Superiority of combination of thiazide with angiotensin-converting enzyme inhibitor or AT\textsubscript{1}-receptor blocker over thiazide alone on renoprotection in l-NAME/SHR

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Zhou, Xiaoyan, Luis C. Matavelli, Hidehiko Ono, and Edward D. Frohlich. Superiority of combination of thiazide with angiotensin-converting enzyme inhibitor or AT\textsubscript{1}-receptor blocker over thiazide alone on renoprotection in l-NAME/SHR, Am J Physiol Renal Physiol 289: F871–F879, 2005. First published May 17, 2005; doi:10.1152/ajprenal.00129.2005.—The renal and glomerular dynamic effects of combining thiazide and angiotensin antagonists have not been reported. The present study was designed to examine the effects of hydrochlorothiazide (HCTZ) alone or in combination with an angiotensin-converting enzyme inhibitor or ANG II type 1-receptor blocker on renal hemodynamics, glomerular dynamics, renal function, and renal histopathology in the \(N^a\)-nitro-L-arginine methyl ester-treated spontaneously hypertensive rat (l-NAME/SHR) model. HCTZ (80 mg·kg\textsuperscript{-1}·day\textsuperscript{-1}) alone or in combination with enalapril (30 mg·kg\textsuperscript{-1}·day\textsuperscript{-1}) or losartan (30 mg·kg\textsuperscript{-1}·day\textsuperscript{-1}) or enalapril (15 mg·kg\textsuperscript{-1}·day\textsuperscript{-1}) plus losartan (15 mg·kg\textsuperscript{-1}·day\textsuperscript{-1}) was administered to l-NAME/SHR (5.0 ± 0.10 mg·kg\textsuperscript{-1}·day\textsuperscript{-1}) for 3 wk. Mean arterial pressure, total peripheral resistance, renal plasma flow, glomerular filtration rate, glomerular hydrostatic pressure, afferent and efferent glomerular arteriolar resistances, single nephron plasma flow, single nephron glomerular filtration rate, serum creatinine concentration, 24-h urinary protein excretion, and glomerular and arteriolar injury scores were determined. HCTZ reduced mean arterial pressure, total peripheral resistance, glomerular hydrostatic pressure, and afferent and efferent glomerular arteriolar resistances \((P < 0.05, \text{ at least})\) but slightly increased renal plasma flow and single nephron plasma flow associated with reduced serum creatinine concentration, urinary protein excretion, and arteriolar injury score compared with l-NAME/SHR control. However, the combination of enalapril and/or losartan with HCTZ markedly improved each of these functions. These results demonstrated minor benefits of HCTZ monotherapy and a marked superiority of its combination with enalapril and/or losartan over HCTZ monotherapy on renoprotection in l-NAME/SHR, thereby providing strong evidence of their clinical benefits for hypertensive patients with renal functional impairment.

thiazide diuretic; ANG II type 1-receptor blocker; renal effect

MATERIALS AND METHODS

Fifty-four male SHR rats, purchased from Charles River Laboratories (Wilmington, MA), were individually housed in a temperature- and humidity-controlled room provided with a 12:12-h dark-light cycle. All rats were given standard chow (PMI Feeds, St. Louis, MO) with free access to tap water, ad libitum. All procedures were conducted in accordance with conventional animal care guidelines, and the experimental protocol was approved in advance by our institutional Animal Care and Use Committee.

At the age of 17 wk, the rats were divided randomly into six experimental groups: \textit{group 1}, control \((n = 10)\); \textit{group 2}, l-NAME \((n = 9; 5.0 ± 0.10 \text{ mg·kg}^{-1}·\text{day}^{-1})\) in drinking water for 3 wk; \textit{group 3}, l-NAME plus HCTZ \((n = 8; 80 \text{ mg·kg}^{-1}·\text{day}^{-1})\) by gastric gavage for 3 wk; \textit{group 4}, l-NAME plus HCTZ plus enalapril \((n = 8; 30 \text{ mg·kg}^{-1}·\text{day}^{-1})\) by gastric gavage for 3 wk; \textit{group 5}, l-NAME plus HCTZ plus losartan \((n = 9; 30 \text{ mg·kg}^{-1}·\text{day}^{-1})\) by gastric gavage for 3 wk; and \textit{group 6} \((n = 9), \text{ l-NAME plus HCTZ plus}

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enalapril (15 mg·kg⁻¹·day⁻¹) plus losartan (15 mg·kg⁻¹·day⁻¹) for 3 wk. Because a one-half dose each of enalapril and losartan cotreatment in preliminary studies was demonstrated to reduce mean arterial pressure (MAP) to the same extent as full doses of either enalapril or losartan alone, we chose a one-half dose of each agent for group 6 to evaluate the effectiveness of combination therapy of HCTZ with enalapril and losartan. L-NAME was purchased from Sigma (St. Louis, MO), and enalapril and losartan were provided by Merck (Rahway, NJ). Before the hemodynamic and renal micropuncture study, 24-h urinary protein and sodium excretions were determined using the Lowry (18) and flame photometric methods, respectively.

Renal micropuncture. Rats were anesthetized with thiobutabarbital sodium (Inactin, 100 mg/kg ip; Sigma-Aldrich, St. Louis, MO) and then placed on a heating pad to maintain rectal temperature at 37°C throughout the study. After tracheal intubation, a polyethylene PE-50 tubing was inserted into the right femoral vein to permit intermittent blood sampling and measurement of MAP and heart rate, which were determined through a transducer (model P23Dx, Statham Instruments, Oxweld, CA) connected to a multichannel polygraph (Sensor Medics 8712, Beckman Instruments, Schiller Park, IL). The right femoral artery was cannulated with a PE-50 tubing for [³H]methoxyinulin (850 μCi/ml) infusion at a rate of 0.1 ml·100 g⁻¹·h⁻¹. The right carotid artery was cannulated with a thermistor microprobe (type IT-18; Physitemp Instruments, Clifton, NJ) connected to a thermaldilution device (Carbothrometer 500, Columbus Instruments, Columbus, OH) to determine cardiac output, which was normalized for body weight and expressed as cardiac index (ml·min⁻¹·kg⁻¹). Total peripheral resistance was calculated as the quotient of MAP and cardiac index. The right jugular vein was also cannulated with a PE-50 tubing for [³H]methoxyinulin (850 μCi/ml) infusion at a rate of 0.1 ml·100 g⁻¹·h⁻¹. The bladder was catheterized with a PE-100 tubing for right renal urine collection. The left kidney was exposed subcostally, its renal surface was bathed in 0.9% NaCl, and its ureter was catheterized with a PE-10 tubing for left renal urine collection. After a 1-h equilibration period, urine was collected over four consecutive 30-min periods with blood samples being withdrawn at their respective midpoints. Simultaneously, the following micropuncture interventions were made: 1) precisely timed (90 s) samples of fluid were collected from four to six randomly selected superficial proximal tubules for determination of single nephron glomerular filtration rate (SNGFR); 2) efferent arteriolar, proximal tubular, and stop-flow pressures were measured directly by a servo-nulling system (Instrumentation for Physiology & Medicine, San Diego, CA); and 3) efferent arteriolar blood was withdrawn directly from two or three superficially located “star vessels” (16, 24, 28). Glomerular capillary pressure was calculated from the sum of the stop-flow pressure and the systemic afferent colloid osmotic pressure. Arterial plasma protein concentration was measured refractometrically, and afferent and efferent colloid osmotic pressures were calculated using the Landis-Pappenheimer equation (8). [³H]methoxyinulin radioactivities of all tubular fluid, plasma, and urine samples were counted to determine SNGFR and glomerular filtration rate. These measurements were used to calculate afferent and effluent glomerular arteriolar resistances. The ultrafiltration coefficient (Kₜ) was calculated as follows: Kₜ = SNGFR/(ΔP - Δt/60), where ΔP is the pressure gradient across glomerular capillary wall and Δt is the transmembrane colloid osmotic pressure difference (16, 24, 28). At the termination of each study, blood was withdrawn to determine serum creatinine and uric acid concentrations using a 747–100 analyzer (Boehringer Mannheim/Hitachi).

Renal histopathology. After fixation in 10% neutral buffered formalin and embedding in paraffin, the kidneys were cut at 2- to 3-μm-thick sections and stained with hematoxylin and eosin, periodic acid-Schiff, or periodic acid-methenamine silver. Histological examination was conducted in a blinded fashion, and glomerular (GIS) and arteriolar (AIS) injury scores were calculated (12, 28). GIS was graded from 0 to 3+ on glomerular injuries and sclerosis: 0 was no injury, 1+ was injury of up to one-third (≤1/3), 2+ was one-third to two-thirds injury (1/3~2/3), and 3+ was injury of more than two-thirds (≥2/3) of glomerular involvement. The AIS was also graded from 0 to 3+ for different arteriolar hyalinosis and sclerosis (arteriolar-losclerosis): 0 was no injury, 1+ demonstrated arteriolar lesions up to 50% of the mural circumference, 2+ demonstrated lesions between 50 and 100% of the wall circumference but without luminal narrowing, and 3+ was complete mural hyalinosis with luminal encroachment. The glomerular pathological profiles were determined at two renal depths, subcapsular and juxtamedullary cortex, each obtained by serial section. Whole-kidney GIS was expressed as the total scores of the subcapsular and juxtamedullary layers. GIS and AIS indicated the total injury scores in 100 glomeruli or arterioles, respectively.

Statistical analyses. All data are presented as means ± SE. One-way ANOVA followed by Duncan’s multiple range tests were used for group comparisons. A value of <5% was considered to be of statistical significance.

RESULTS

Body and organ weights. L-NAME significantly reduced body weight and increased mass of left ventricle, left kidney, and aorta, which were prevented, for the most part, by the HCTZ alone or in combination with enalapril and/or losartan. Of particular interest, coadministration of HCTZ with enalapril plus losartan did not prevent the L-NAME-induced body weight loss (Table 1).

Systemic and renal hemodynamics and glomerular dynamics. HCTZ alone or in combination with enalapril and/or losartan significantly decreased MAP and total peripheral resistance while increasing cardiac index. Hematocrit was significantly reduced by treatment with losartan. The HCTZ monotherapy and its combination therapies increased renal plasma flow, glomerular filtration rate, and filtration fraction and reduced renal vascular resistance, although HCTZ alone did not affect renal plasma flow significantly. However, HCTZ alone significantly increased SNGFR and reduced afferent and

Table 1. Body and organ weights

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<tr>
<td>Body weight, g</td>
<td>340 ±5.7</td>
<td>321 ± 8.2</td>
<td>350 ± 3.4</td>
<td>342 ±7.0e</td>
<td>347 ±9.0e</td>
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<td>Left ventricle, mg/g</td>
<td>2.76 ±0.04</td>
<td>3.55 ±0.09p</td>
<td>3.05 ±0.07p</td>
<td>3.25 ±0.03bde</td>
<td>2.29 ±0.06bde</td>
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<tr>
<td>Right ventricle, mg/g</td>
<td>0.59 ±0.03</td>
<td>0.66 ±0.02a</td>
<td>0.57 ±0.01a</td>
<td>0.57 ±0.02a</td>
<td>0.58 ±0.01a</td>
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<tr>
<td>Left kidney, mg/g</td>
<td>3.41 ±0.12</td>
<td>4.00 ±0.14p</td>
<td>3.70 ±0.05p</td>
<td>3.70 ±0.06e</td>
<td>3.74 ±0.06e</td>
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<tr>
<td>Aorta mg·mm⁻¹·kg⁻¹</td>
<td>3.13 ±0.11</td>
<td>3.99 ±0.20p</td>
<td>3.46 ±1.0a</td>
<td>2.78 ±0.05abe</td>
<td>2.67 ±0.16abe</td>
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Values are means ± SE. SHR, spontaneously hypertensive rats; L-NAME, N⁵-nitro-l-arginine methyl ester; HCTZ, hydrochlorothiazide. *P < 0.05, **P < 0.01 vs. SHR control; *P < 0.05, **P < 0.01 vs. L-NAME/SHR; *P < 0.01 vs. L-NAME/SHR + HCTZ.
efferent glomerular arteriolar resistances and glomerular hydrostatic pressure; single nephron plasma flow, single nephron filtration fraction, and $K_f$ were insignificantly changed. The combinations of HCTZ with enalapril and/or losartan significantly improved each of these glomerular functions. Of special note, any combination, either HCTZ with enalapril or with losartan or with both enalapril and losartan, was markedly superior to HCTZ monotherapy in improving systemic and whole kidney hemodynamics as well as glomerular dynamics, even though there were no further benefits for combining enalapril plus losartan with HCTZ compared with either enalapril or losartan with HCTZ. (Figs. 1–4)

**Renal function and histopathology.** HCTZ alone markedly reduced the serum creatinine and uric acid concentrations and 24-h urinary protein excretion, as did its combination with enalapril and/or losartan. Histological examination demonstrated that L-NAME induced severe nephrosclerosis, as indicated by significantly increased GIS (both subcapsular and juxtamedullary nephrons) and AIS. HCTZ alone significantly reduced AIS but did not alter GIS; however, its combination with enalapril and/or losartan produced added benefits in preventing renal functional and histological alterations. The combination of enalapril plus losartan with HCTZ did not provide further benefits on renal function or histopathology compared with either enalapril or losartan with HCTZ. (Figs. 5–8)

**DISCUSSION**

The results of this study demonstrated a slightly salutary effect of HCTZ alone but a marked superiority of its combination with enalapril or losartan in improving systemic and whole kidney hemodynamics, glomerular dynamics, renal function, and histopathology in L-NAME/SHR. Therefore, this study further confirmed previous reports (5, 6, 9, 14, 31, 37, 38, 40) that an ACE inhibitor or ARB combined with the thiazide provides better blood pressure control. Moreover, this study is the first to provide clear evidence of the profound benefits of combination therapy of HCTZ with enalapril and/or losartan on glomerular dynamics and renal functions in an experimental model of hypertension and severe renal injury. Thus the addition of an ACE inhibitor and/or an ARB to a thiazide provides the additional benefit of blocking the effects of ANG II, when renal hemodynamics and histopathology are severely impaired by L-NAME (15, 27, 39, 41). Recently, increasing evidence has supported further the renoprotective benefits of combined treatment with an ACE inhibitor and an ARB in patients with chronic renal diseases (2, 10, 17, 21, 25, 30), presumably because each of the two agents has different pharmacological and physiological profiles. The ARB antagonizes the actions of all ANG II at the receptor level, whereas the ACE inhibitor is ineffective in blocking ANG II generated by alternative non-ACE pathways such as chymases and endopeptidases (20, 29). Moreover, unopposed upregulation of the ANG II type 2 receptor following administration of ARB may also exert an antiproliferative effect (19, 23, 32, 34) and the ACE inhibitor also increases bradykinin (11, 35), a potent vasodilator (13). Because of these theoretical considerations, we studied the effectiveness of the combined therapy of enalapril and losartan (one-half dose of each agent) with HCTZ (full dose) in group 6. Our data, however, provided no additional benefit in this group compared with either enalapril or losartan alone together.

**SYSTEMIC HEMODYNAMICS**

![Graphs showing systemic hemodynamics](http://ajprenal.physiology.org/)

Fig. 1. Effects of hydrochlorothiazide (HCTZ) alone and its combination with enalapril and/or losartan on systemic hemodynamics in N^o^-nitro-l-arginine methyl ester-spontaneously hypertensive rats (L-NAME/SHR). MAP, mean arterial pressure; TPR, total peripheral resistance. Values are means ± SE. *$P<0.05$ (at least) vs. SHR control; †$P<0.05$ (at least) vs. L-NAME/SHR; #$P<0.05$ (at least) vs. L-NAME/SHR + HCTZ.

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Fig. 2. Effects of HCTZ alone and its combination with enalapril and/or losartan on renal hemodynamics in L-NAME/SHR. RPF, renal plasma flow; GFR, glomerular filtration rate; RVR, renal vascular resistance; FF, filtration fraction. Values are means ± SE. *P < 0.05 (at least) vs. SHR control; †P < 0.05 (at least) vs. L-NAME/SHR; #P < 0.05 (at least) vs. L-NAME/SHR + HCTZ.

Fig. 3. Effects of HCTZ alone and its combination with enalapril and/or losartan on glomerular dynamics in L-NAME/SHR. SNPF, single nephron plasma flow; SNGFR, single nephron glomerular filtration rate; SNFF, single nephron filtration fraction; Kᵢ, ultrafiltration coefficient. Values are means ± SE. *P < 0.05 (at least) vs. SHR control; †P < 0.05 (at least) vs. L-NAME/SHR.
Fig. 4. Effects of HCTZ alone and its combination with enalapril and/or losartan on glomerular dynamics in L-NAME/SHR. \( R_A \) and \( R_E \), afferent and efferent glomerular arteriolar resistances, respectively; \( P_G \), glomerular capillary pressure; SFP, stop-flow pressure. Values are means ± SE. \( *P < 0.05 \) (at least) vs. SHR control; \( †P < 0.05 \) (at least) vs. L-NAME/SHR; \( #P < 0.05 \) (at least) vs. L-NAME/SHR + HCTZ.

Fig. 5. Effects of HCTZ alone and its combination with enalapril and/or losartan on urinary protein, serum creatinine, urinary sodium, and serum uric acid in L-NAME/SHR. Values are means ± SE. \( *P < 0.05 \) (at least) vs. SHR control; \( †P < 0.05 \) (at least) vs. L-NAME/SHR; \( #P < 0.05 \) (at least) vs. L-NAME/SHR + HCTZ.
Fig. 6. Effects of HCTZ alone and its combination with enalapril and/or losartan on renal histopathology in L-NAME/SHR. GIS, glomerular injury score; AIS, arteriolar injury score. Values are means ± SE. *P < 0.05 (at least) vs. SHR control; †P < 0.05 (at least) vs. L-NAME/SHR; #P < 0.05 (at least) vs. L-NAME/SHR + HCTZ.

Fig. 7. Light microscopic findings in the glomeruli. Top: SHR control shows the almost normal glomerulus, L-NAME/SHR and L-NAME/SHR treated with HCTZ alone show significantly increased mesangial matrix in glomeruli. Bottom: HCTZ combined with enalapril (left), losartan (middle), and both enalapril and losartan (right), demonstrating the markedly improved glomerular changes.
with HCTZ, supporting our previous finding of similar renoprotection of angiotensin type 1-receptor antagonism and ACE inhibition (24). However, in an earlier report dealing with the coronary circulation, the combination of an ACE inhibitor with an ARB provided significantly more cardioprotection in SHR rats (26). Another study by de Gasparo et al. (4) demonstrated that combination of low-dose valsartan and enalapril significantly improved endothelial function and increased coronary reserve in L-NAME/SHR (4).

The present study was not designed to determine the mechanism for the short-term beneficial renal effects of HCTZ alone or with enalapril and/or losartan. However, this study demonstrated a far less impressive improvement in glomerular dynamics when HCTZ was used alone and the greater nephroprotective benefits of HCTZ combined with inhibition of the renin-angiotensin system, suggesting the greater specificity with these agents. Our earlier study demonstrated that ANG II inhibition with either candesartan or enalapril produced striking renoprotection (prevention as well as reversal) in the L-NAME/SHR with severe hypertensive nephrosclerosis (24).

Combined treatment of HCTZ with enalapril and/or losartan in the present study produced similar renal beneficial effects to those of candesartan or enalapril, suggesting that combination of HCTZ with agents that inhibit the renin-angiotensin system is both safe and highly effective in treating hypertension with renal injury. These findings are clinically important under those circumstances whereby combination therapy is needed and especially when a thiazide diuretic is necessary. This combination is the indispensable basis for treatment of patients with hypertension and renal functional impairment.

It is worthwhile to mention that losartan decreased hematocrit in the L-NAME/SHR. Previous studies from ours and other laboratories reported that hematocrit was reduced in rats treated with another ARB, candesartan (22, 24, 36). Naeshiro et al. (22) suggested that the cause of the anemia induced by candesartan was suppression of erythropoietin production due to increased renal blood flow produced by candesartan treatment. However, the plasma and renal erythropoietin levels were not significantly changed in SHR treated chronically with candesartan (24), and candesartan did not directly affect hypoxia-induced erythropoietin production in our previous study (36). Thus it appears that the underlying mechanism of the reduced hematocrit induced by an ARB needs further study. In addition, adding enalapril and losartan to HCTZ in L-NAME/SHR did not increase body weight, and we did not investigate the reason for this in the present study. However, complete inhibition of ANG II by combination of the ACE inhibitor and ARB could produce metabolic alterations produced by inhibiting adipocyte growth (6).

With respect to our dosage selection, 80 mg·kg⁻¹·day⁻¹ of HCTZ in the present study was based on the previous findings of our group’s two studies (16, 28). This dose produced detrimental renal effects even when arterial pressure was significantly reduced (28). We therefore chose the same dose of 80 mg·kg⁻¹·day⁻¹ in the present study for the groups of HCTZ alone and its combination with an ACE inhibitor and/or an ARB to evaluate whether the combination would produce better blood pressure control and, additionally, afford renal...

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**LIGHT MICROGRAPHS OF THE ARTERIOLES**

![SHR Control](image1)

![SHR+L-NAME](image2)

![L-NAME+HCTZ](image3)

![L-NAME+HCTZ+Enalapril](image4)

![L-NAME+HCTZ+Losartan](image5)

![L-NAME+HCTZ+Enalapril+Losartan](image6)

**Fig. 8. Light micrographic changes in arterioles.** Top: afferent arteriolar architecture with normal findings in SHR control (left), severe fibrinoid necrosis in the L-NAME/SHR (middle), and slight improvement in the L-NAME/SHR treated with HCTZ alone (right). Bottom: striking improvement of the arteriolar changes by combination of HCTZ with enalapril or losartan or both enalapril and losartan.
Injury in L-NAME/SHR (28), and induced efferent glomerular arteriolar constriction in SHR (16), these findings were different from those provided in the present report. The earlier study (28) was conducted over 9 yr ago; the rats were purchased from the same laboratory and were of the same age, gender, and strain; and our methods and techniques were identical. However, the doses of consumed L-NAME were different in our two studies, higher in the former (7.6 ± 0.7 mg·kg⁻¹·day⁻¹) than in the present (5.0 ± 0.10 mg·kg⁻¹·day⁻¹) study. Furthermore, we may be dealing with two genetically different SHR groups in whom mutations after over 9 yr are unknown. Moreover, susceptibility to nitric oxide inhibition and response to the thiazide may vary in the different SHR generations. This only underscores the importance of having specific control groups for all experimental studies, even when emanating from the same laboratory.

In summary, HCTZ alone provided minor benefits to the kidney; however, the addition of an ACE inhibitor and/or an ARB to HCTZ produced not only significantly greater reductions in arterial pressure and but also additional improvements in renal hemodynamics and glomerular dynamics as well as renal functions and histopathology.

The present findings in the L-NAME/SHR model provide strong evidence of the pathophysiological, clinical, and therapeutic benefits of combination therapies for hypertensive patients with preexisting renal functional impairment.

REFERENCES


