaala,
Eero Et Al Journal of the american society of nephrology (Aug . 1999) Vol
10, N° 8, pp 1669-1680

- 3 2 Copia del abstract del artículo "Safety of the combination of valsartan and
benazepril in patients with chronic renal disease" Rullope L M et al Journal
of hypertension, current science, Philadelphia, PA, US, Vol 18, N° 1 January
2000, pp 89-95

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Angiotensin-Converting Enzyme Inhibition and AT₁ Receptor Blockade Modify the Pressure-Natriuresis Relationship by Additive Mechanisms in Rats with Human Renin and Angiotensinogen Genes

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Abstract The intrarenal factors responsible for hypertension in double-transgenic rats (dTGR) harboring human renin and human angiotensinogen genes are unclear. The pressure-natriuresis and -diuresis relationships in response to chronic angiotensin converting enzyme (ACE) inhibition and AT₁ receptor blockade were evaluated. Renal renin-angiotensin and nitric oxide (NO) system gene expression was also investigated. Six week-old dTGR were treated for 3 wk with submaximal doses of cilazapril (10 mg/kg orally) or losartan (10 mg/kg orally) or with the drug combination. In untreated dTGR, pressure-natriuresis relationships were maximally shifted rightward by approximately 70 to 80 mmHg, and both renal blood flow (RBF) and GFR were markedly decreased. Submaximal cilazapril and losartan dosages both decreased systolic BP by 30 mmHg and shifted the pressure-natriuresis curves leftward by 25 to 30 mmHg. Cilazapril increased RBF and GFR to values observed in normotensive control animals but did not significantly affect fractional sodium excretion (FENa) or fractional water excretion (FEH₂O) curves. In contrast, losartan had no significant effect on RBF or GFR but shifted the FENa and FEH₂O curves leftward. The cilazapril and losartan com-

bination completely normalized BP and shifted the pressure-natriuresis curves leftward more than did either drug alone. When cilazapril and losartan were administered at higher doses (30 mg/kg, orally), the two drugs equally shifted the pressure-natriuresis curves leftward by 50 mmHg. Both drugs increased RBF and GFR; however, only losartan shifted FENa and FEH₂O curves leftward. Human and rat renin and angiotensinogen genes were downregulated in dTGR and were increased by losartan and cilazapril treatments, whereas no changes in the expression of rat ACE and AT_{1A} receptor genes were observed. Endothelial NO synthase expression was increased by cilazapril but not by losartan. Neither inducible NO synthase nor neuronal NO synthase gene expression was affected by drug treatments. Therefore, submaximal ACE inhibition enhanced sodium excretion mainly by increasing RBF and GFR, whereas submaximal AT₁ receptor blockade decreased tubular sodium and water reabsorption. The combination of the two drugs produced an additive effect. The ACE inhibitor effects may involve increased endothelial NO synthase expression, perhaps related to the inhibition of bradykinin degradation.

The renin-angiotensin system (RAS) is important in the regulation of BP and is a key determinant of renal sodium and water excretion. In rats, physiologic and pharmacologic experiments designed to explore the role of the RAS in hypertension are hampered by the species specificity of the renin-angiotensinogen interaction. The hypertension of double-transgenic rats (dTGR) with the human renin (hRen) and human angiotensino-

gen genes is dependent on the human RAS components (1). These rats offer a unique opportunity to study the human RAS and pharmacologically induced changes in the human RAS in an experimental animal model. dTGR are also suitable for studying the lifelong effects of RAS overexpression, whereas experimental protocols using chronic angiotensin II (AngII) infusion via osmotic minipumps are usually limited to experimental periods of 1 to 2 wk. dTGR develop severe hypertension and nephrosclerosis at the age of 8 to 10 wk because of the human components of the RAS (1). We showed earlier that at 6 wk of age, when the kidneys of dTGR were histologically unaffected, the pressure-natriuresis and -diuresis relationships were already shifted toward higher operating BP levels (2). The altered renal excretory function in dTGR seemed to be intrinsically localized to the kidney because clamping of the

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neurohumoral factors by renal denervation and infusions of aldosterone, 17-hydroxycorticosterone, norepinephrine, or vasopressin did not change the renal pressure-natriuresis/diuresis relationships in these animals (2). The RAS also exerts multiple effects inside the kidney. AngII decreases sodium and water excretion by decreasing renal blood flow (RBF) and GFR. In addition, AngII directly stimulates sodium reabsorption in the proximal and distal tubules (3-5). To more specifically evaluate the pathophysiologic renal mechanisms responsible for the altered pressure-natriuresis relationships in dTGR, as well as to observe the effects on gene and transgene expression, we directly blocked AT₁ receptors or we inhibited the angiotensin-converting enzyme (ACE). We observed that ACE inhibition and AT₁ receptor blockade operated by distinct additive mechanisms.

Materials and Methods

Animal Protocols

Protocol 1. Experiments were conducted in 43 male dTGR (175 to 250 g) bearing the hRen gene and human angiotensinogen gene and in 10 normotensive control rats bearing only the hRen gene (hRen rats). The rats were purchased from Biological Research Laboratories (Füllinsdorf, Switzerland). The procedures and experimental protocols were approved by the local Council on Animal Care, whose standards correspond to those of the American Physiological Society. The rats were allowed free access to standard rat chow (0.3% sodium SSNIFF Spezialdiäten, Soest, Germany) and drinking water.

In a preliminary study, we treated 6-wk-old dTGR ($n = 6$ in each group) with a single oral dose of cilazapril or losartan (10 mg/kg, 4 h before kidney function testing) and found no effects on pressure-natriuresis/diuresis curves, RBF, or GFR. These findings were in agreement with previous reports demonstrating no renal responses to AT₁ blockade after acute treatment (6,7). We therefore decided to administer losartan and cilazapril chronically (10 mg/kg, by gavage, once each day for 3 wk). We divided the 6-wk-old BP and body weight-matched dTGR into three treatment groups, which received either cilazapril ($n = 10$), losartan ($n = 10$), or vehicle ($n = 15$). Because neither cilazapril nor losartan completely normalized BP, another group of 6-wk-old dTGR ($n = 8$) were treated with the drug combination (both drugs at 10 mg/kg, by gavage) for 3 wk, in an additional study. Control dTGR and normotensive hRen rats received vehicle (5% gum arabicum). BP was measured by the tail-cuff method at the beginning of the study for matching of the rats.

There were no differences among the treatment groups with respect to body weight gain during the follow-up period (ANOVA, $P = 0.76$). At the age of 9 wk, the rats were prepared as described previously (8). Because clamping of the neurohumoral factors by renal denervation and infusions of aldosterone, 17-hydroxycorticosterone, norepinephrine, or vasopressin did not change the renal pressure-natriuresis/diuresis relationships in dTGR in our previous study (2), only the protocol without neurohumoral clamping was used in this study. Briefly, the rats were anesthetized with an intramuscular injection of 35 mg/kg ketamine (Parke-Davis, Berlin, Germany) and an intraperitoneal injection of 65 mg/kg mactin (Research Biochemicals, Natick, MA). The rats were placed on a heated table to maintain their body temperatures at 37°C. Cannulae were inserted into the trachea to facilitate breathing, into the carotid and femoral arteries for measurement of renal perfusion pressure (RPP), into the jugular vein for compound infusion, and into the left ureter for urine collection. The right kidney was removed through a midline incision. An adjustable

clamp was placed around the aorta above the left renal artery, and ligatures were loosely placed around the celiac and mesenteric arteries and around the aorta below the left renal artery for later occlusion. During preparation, the rats were infused with a 0.9% saline solution containing 1% bovine albumin at a rate of 50 μ l/min per 100 g body wt. Inulin (20 mg/ml, Sigma) and para-aminohippurate (1.2 mg/ml, Merck Sharp & Dohme) were then included in the infusion for measurement of GFR and renal plasma flow, and the infusion rate was reduced to 33 μ l/min per 100 g body wt.

After surgical preparation and an equilibration period of 60 min, RPP was lowered to 69 mmHg in hRen rats and 100 mmHg in untreated dTGR. After an additional 30-min equilibration period, urine flow, sodium excretion, GFR, and RBF were determined in two 20- to 30-min collection periods, depending on the magnitude of the urine flow. The supra-aortic occluder was then released to increase RPP by approximately 30 mmHg. Again, urine and plasma samples were obtained after a 25- to 30-min equilibration period. RPP was finally increased to approximately 124 mmHg in hRen rats and 207 mmHg in untreated dTGR. After a 10-min equilibration period, urine and plasma samples were collected in two 5-min periods. In cilazapril- and losartan-treated dTGR, we were able to first decrease RPP to a level of 73 to 87 mmHg. We then increased RPP to 112 to 118 mmHg and finally to 152 to 175 mmHg. At these pressure levels, urine samples were collected in a manner similar to that used for untreated rats. In dTGR treated with the drug combination, RPP was first decreased to 70 mmHg, RPP was then increased to 102 mmHg and finally to 138 mmHg.

Protocol 2. Four-week-old BP and body weight-matched dTGR were divided into three groups, which received either high-dose cilazapril (30 mg/kg, orally) ($n = 5$), high-dose losartan (30 mg/kg, orally) ($n = 5$), or vehicle ($n = 7$) for 3 wk. After surgical preparation as described above and a 60-min equilibration period, RPP in the dTGR control group was first lowered to 118 mmHg; RPP was then increased to 155 mmHg and finally to 199 mmHg. In cilazapril- and losartan-treated dTGR, RPP was first decreased to a level of 68 to 70 mmHg. We then increased RPP to 112 to 123 mmHg and finally to 144 to 149 mmHg. At these pressure levels, urine flow, sodium excretion, GFR, and RBF were determined as described for protocol 1.

General Procedures

Mean arterial BP was continuously measured throughout the experiment and was recorded on a computer system (TSE, Bad Homburg, Germany). Representative mean arterial BP values were determined before the start of pressure-natriuresis experiments (to characterize the BP levels of hRen rats and dTGR) and during each urine collection period, by averaging all values recorded during that period. Urine flow was determined gravimetrically. Inulin and para-aminohippurate concentrations of urine and plasma samples were determined according to methods outlined elsewhere (9,10). GFR was calculated as urine/plasma inulin concentration ratio $\times$ urine flow. RBF was calculated as renal plasma flow/(1 - hematocrit). Urinary (FLM3, Radiometer, Copenhagen, Denmark) and plasma (Cobas Mira Plus, Roche, Basel, Switzerland) sodium concentrations were determined by flame photometry. Urine flow, sodium excretion, GFR, and RBF were normalized per gram of kidney wet weight. For estimation of left ventricular hypertrophy, the heart was excised, the great vessels, the atria, and the free wall of the right ventricle were dissected, and the left ventricular mass was measured. The left ventricular wet weight/body weight was calculated as an index of left ventricular hypertrophy.

Tissue Preparation for Morphologic Analysis

For conventional morphologic analysis, the kidneys were removed, cut sagittally and fixed in 4% buffered paraformaldehyde, at room temperature. The tissue was dehydrated in graded alcohols and embedded in paraffin. Sections of 2 to 3 μ m thickness were cut using a Leitz microtome (Leitz 1512 Leitz, Wetzlar, Germany). The sections were deparaffinized and rehydrated before being stained as outlined previously (11). The tissue was examined without knowledge of the identity of the rats.

RNAse Protection Assays and In Situ Hybridization

For RNase protection assays, one half of the right kidney was snap frozen in isopentane (-35°C) and subjected to RNA isolation. The tissue was homogenized and the RNA was isolated by a standard lithium chloride/urea precipitation technique as described in detail elsewhere (1). mRNA specific for human angiotensinogen, rat angiotensinogen, rat renin, hRen, and β -actin were identified by RNase protection assays with an Ambion RPA III kit (ITC Biotechnology, Austin, TX), according to the protocols provided by the manufacturer. For the detection of rat renin, hRen, and rat and human angiotensinogen mRNA, the probes and protocols described by Bohlender *et al.* (1) were used. For the detection of ACE and $\text{AT}_{1\text{A}}$ receptor mRNA, the probes described by Gross *et al.* (12) were used. The probes and procedures used for the detection of endothelial inducible and neural nitric oxide (NO) synthases were described in detail elsewhere (13). *In situ* hybridization was performed for the localization of ACE and $\text{AT}_{1\text{A}}$ receptor genes in the kidney. The probes and the *in situ* hybridization procedures were described in detail previously (12, 14).

Statistical Analyses

Data are presented as means $\pm$ SEM. Statistically significant differences in mean values were tested using two-way ANOVA for repeated measures and the Duncan multiple-range test. Linear regression lines were calculated using the least-squares method. Values of $P < 0.05$ were considered statistically significant.

Results

Mortality Rates, BP, and Left Ventricular Hypertrophy with Protocols 1 and 2

With protocol 1, the mortality rate for untreated dTGR during the experiment was 53% (8 of 15). The mean arterial BP of anesthetized untreated dTGR (160 ± 8 mmHg) was significantly higher than that of normotensive hRen controls (108 ± 4 mmHg) and the hearts of untreated dTGR showed pronounced left ventricular hypertrophy (Figure 1, A and B). No deaths were observed in the drug-treated groups. Compared with results for untreated dTGR, both cilazapril and losartan decreased mean arterial BP by 30 mmHg, to the level of 130 mmHg, and prevented the development of left ventricular hypertrophy (Figure 1, A and B). The combination of cilazapril and losartan completely normalized BP and myocardial size. There was a close correlation between mean arterial BP and left ventricular hypertrophy (Figure 1C). With protocol 2, the mean arterial BP of anesthetized untreated dTGR was 157 ± 9 mmHg. Compared with results for untreated dTGR, cilazapril and losartan decreased mean arterial BP by approximately 50 mmHg (mean arterial BP of 99 ± 4 mmHg in the cilazapril-treated group and 104 ± 5 mmHg in the losartan-treated group, $P < 0.05$ compared with control dTGR). Cilazapril and losar-

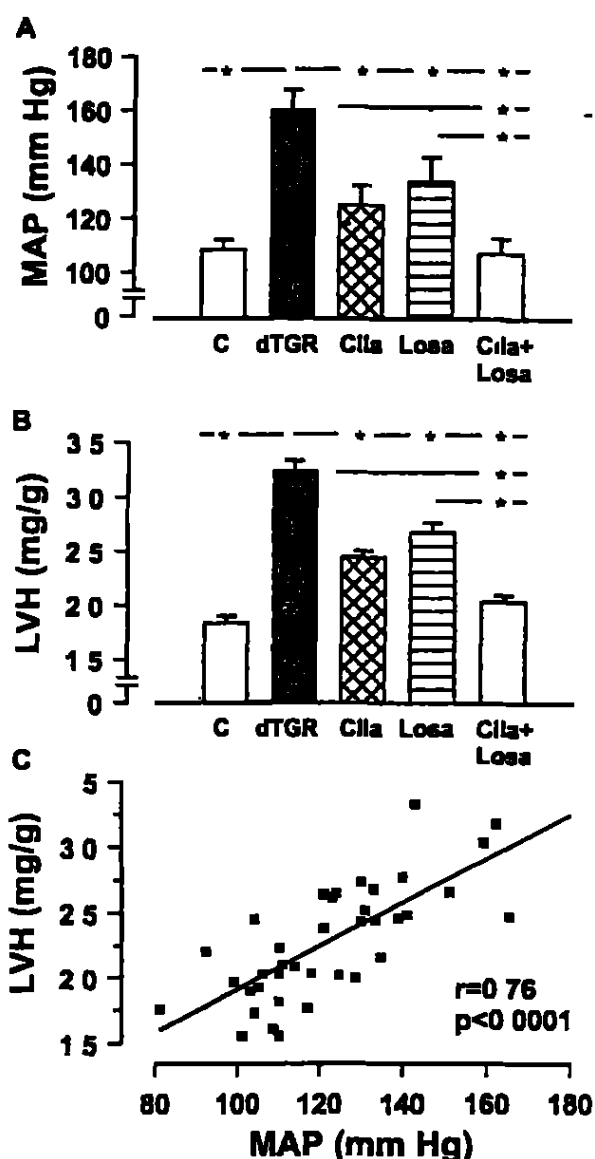


Figure 1 (A and B) Mean arterial pressure (MAP) (A) and left ventricular hypertrophy (LVH) (B) at the end of the experiment in normotensive human renin (hRen) control rats (C), double transgenic rats (dTGR), and dTGR treated with either cilazapril (Cila) (19 mg/kg), losartan (Losa) (10 mg/kg) or the drug combination Cilazapril and losartan only partially decreased BP and prevented the development of LVH. The drug combination produced complete normalization of BP and myocardial size. (C) Graph showing the strong correlation between mean arterial pressure and left ventricular hypertrophy.

tan also prevented the development of left ventricular hypertrophy (3.697 ± 0.115 , 2.191 ± 0.08 , and 2.416 mg/g in the control, cilazapril-treated, and losartan-treated groups, respectively, $P < 0.05$ compared with control dTGR).

Pressure-Natriuresis and Diuresis Curves with Protocol 1

Figure 2 shows pressure-natriuresis and -diuresis responses of hRen rats and dTGR. We did not include fiducial limits for

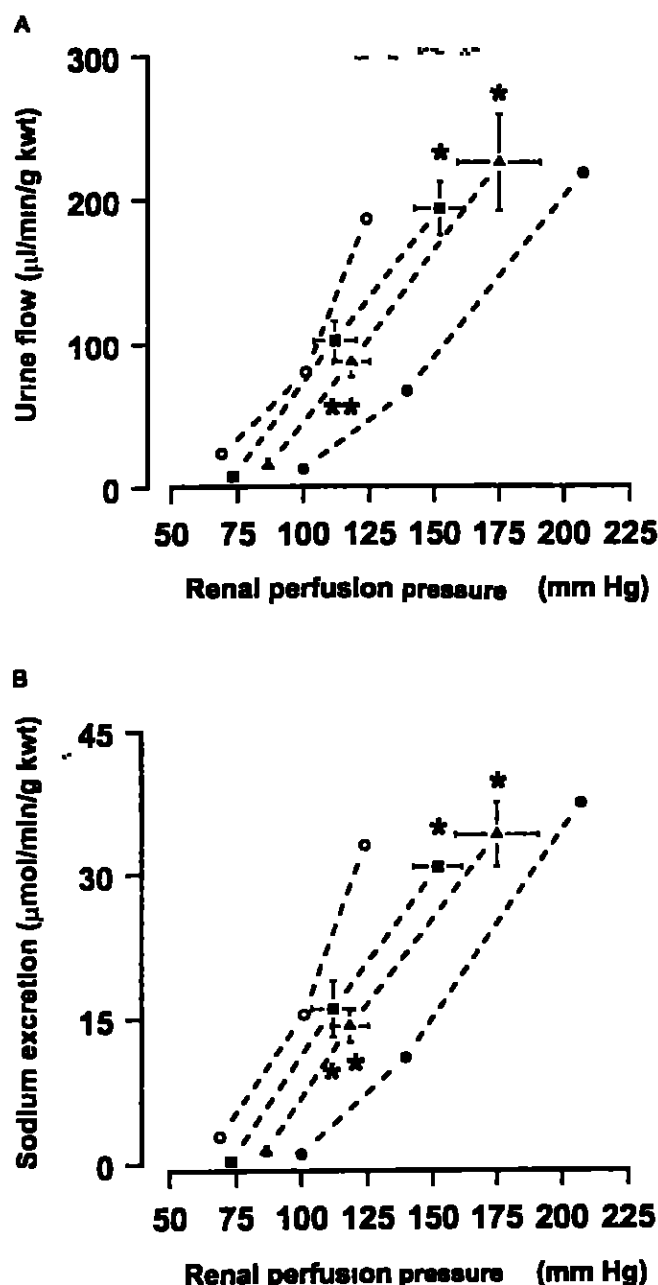


Figure 2 Relationship between renal perfusion pressure (RPP) and urine flow (A) and sodium excretion (B) for normotensive hRen rats (○), dTGR (●), and dTGR treated with either cilazapril (10 mg/kg) (■) or losartan (10 mg/kg) (▲). We did not include fiducial limits for the two untreated groups, so that the data could be more easily observed. * $P < 0.05$ for untreated dTGR versus dTGR treated with cilazapril or dTGR treated with losartan; values were compared at equivalent RPP levels. Pressure-diuresis and -natriuresis curves were shifted to the right for dTGR. Both cilazapril and losartan shifted the pressure-natriuresis curves leftward by approximately 25 to 30 mmHg.

the two untreated groups, so that the data could be more easily observed. In hRen rats, urine flow and sodium excretion were increased from 24 ± 9 to $187 \pm 31 \mu\text{l/min per g kidney wt}$ and from 2.8 ± 1.2 to $33.1 \pm 4.2 \mu\text{mol/min per g kidney wt}$

respectively as RPP was increased from 69 to 124 mmHg. The pressure-natriuresis and -diuresis curves for untreated dTGR were maximally shifted rightward by 70 to 80 mmHg. In untreated dTGR, urine flow and sodium excretion averaged $13 \pm 3 \mu\text{l/min per g kidney wt}$ and $1.0 \pm 0.3 \mu\text{mol/min per g kidney wt}$, respectively at the RPP level of 100 mmHg. Increasing the RPP to 207 mmHg in these rats increased urine flow and sodium excretion to $218 \pm 24 \mu\text{l/min per g kidney wt}$ and $37.5 \pm 3.3 \mu\text{mol/min per g kidney wt}$, respectively. Consequently, the slopes of the pressure-diuresis/natriuresis curves were not different, despite the rightward shift in the untreated dTGR pressure-natriuresis and -diuresis curves. Both cilazapril and losartan shifted the pressure-natriuresis and -diuresis curves back to the left by approximately 25 to 30 mmHg.

Figure 3 shows the relationships between RPP, RBF, and GFR. RBF in hRen rats averaged between 3.9 ± 0.4 and $7.5 \pm 0.9 \text{ ml/min per g kidney wt}$. In untreated dTGR, RBF averaged between 3.3 ± 0.4 and $5.8 \pm 1.0 \text{ ml/min per g kidney wt}$ and the curve was shifted to the right in the pressure range investigated. The GFR in hRen control rats averaged approximately $1 \text{ ml/min per g kidney wt}$. In untreated dTGR, the GFR was decreased, ranging between 0.56 and 0.87 ml/min per g kidney wt, and the curve was shifted to the right. Cilazapril increased RBF and GFR to 4.8 to 8.6 and to 0.85 to 1.24 ml/min per g kidney wt, respectively, so that hRen rats and cilazapril-treated dTGR were not different. Losartan had no significant effect on RBF or GFR.

Figure 4 shows fractional sodium excretion (FENa) and fractional water excretion (FEH₂O) data. In hRen rats, FENa and FEH₂O averaged 2.6 ± 1.1 and $3.6 \pm 1.1\%$, respectively at the RPP level of 69 mmHg and were increased to 21.1 ± 2.4 and $17.7 \pm 2.8\%$ when RPP was increased to 124 mmHg. In untreated dTGR, both curves were shifted, in parallel, approximately 15 mmHg rightward. Losartan shifted the FENa and FEH₂O curves leftward, whereas cilazapril had no significant effects on the tubular absorption of sodium and water.

The combination of cilazapril and losartan shifted the pressure-natriuresis curves leftward by approximately 70 mmHg (data not shown), which was greater than the effects observed with the cilazapril or losartan monotherapies (25 to 30 mmHg). GFR and RBF reached $1.3 \text{ ml/min per g kidney wt}$ and $6.0 \text{ ml/min per g kidney wt}$, respectively, which were within the ranges found in hRen control rats. FENa and FEH₂O reached 0.6% and 0.9%, respectively, at an RPP of 70 mmHg. When RPP was increased to 102 mmHg, FENa and FEH₂O were increased to 8.0 and 5.7%, respectively. These values were not different from those observed in hRen rats with the same RPP levels. hRen rats were able to further increase FENa and FEH₂O as the RPP was increased; however, in dTGR treated with the drug combination, FENa and FEH₂O remained at the level observed at 102 mmHg as the RPP was further increased.

Pressure-Natriuresis and -Diuresis Curves with Protocol 2

Figure 5 A and B shows the effects of high-dose cilazapril and high-dose losartan treatments on pressure-natriuresis and -diuresis responses in dTGR. In untreated dTGR, urine flow

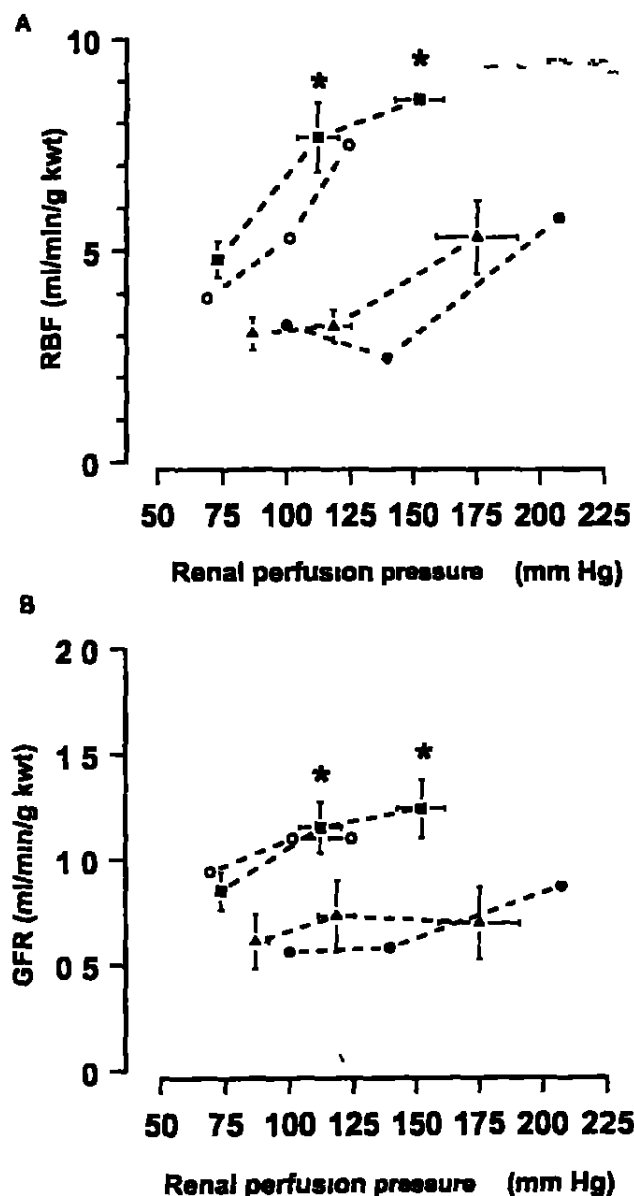


Figure 3 Relationship between RPP and renal blood flow (RBF) (A) and GFR (B) for normotensive hRen rats (O), dTGR (●) and dTGR treated with either cilazapril (10 mg/kg) (■) or losartan (10 mg/kg) (▲). We did not include fiducial limits for the two untreated groups, so that the data could be more easily observed. * $P < 0.05$ for cilazapril-treated dTGR versus untreated dTGR or dTGR treated with losartan values were compared at equivalent RPP levels. Relationships between RPP RBF and GFR were shifted to the right for dTGR. Cilazapril increased RBF and GFR to the levels found in normotensive rats. Losartan had no effect on RBF or GFR.

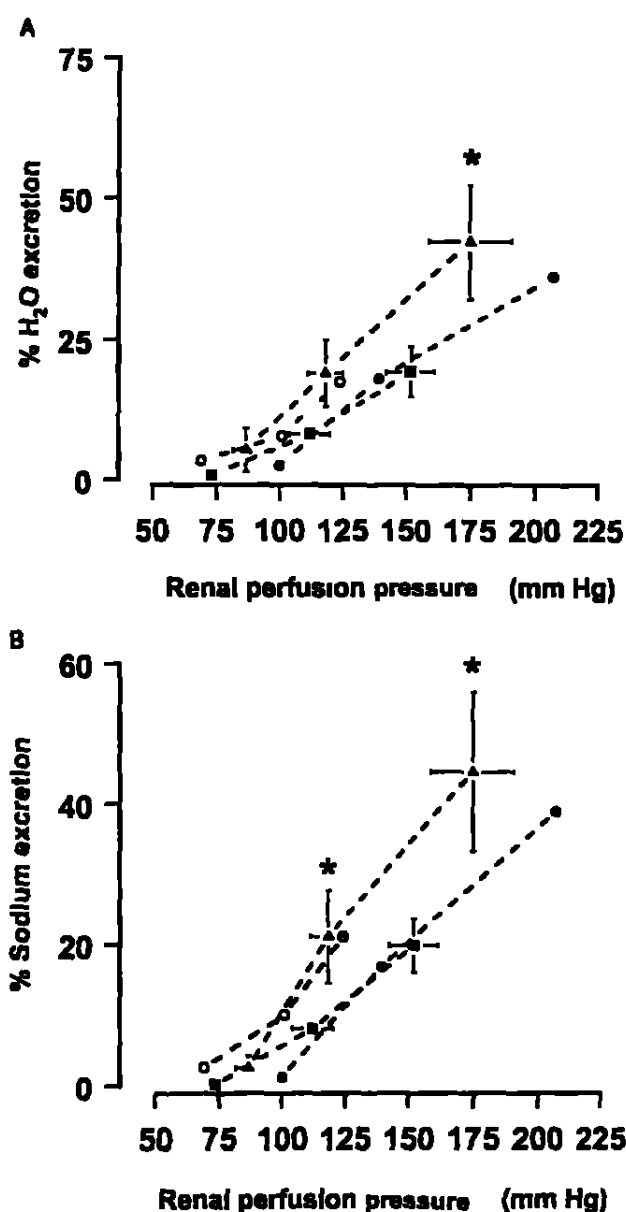


Figure 4 Relationship between RPP and fractional sodium excretion (FENa) (A) and fractional water excretion (FEH₂O) (B) for normotensive hRen rats (O), dTGR (●), and dTGR treated with either cilazapril (10 mg/kg) (■) or losartan (10 mg/kg) (▲). We did not include fiducial limits for the two untreated groups, so that the data could be more easily observed. * $P < 0.05$ for losartan treated dTGR versus untreated dTGR or dTGR treated with cilazapril values were compared at equivalent RPP levels. FENa and FEH₂O curves were shifted to the right for dTGR. Losartan shifted the curves leftward, whereas cilazapril did not have any effect on FENa or FEH₂O.

and sodium excretion averaged $5 \pm 0.8 \mu\text{l/min per g kidney wt}$ and $0.4 \pm 0.2 \mu\text{mol/min per g kidney wt}$, respectively at the RPP level of 118 mmHg. Increasing the RPP to 199 mmHg in these rats increased urine flow and sodium excretion to $130 \pm 11 \mu\text{l/min per g kidney wt}$ and $33.0 \pm 5.5 \mu\text{mol/min per g kidney wt}$ respectively. Both cilazapril and losartan shifted the

pressure-natriuresis and -diuresis curves back to the left by approximately 50 mmHg. Figure 5 C and D shows the relationships between RPP RBF and GFR. In untreated dTGR, RBF averaged between 2.7 ± 0.4 and $4.2 \pm 1.1 \text{ ml/min per g kidney wt}$ and GFR between 0.6 and 1.1 ml/min per g kidney

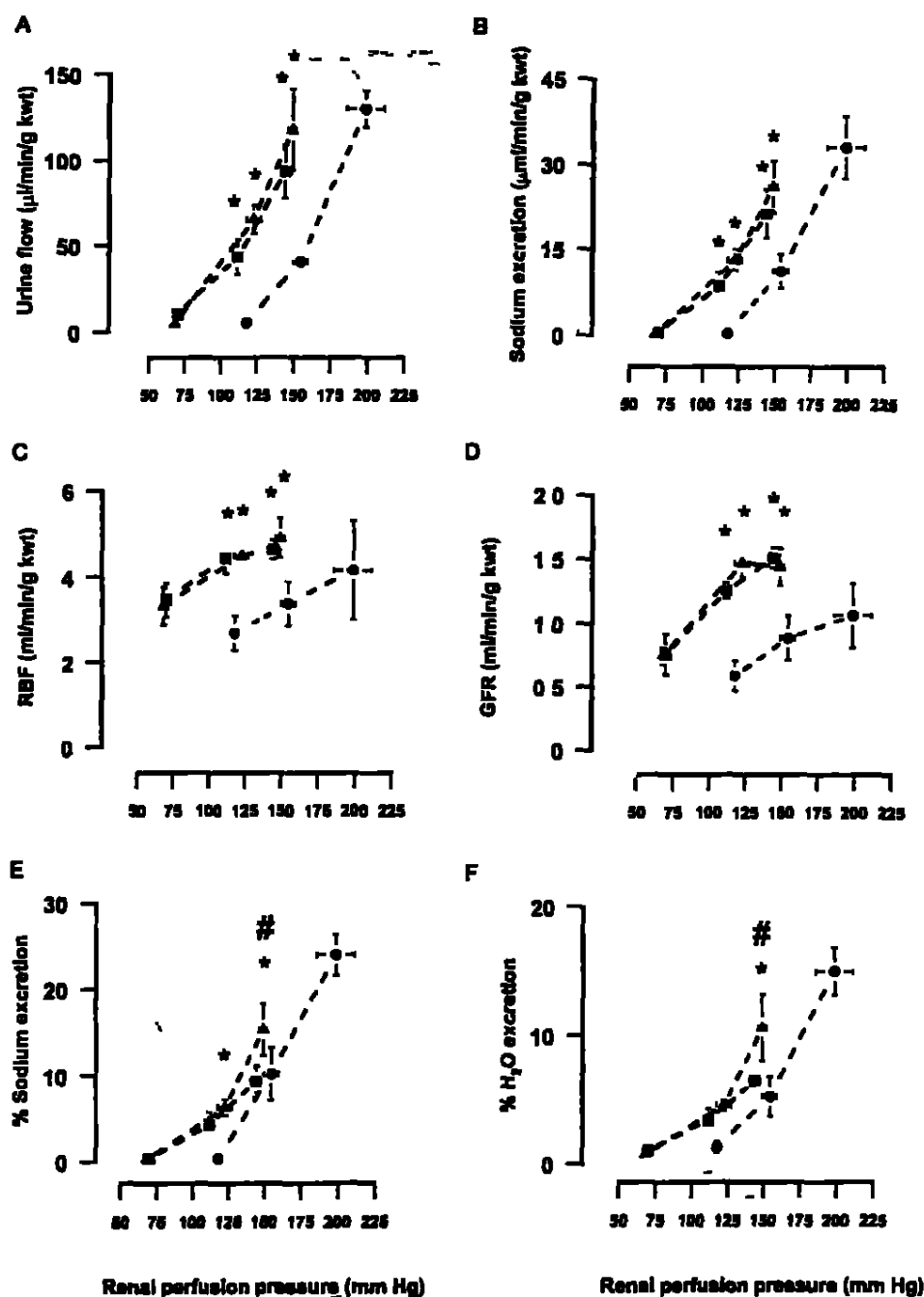


Figure 5 Effects of high-dose cilazapril (■) and high-dose losartan (▲) (both drugs at 30 mg/kg) on pressure natriuresis mechanisms in dTGR. ● Untreated dTGR. Both high-dose cilazapril and high-dose losartan shifted the pressure-natriuresis curves leftward by approximately 50 mmHg (A and B) and increased RBF (C) and GFR (D). Losartan shifted the FENa (E) and FEH₂O (F) curves more leftward than did cilazapril. **P* < 0.05 for losartan-treated dTGR and cilazapril-treated dTGR versus untreated dTGR. #*P* < 0.05 for losartan-treated dTGR versus cilazapril-treated dTGR.

wt. Cilazapril and losartan equally increased both RBF and GFR. Figure 5 E and F shows FENa and FEH₂O data. In untreated dTGR, FENa and FEH₂O averaged 0.3 ± 0.1 and $1.3 \pm 0.5\%$, respectively at the RPP level of 118 mmHg and increased to 24.0 ± 2.4 and $15.0 \pm 1.8\%$ when the RPP was increased to 199 mmHg. Losartan shifted the FENa and

FEH₂O curves leftward, whereas cilazapril had no significant effects on the tubular absorption of sodium and water.

In a separate pilot study we also examined the effects of volume depletion on pressure-natriuresis relationship in dTGR by removing the 1% albumin solution from our infusion protocol. In volume-depleted dTGR, the pressure-natriuresis/di-

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uresis relationship was markedly blunted. Urine flow and sodium excretion averaged $2.7 \mu\text{l}/\text{min}$ per g kidney wt and $0.2 \mu\text{mol}/\text{min}$ per g kidney wt, respectively, at the RPP level of 120 mmHg. Increasing the RPP to 147 mmHg in these rats increased urine flow and sodium excretion to $9.3 \mu\text{l}/\text{min}$ per g kidney wt and $1.0 \mu\text{mol}/\text{min}$ per g kidney wt, respectively. When the RPP was further increased to 168 mmHg, urine flow and sodium excretion were increased to $44 \mu\text{l}/\text{min}$ per g kidney wt and $9.2 \mu\text{mol}/\text{min}$ per g kidney wt, respectively. RBF averaged 2.5 to 3.7 ml/min per g kidney wt and GFR averaged

0.26 to 1.25 ml/min per g kidney wt. The hematocrit values were significantly higher (approximately 0.50) in volume-depleted dTGR, compared with non-volume-depleted TGR.

Renal Morphologic Features with Protocol 1

Histologic sections from kidneys are shown in Figure 6. The vessels of 9-wk-old untreated dTGR (Figure 6A) showed increased intimal and medial thickening as well as hyaline deposits. The glomeruli were preserved. The tubules were

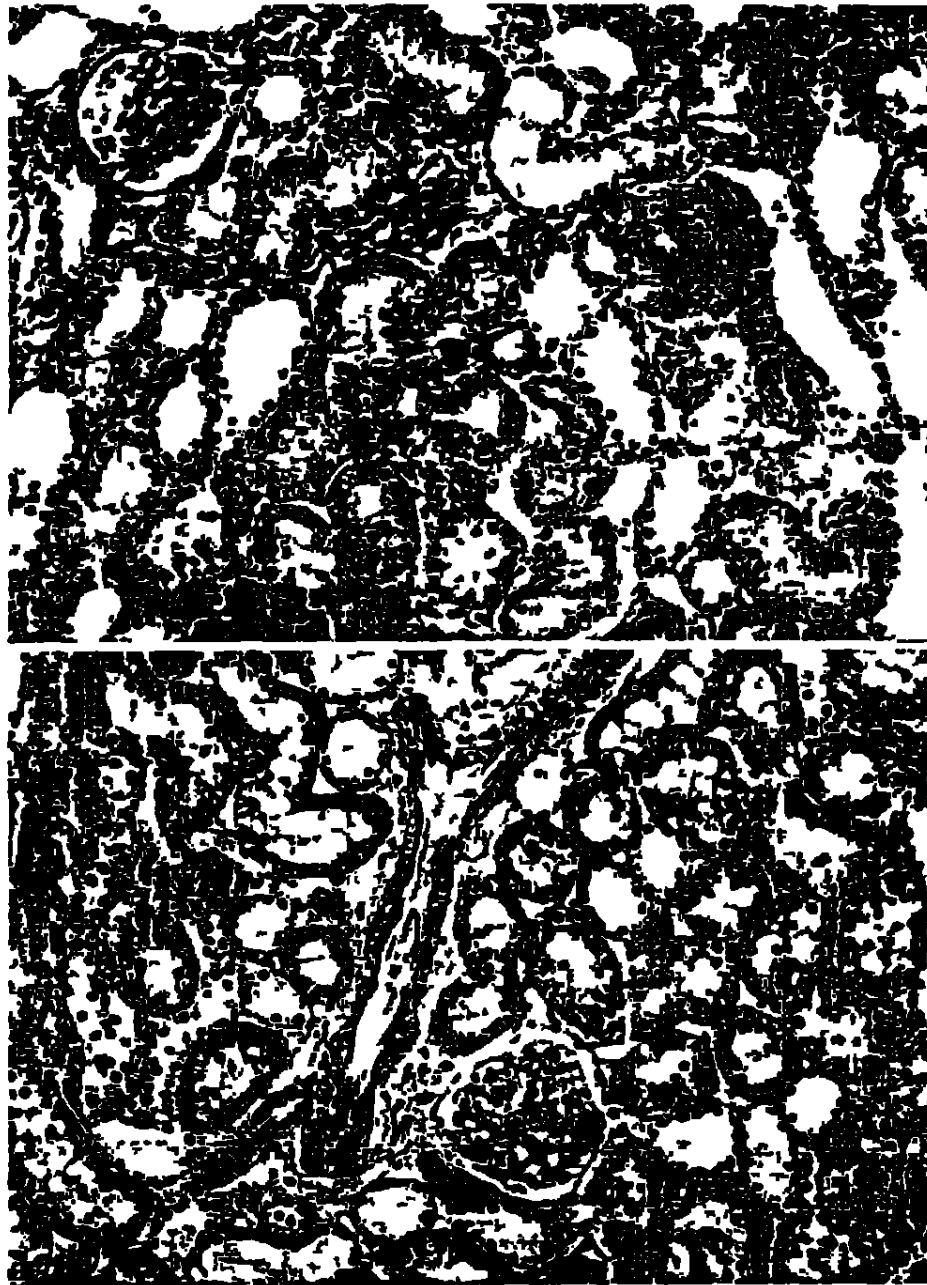


Figure 6. Histologic sections from kidneys of untreated 9-wk-old dTGR (A) and cilazapril treated dTGR (B). In untreated dTGR, medial thickening and hyaline depositions were seen in arterioles. The interstitium showed moderate infiltration. Cilazapril treatment (10 mg/kg) as well as treatment with losartan (10 mg/kg) and the drug combination, blocked these changes. Periodic acid-Schiff reaction. Magnification, $\times 500$.

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frequently filled with proteinaceous material and the interstitium showed moderate infiltration. Chronic treatment with cilazapril (Figure 6B) as well as with losartan and the drug combination (data not shown) was able to block the aforementioned morphologic changes in the kidney.

RNAse Protection Assays and In Situ Hybridization

The expression of hRen, rat renin and angiotensinogen genes in the kidney is shown in Figure 7. The results are

provided in arbitrary units. Both rat renin and hRen mRNA levels were significantly higher in hRen rats compared with untreated dTGR. Cilazapril and losartan treatments increased renin gene expression similarly. Rat angiotensinogen mRNA levels were decreased in untreated dTGR, compared with hRen rats. Both cilazapril and losartan increased rat and human angiotensinogen gene expression in the kidney. *In situ* hybridization experiments showed that the ACE gene was mainly localized in the tubular cells of the cortex.

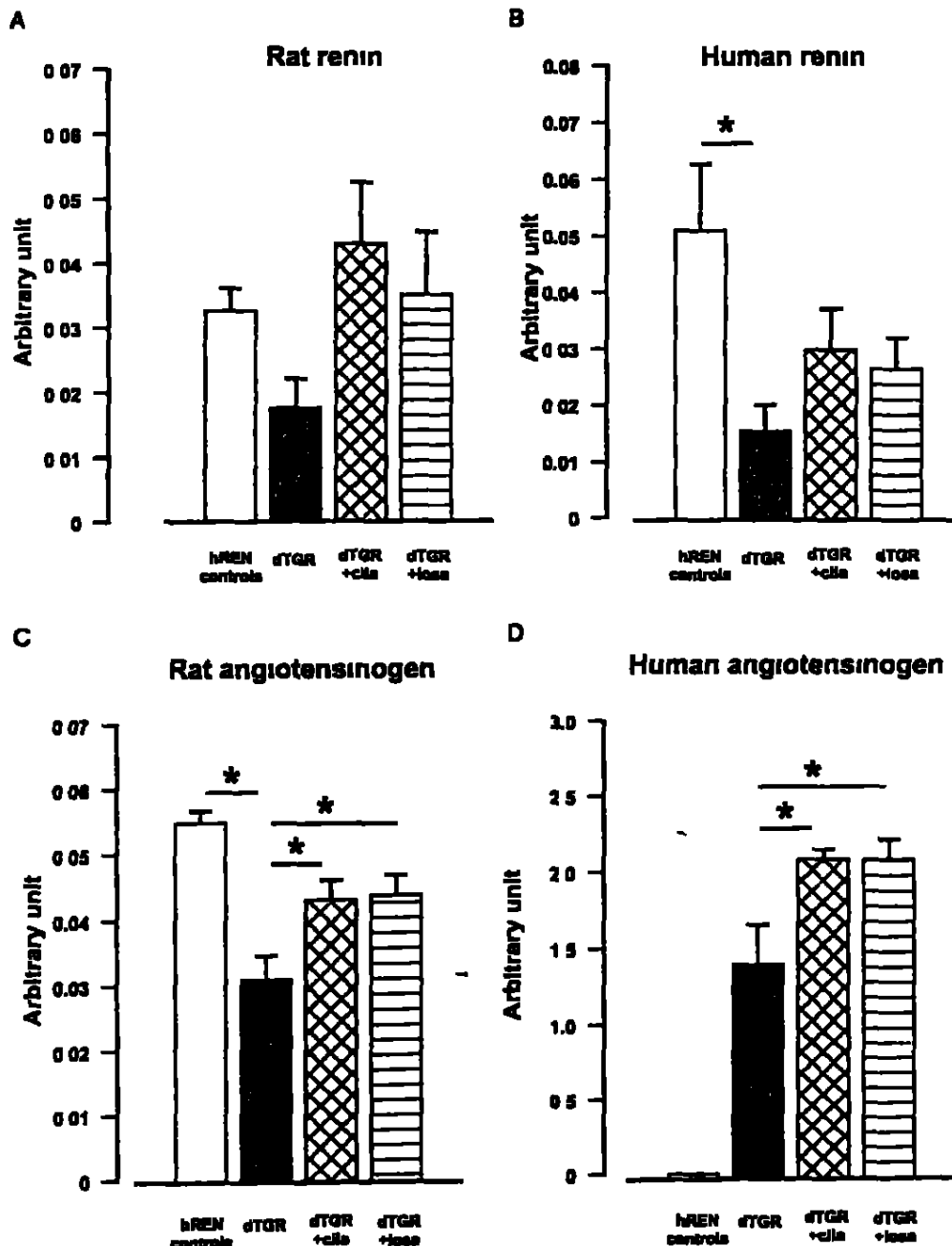


Figure 7 Semiquantitative analysis of rat renin mRNA (A), hRen mRNA (B), rat angiotensinogen mRNA (C) and human angiotensinogen mRNA (D) expression in the kidneys of normotensive hRen rats, untreated dTGR, and dTGR treated with either cilazapril (cila) (10 mg/kg) or losartan (losa) (10 mg/kg). Both hRen and rat renin genes were downregulated in dTGR and were increased by losartan and cilazapril treatments. Drug treatments increased rat and human angiotensinogen gene expression. * $P < 0.05$.

whereas the AT_{1A} receptor gene was mainly expressed in the medulla (data not shown). There were no differences between the treatment groups with respect to ACE or AT_{1A} receptor gene expression in the kidney (Figure 8). The expression of endothelial NO synthase mRNA in the kidney is shown in Figure 9. Cilazapril treatment significantly increased renal endothelial NO synthase gene expression compared with losartan-treated and untreated dTGR. The endothelial NO synthase gene expression in hRen rats was lower than that in dTGR. Cilazapril and losartan did not affect inducible or neural NO synthase gene expression in the kidney (data not shown).

Discussion

The high-hRen dTGR model of hypertension features moderate hypertension with severe histologic damage in the kid-

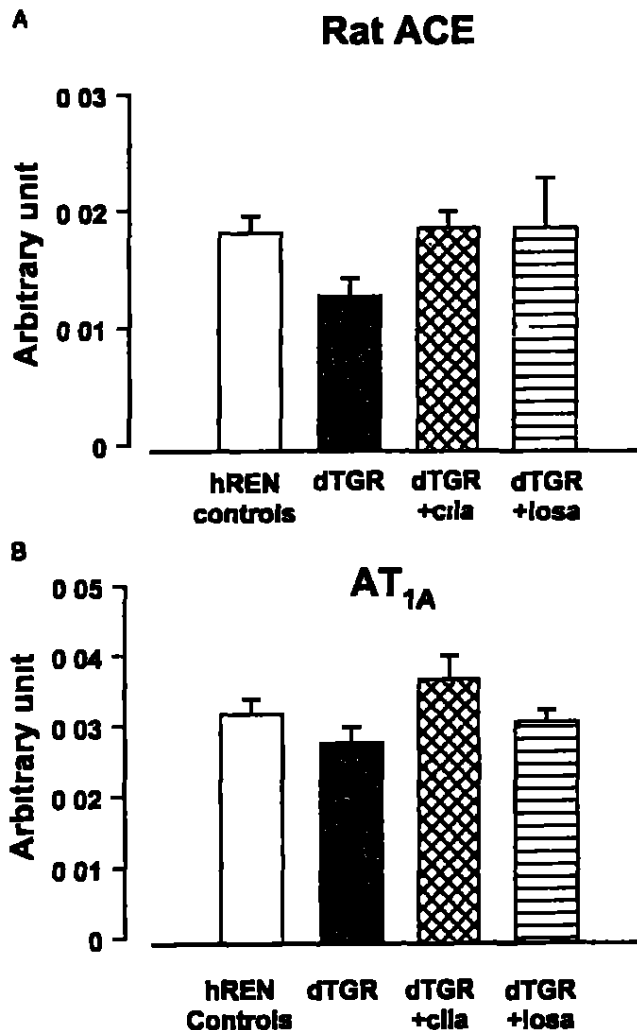


Figure 8. Semiquantitative analysis of rat angiotensin-converting enzyme (ACE) mRNA (A) and AT_{1A} receptor mRNA (B) expression in the kidneys of normotensive hRen rats, untreated dTGR, and dTGR treated with either cilazapril (10 mg/kg) or losartan (10 mg/kg). There were no differences between the treatment groups in ACE or AT_{1A} receptor gene expression.

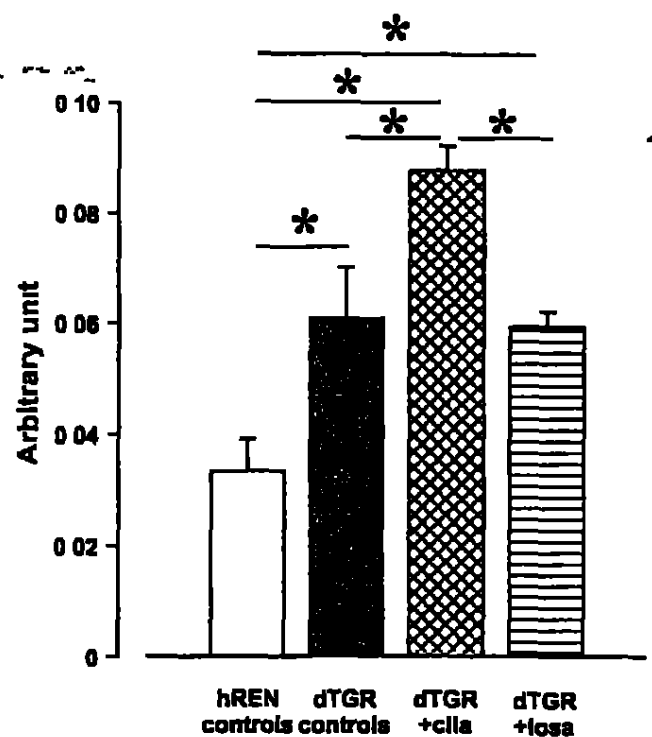


Figure 9. Semiquantitative analysis of endothelial nitric oxide (NO) synthase mRNA expression in the kidneys of normotensive hRen rats, untreated dTGR, and dTGR treated with either cilazapril (10 mg/kg) or losartan (10 mg/kg). Cilazapril but not losartan markedly increased endothelial NO synthase mRNA expression in dTGR. Endothelial NO synthase gene expression was lower in hRen rats than in dTGR. $P < 0.05$.

neys and the heart, resulting in 50% mortality rates at 7 to 9 wk of age (1, 15, 16). We showed earlier that the rightward shift in the pressure-natriuresis and -diuresis curves for dTGR depends on mechanisms inherent to the kidneys themselves (2). Here we show that blockade of AngII generation with cilazapril and blockade of AT_1 receptors with losartan are both able to shift the pressure-natriuresis and -diuresis curves toward normal ranges in volume-expanded dTGR. At the doses tested (10 mg/kg), neither BP reductions nor pressure-natriuresis curve shifts were completely reversed to normal values. In contrast, when submaximal ACE inhibition and AT_1 receptor blockade were combined, the BP and left ventricular mass were completely normalized, and the pressure-natriuresis curves were shifted more than with cilazapril or losartan alone. Our most novel finding was that submaximal ACE inhibition and AT_1 receptor blockade shifted the pressure-natriuresis and -diuresis curves leftward in dTGR by additive mechanisms. ACE inhibition increased RBF and GFR but had no significant effects on fractional sodium or water reabsorption. On the other hand, AT_1 receptor blockade shifted the curves for fractional sodium and water reabsorption to the left but had relatively modest effects on RBF and GFR. Our findings indicate that both AngII-induced tubular mechanisms and AngII-mediated hemodynamic mechanisms are responsible for the resetting of pressure-natriuresis/diuresis curves in volume-expanded dTGR.

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The RAS exerts multiple effects inside the kidney. AngII decreases RBF and GFR. In this respect, the sodium-retaining action of AngII is attributable in part to changes in the peritubular capillary physical forces. AngII also stimulates sodium reabsorption directly in the proximal and distal tubules (3-5). Together, these functions are responsible for the shift to higher operating arterial BP levels, as clearly demonstrated in the rat model of chronic AngII infusion (17,18) and in the mouse renin (mRen-2)27 transgenic rat model (19). We recently demonstrated that hypertension in dTGR clearly depends on the AngII-induced reduction in sodium and water excretion, which is intrinsic to the kidney (2). To clarify the importance of the RAS in pressure-dependent sodium excretion, we blocked AngII generation with cilazapril or blocked its effects on AT_1 receptors with losartan. Cowley (20) showed earlier that the pressure-natriuresis mechanism is dependent on the volume state: the pressure-natriuresis relationship is markedly enhanced under conditions of volume expansion, whereas volume depletion substantially reduces the sensitivity of the pressure-natriuresis relationship. In our pilot study, we examined the effects of the volume state on the pressure-natriuresis relationship in dTGR. The volume-depleted dTGR showed marked blunting of the pressure-natriuresis relationship compared with volume-expanded dTGR. Therefore, we emphasize that this study describes the effects of cilazapril and losartan only in volume-expanded animals, and volume expansion might have reduced the differences between treatment groups. In addition to suppressing both intrarenal and circulating AngII levels, ACE inhibitors have been reported to stimulate prostaglandin biosynthesis, diminish endothelin-1 generation, and potentiate bradykinin effects (21,22). Activation of endothelial B_2 kinin receptors leads to the formation of NO, prostacyclin, and platelet-activating factor (reviewed in reference (23)). Therefore, kinins could influence renal excretory function either directly or by interactions with other endocrine and paracrine systems (24-26). We found that cilazapril, but not losartan, markedly induced endothelial NO synthase gene expression in the kidneys of dTGR, supporting the notion that kinins contribute to the cardiovascular and renal actions of ACE inhibitors. Suppression of AngII generation together with the multiple effects of cilazapril on the kallikrein-kinin system improved sodium excretion in cilazapril-treated dTGR so that, at the RPP levels investigated, sodium and water excretion were markedly increased. The fact that cilazapril shifted the pressure-natriuresis/diuresis curves without affecting FENa or FEH_2O suggests that the resetting of the renal function curves by ACE inhibition may be primarily related to hemodynamic factors. This interpretation is consistent with increased RBF and GFR in cilazapril-treated dTGR and is in agreement with the view that ACE inhibitors have important effects on renal hemodynamics and GFR (27-31).

In contrast to cilazapril, chronic treatment with losartan at a submaximal dose (10 mg/kg, orally) had no significant effect on RBF or GFR in dTGR. On the other hand, the curves for FENa and FEH_2O were clearly positioned to the left of the corresponding curves in cilazapril-treated dTGR. Our findings indicate that a net decrease in tubular sodium reabsorption

might have been responsible for the improvements in sodium excretion and BP reduction in losartan-treated dTGR. Consistent with our findings, other authors also suggested that the antihypertensive effects of long-term AT_1 receptor blockade are related to direct inhibitory effects on sodium reabsorption in renal tubular cells (5,6). However, when losartan was administered to dTGR at a significantly higher dose (30 mg/kg, orally), not only FENa and FEH_2O but also RBF and GFR were markedly increased, indicating that the effects of AT_1 receptor blockade on renal hemodynamics are clearly dose-dependent and may occur only at very high doses. Consistent with our findings, Wang *et al* (18) recently showed that in rats with AngII-induced hypertension, chronic treatment with high doses of losartan prevented the AngII-induced decreases in GFR and RBF and the AngII-induced increases in BP. However, we emphasize that although both cilazapril and losartan markedly improved renal hemodynamics when administered at higher doses, impaired autoregulation persisted. The reasons for this relatively poor autoregulation in dTGR in this study and our earlier report (2) remain unclear.

We confirmed our previous findings (1,2) that both transfect genes are expressed in the kidney. Both hRen and rat renin genes were physiologically regulated in 9-wk-old hypertensive dTGR; this is similar to the results we reported for 6-wk-old dTGR (2). The lower expression of hRen and rat renin genes in dTGR is probably related to higher BP and higher AngII levels in these rats. The expression of both renin genes was also increased after long-term treatment with cilazapril and losartan, in parallel with the drug-induced BP reduction in these rats. These observations are different from those reported for the (mRen-2)27 transgenic rat model. In those transgenic rats, which bear the salivary (mRen-2)27 gene, the mRen gene is expressed in the kidney but is relatively impervious to even lifelong ACE inhibition, with normalization of BP (14).

Rat angiotensinogen gene expression in the kidney was lower in untreated dTGR compared with control animals. Moreover, both cilazapril and losartan increased rat as well as human angiotensinogen gene expression in the kidney. Our findings are consistent with results from previous studies (14,32,33) and suggest that AngII and BP are physiologic regulators of angiotensinogen gene expression in the kidney. This study is the first to describe rat ACE and AT_{1A} receptor gene expression in dTGR. We found no differences between normotensive control rats and dTGR in the expression or localization of these genes in the kidney. Neither chronic ACE inhibition nor AT_1 receptor blockade had any effect on the expression of these genes. We did not measure renal AT_1 receptor protein density in this study. Therefore, we cannot speculate on whether the high circulating AngII levels in dTGR are able to reduce the density of AT_1 receptors in the kidneys without altering the renal expression of the AT_{1A} receptor gene, as shown by Sechi *et al* (34).

In summary, our experiments indicate that pressure-natriuresis/diuresis curves are shifted to higher RPP ranges in dTGR, compared with normotensive control rats, and that inhibition of AngII generation by cilazapril or blockade of AT_1 receptors by

losartan is accompanied by a shift of these renal function curves to lower operating arterial BP levels. When administered at submaximal doses, the two drugs exert similar effects on systemic BP and pressure-natriuresis/diuresis curves; however, cilazapril improved sodium and water excretion mainly by increasing RBF and GFR, whereas losartan decreased tubular sodium and water reabsorption without markedly affecting renal hemodynamics. A combination of ACE inhibition and AT₁ receptor blockade induced an additive effect. Our results suggest that the hypertensive rightward shift in the pressure-natriuresis curve in dTGR is dependent on AngII-induced changes in renal hemodynamics, as well as on disturbances in tubular function.

Acknowledgments

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Safety of the combination of valsartan and benazepril in patients with chronic renal disease European Group for the Investigation of Valsartan in Chronic Renal Disease

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OBJECTIVE Several experimental and clinical studies indicate that the renin system may play a pivotal role in progressing renal disease. The combination of an angiotensin-converting enzyme inhibitor and an angiotensin receptor blocker could provide a higher degree of blockade of the renin-angiotensin system than either agent alone. Such enhanced suppression might be of benefit for patients exhibiting a progressive decline in renal function because of chronic renal disease.

METHODS A pilot multinational multicentre, randomized active-controlled parallel group open-label study has been conducted in a group of patients with progressive chronic renal failure (creatinine clearance 20-45 ml/min) either with or without proteinuria and hypertension. The primary aim of the study was to investigate the safety and tolerability of the combination of valsartan and benazepril. Patients were randomly assigned to one of three groups: group 1 received valsartan 160 mg once daily (n = 22); group 2 received valsartan 80 mg once daily plus benazepril 5 or 10 mg once daily (n = 42); group 3 received valsartan 160 mg once daily plus benazepril 5 or 10 mg once daily (n = 44). The study lasted for 5 weeks and in groups 2 and 3 benazepril was added on top of valsartan after the first week of therapy with the angiotensin receptor blocker.

RESULTS Serum creatinine increased in all three groups (mean change within a group: 11 micromol/l in group 1, P = 0.045; 9 micromol/l in group 2, P = 0.030; 15 micromol/l in group 3, P = 0.0006). Serum potassium also increased in all three groups of patients (mean change within a group: 0.28 mmol/l in group 1, P = 0.28; 0.48 mmol/l in group 2, P = 0.0008; 0.36 mmol/l in group 3, P = 0.02). After 5 weeks of treatment the largest decrease in blood pressure was observed in group 3 (the mean change from baseline in seated diastolic blood pressure (SDBP) and seated systolic blood pressure (SSBP) respectively were -2.0 and -11.5 mmHg in group 1, -7.6 and -15.4 mmHg in group 2, -12.6 and -21.6 mmHg in group 3). In addition both combination treatments resulted in the reduction of proteinuria. The total number of patients with adverse experiences were 10 (45.5%), 14 (33.3%) and 11 (25%) in groups 1, 2 and 3 respectively. In six patients (5.6%) therapy was discontinued as a result of adverse experiences. Only one patient in each of the combined therapy groups withdrew from the study because of hyperkalaemia and no patients were forced to withdraw because of an increase in serum creatinine, acute renal failure or hospitalization.

CONCLUSIONS These results indicate that short-term combination of an angiotensin-converting enzyme inhibitor and an angiotensin receptor blocker is safe and well tolerated in patients with moderate chronic renal failure.

Publication types

- Clinical Trial
- Multicenter Study
- Randomized Controlled Trial

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