Effects of angiotensin-converting-enzyme inhibitors in combination with diuretics on blood pressure and renal injury in nitric-oxide-deficiency-induced hypertension in rats

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ABSTRACT

The present study investigates the effects of chronic administration of ACEIs (angiotensin-converting-enzyme inhibitors; either zofenopril or enalapril) in combination with a diuretic (hydrochlorothiazide) on BP (blood pressure) increase and renal injury induced by L-NAME (N\textsuperscript{G}-nitro-L-arginine methyl ester), an inhibitor of NO (nitric oxide) synthesis. Rats were untreated or received L-NAME alone, L-NAME + zofenopril + hydrochlorothiazide or L-NAME + enalapril + hydrochlorothiazide for 8 weeks. L-NAME treatment resulted in marked elevation in BP and mortality. Treatment with either ACEI and diuretic prevented the increase in BP induced by L-NAME, reduced the death rate and improved excretory parameters. Renal injury in the L-NAME group was severe, but, in the groups treated with either ACEI and diuretic, glomerular and tubulointerstitial lesions were not observed and the intensity, number and size of vessels affected was reduced. However, the efficacy of zofenopril + diuretic was superior to that of enalapril + diuretic in reducing vascular alterations. Oxidative stress indices and the expression of NO synthase and nitrotyrosine were normalized by the treatments. In conclusion, the combined treatment of zofenopril or enalapril with hydrochlorothiazide completely prevented the development of arterial hypertension induced by L-NAME. Renal morphological and functional alterations in the hypertensive animals were also almost completely normalized, but the treatment with zofenopril + diuretic produced a more complete organ protection. The protective effect is related to an activation of endothelial NO synthase expression and to a normalization of the oxidative stress parameters due to the inhibition of angiotensin II.

Key words: angiotensin-converting enzyme (ACE), arterial hypertension, nitric oxide, N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME), nitrotyrosine, renal injury.

Abbreviations: ACE, angiotensin-converting enzyme; ACEI, ACE inhibitor; BP, blood pressure; ENAL, enalapril; HA, hyaline arteriopathy; HCTZ, hydrochlorothiazide; L-NAME, N\textsuperscript{G}-nitro-L-arginine methyl ester; MAP, mean arterial pressure; MH, myointimal hyperplasia; NO, nitric oxide; NOS, NO synthase; PAS, periodate–Schiff; TBARS, thiobarbituric acid reactive substances; ZOFE, zofenopril.

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INTRODUCTION

NO (nitric oxide) is a very important vasodilator substance released by the vascular endothelium which plays an important role in the control of vascular and renal function. Inhibition of NO has been shown to induce vasoconstriction and arterial hypertension in experimental animals or human subjects [1–6]. In hypertensive patients, many studies suggest that endothelial dysfunction, resulting from a lower production of NO, may participate in the development and maintenance of arterial hypertension [1]. One of the most frequently used experimental models to analyse the role of NO in hypertension is that induced by the oral administration of NOS (NO synthase) inhibitors, such as l-NAME (N\textsubscript{G}-nitro-l-arginine methyl ester), in rats.

The mechanisms that participate in the renal alterations observed in the l-NAME-induced hypertension rat model have not been fully characterized, but it is now established that the local renin–angiotensin system plays an important role. In fact, arterial hypertension and renal alterations are prevented, or almost completely corrected, by inhibition of ACE (angiotensin-converting enzyme) or AT1s (type 1 angiotensin II receptors) [7]. The resulting vasoconstriction has been associated with enhanced sodium and water renal reabsorption [8], renal vasoconstriction [5], elevation in oxidative stress [9–11] and enhanced calcium signalling in smooth muscle cells [12,13]. In addition to its pronounced effect on circulatory function, chronic NOS inhibition can profoundly affect the structure of different organs, including blood vessels and renal, myocardial and nervous tissue. This is observed particularly in the kidney, where a variety of lesions characteristic of renal injury, such as glomerulosclerosis, glomerular ischaemia, glomerular segmental necrosis, interstitial expansion and microvascular lesions, are usually found after a prolonged increase in BP (blood pressure). All these types of renal injury are associated with progressive albuminuria, indicating that functional impairment of the glomerular wall barrier also occurs in this hypertension model. It is not known if these morphological alterations are produced by the arterial hypertension or by additional factors, such as the increased levels of angiotensin II or the enhanced renal oxidative status [14,15]. Therefore, in the present study, we have analysed these factors and the potential effect of the combination of the diuretic hydrochlorothiazide (HCTZ) with the sulphydrylic ACEI (ACE inhibitor) zofenopril (ZOFE), which, similar to captopril, possesses radical-scavenging capabilities [16,17], or with the carboxylic ACEI enalapril (ENAL). Due to the incomplete normalization produced by single antihypertensive therapy in this model [7], we have analysed the effect of combination therapy on l-NAME-induced hypertension and renal injury. In addition, it is known that the antihypertensive effect of single therapy is enhanced by a combination of drugs. This is used in moderate, but also in severe hypertensive states, and in non-responders to single therapy [18].

METHODS

Animals and experimental groups

Male Sprague–Dawley rats, born and raised at the Animal Centre of the University of Murcia, were used. The experiments were performed according to European Union Guidelines for the Ethical Care of Animals. Rats that initially weighed 200–250 g were randomly assigned to different experimental groups. All animals had free access to standard rat diet with a sodium content of 0.5% (rodent toxicology diet; B&K) and tap water ad libitum.

Rats were divided randomly into four groups: (i) control (n=10), (ii) l-NAME treated (20 mg·day\textsuperscript{-1}·kg\textsuperscript{-1} of body weight; n=11), (iii) l-NAME + ZOFE + HCTZ (20 mg·day\textsuperscript{-1}·kg\textsuperscript{-1} of body weight of l-NAME and 10 day\textsuperscript{-1}·kg\textsuperscript{-1} of body weight of ZOFE and HCTZ; n=11), and (iv) l-NAME + ENAL + HCTZ (20 mg·day\textsuperscript{-1}·kg\textsuperscript{-1} of body weight of l-NAME and 10·day\textsuperscript{-1}·kg\textsuperscript{-1} of body weight of ENAL and HCTZ; n=9). All the drugs were given in the drinking water, such that the concentrations were adjusted daily according to body weight and water intake. All treatments were started at the same time and were maintained for 8 weeks. Zofenopril calcium (Menarini IFR), ENAL (Sigma) and HCTZ (Pliva) were kindly provided by Menarini Ricerche.

Experimental protocol

Body weight and tail BP were determined every 2 weeks during the course of the experiment. BP was determined by the tail cuff method (Cibertec) by using MacLab software (AD Instruments) on a Macintosh LCII computer. This approach allowed the estimation of systolic and diastolic BPs and MAP (mean arterial pressure). After the time-course study, all animals were housed in metabolic cages with free access to food and their respective drinking fluids. After 2 days of adaptation, food and water intake and urine values were gathered during two consecutive days. Values obtained on each experimental day were averaged for statistical purposes. The urinary variables measured were: diuresis, natriuresis, creatinine and proteinuria. After the metabolic study was completed, animals were anaesthetized (50 mg of inactin/kg of body weight; intraperitoneally) and blood samples were taken into heparinized tubes from the abdominal aorta. Kidneys were removed for histological study.

Analytical procedures

Plasma and urine creatinine were measured by the Jaffe reaction using a commercially available kit (Boehringer etc.).
Mannheim). Sodium concentration was measured by an ion-selective electrode. Proteinuria was measured by the method described by Bradford [19].

TBARS (thiobarbituric acid reactive substances) in kidney tissue were determined as a measure of lipid peroxidation by using a colorimetric method [20]. Briefly, 0.5 ml of PBS was mixed with 50 µl of kidney lysate (between 1–3 mg of protein) and 1 ml of reagent [1 mmol/l deferoxamine mesylate, 7.5 % (w/v) trichloroacetic acid, 0.25 mol/l HCl and 0.37 % thiobarbituric acid] was added. The mixture was vortex-mixed and heated for 45 min in boiling water. After the mixture had returned to room temperature, TBARS from standards (prepared from 1,1,3,3-tetraethoxypropane) and samples were extracted into 1 ml of butanol. After a vigorous vortex-mixing and a brief centrifugation (1000 × g for 5 min), the absorbance of the butanol layer was read at 532 nm in a spectrophotometer (Varian 634), and the value was expressed as µg of TBARS/mg of protein.

Western blotting

Frozen kidneys were homogenized in lysis buffer [150 mmol/l NaCl, 5 mmol/l EDTA, 20 mmol/l Tris/HCl (pH 7.4) supplemented with detergents (0.5 % Igepal CA-630 and 1 % Triton X-100) and a cocktail of protease inhibitors (2 µg/ml aprotinin, 1 µg/ml pepstatin A, 10 µg/ml leupeptin, 500 µmol/l Na3VO4 and 1 mmol/l PMSF)]. The tissue homogenate was centrifuged (10 min, 10,000 × g), the supernatant was kept as the kidney tissue lysate. Protein samples (100 µg) were mixed (1:1) in 2× sample buffer [2 % (v/v) 2-mercaptoethanol, 4 % (w/v) SDS, 20 % (v/v) glycerol, 0.001 % Bromophenol Blue and 500 µmol/l Tris/HCl (pH 7.4)] and boiled for 5 min. Proteins were then separated by electrophoresis at constant voltage (100 V) on SDS/8 and 11 % (w/v) polyacrylamide gels in 25 mmol/l Tris/192 mmol/l glycine/0.1 % SDS. Proteins were transferred onto a 0.45 µm PVDF membrane (Millipore) by wet electrophoretic blotting in 25 mmol/l Tris/192 mmol/l glycine. Non-specific binding was blocked for 2 h in a TBS-T blocking buffer [20 mmol/l Tris/HCl (pH 7.5), 500 mmol/l NaCl and 0.1 % Tween 20] with 3 % (w/v) BSA. Western blot analysis was performed with specific monoclonal anti-eNOS (BD Transduction) or polyclonal anti-nitrotyrosine (Cayman Chemical) antibodies. Blots were incubated in TBS-T containing 1 % (w/v) BSA with the primary antibodies (1:1000 dilution) overnight at 4 °C. After incubation, the blots were washed and then incubated for 1 h in TBS-T containing 1 % (w/v) BSA with the secondary antibody [anti-(mouse IgG) or anti-(rabbit IgG) conjugated to HRP (horseradish peroxidase); 1:1000 dilution]. Following incubation, the blots were washed prior to detection of the proteins. Pre-stained protein markers (Bio-Rad Laboratories) were used for molecular-mass determination. Lysates from human aortic endothelium and nitrotyrosine BSA were used as positive controls for eNOS and nitrotyrosine respectively. Both protein markers and positive controls were run in parallel with the samples. Detection of specific proteins (140 kDa for eNOS and approx. 70 kDa for nitrotyrosine) was carried out using ECL® (Amershan Biosciences). The bands corresponding to the different proteins were scanned and the relative expression of NOS protein was quantified by densitometric analysis. Densitometric results are reported as integrated values (area × density of the band, corrected by protein loading) and expressed as a percentage of the controls (100 %). To determine protein loading, the blots were stained with 0.1 % Ponceau Red (Sigma).

Histological techniques

For conventional morphology, buffered 4 % (v/v) formaldehyde-fixed paraffin-embedded longitudinal kidney sections in sagittal plane were stained with haematoxylin and eosin and PAS (periodate–Schiff) stain. The extent of vascular injury [stenosis, HA (hyaline arteriopathy) and MH (myointimal hyperplasia)] was assessed by examining profiles of arteries and arterioles in a single kidney section and by counting the number of vessels affected. The presence of glomerular lesions (glomerulosclerosis and capsular fibrosis) was evaluated in at least 200 glomeruli. Tubular atrophy and tubular casts were also evaluated. The morphological study was done in blinded fashion on 4-µm-thick sections with light microscopy, using the most appropriate stain for each lesion. The values were expressed as the percentage of rats with lesions in each group, and the severity of lesions was calculated semi-quantitatively using a 0–3 scale [0, absence; 1, mild (<10 % of vessel, tubules or glomeruli involved); 2, moderate (10–25 %); and 3, severe (>25 %)]. In addition, the number of vessels affected by HA per section were counted.

Statistical analyses

Data are means ± S.E.M. A repeated measures ANOVA was used to obtain the statistical significance between and within groups. Differences were considered statistically significant at a P value < 0.05.

RESULTS

1-NAME intake induced a marked increase in BP (Figure 1) and either ZOFE or ENAL in combination with HCTZ completely and significantly (P < 0.01) prevented this rise in BP associated with chronic inhibition of NOS from the first BP measurement 15 days after the start of the treatment (Figure 1). The hypotensive effects of both ACEI combinations remained stable during the whole observation period up to 60 days of
treatment (Figure 1). A trend of decreased body weight compared with controls was observed in all of the three groups that received l-NAME, but no statistical differences in body weight were found between these groups during the observation period (results not shown).

l-NAME intake induced a noticeable mortality, which was prevented by the concomitant treatment with both combinations of ACEIs and diuretic (Table 1).

As shown in Table 1, there was a significant decrease ($P < 0.05$) in creatinine clearance and urinary nitrite concentration in the l-NAME-treated animals, which was completely prevented by both ACEIs plus diuretic. Diuresis increased in the group treated with l-NAME compared with controls, and was normalized only in the group treated with ZOFE + HCTZ, indicating a better protection of kidney function produced by this sulphydryl ACEI in combination with the diuretic (Table 1). Natriuresis was decreased significantly in the l-NAME-treated group and both treatments with ACEIs and diuretic increased it, probably due to the inhibitory reabsorption effect of HCTZ (Table 1).

Figure 2 shows the protein expression of eNOS and nitrotyrosine measured as a percentage of controls (100%). There was a significant reduction in eNOS expression in the l-NAME-treated animals, which was corrected well above basal levels by both combinations of antihypertensive treatment. Nitrotyrosine expression was significantly reduced by l-NAME treatment, and combinations of either ZOFE or ENAL with the diuretic prevented this decrease.

A significant increase ($P < 0.05$) in the concentration of TBARS was observed in kidneys of animals chronically treated with l-NAME compared with controls (1.41 ± 0.23 compared with 0.57 ± 0.19 nmol/mg of protein respectively), which was normalized by treatment

<table>
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<tr>
<th>Table 1</th>
<th>Mortality and renal functional parameters in the experimental groups</th>
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<tr>
<td>Values are means ± S.E.M. of 9–11 rats per group. *$P &lt; 0.05$ compared with control.</td>
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<tr>
<td>l-NAME</td>
<td>Control</td>
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<td>Rats survived/total (n)</td>
<td>9/10</td>
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<tr>
<td>Creatinine clearance (ml·min$^{-1}$·100 g$^{-1}$)</td>
<td>0.98 ± 0.22</td>
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<tr>
<td>Proteinuria (mg·day$^{-1}$·100 g$^{-1}$)</td>
<td>1.20 ± 0.18</td>
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<td>Urinary nitrate concentration (µmol/l)</td>
<td>11.64 ± 2.69</td>
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<tr>
<td>Diuresis (ml·day$^{-1}$·100 g$^{-1}$)</td>
<td>6.83 ± 0.24</td>
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<tr>
<td>Natriuresis (µEq·day$^{-1}$·100 g$^{-1}$)</td>
<td>347.4 ± 42.8</td>
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Effect of ACE inhibitors and diuretics on NO-induced hypertension

Figure 3  Histological sections of kidneys in the experimental groups
(A) Control rats: an absence of glomerular, vascular or tubulointerstitial lesions was observed. (B) L-NAME-treated rats: an intense vascular lesion with HA, severe MH and vascular thrombosis in medium-sized vessels (interlobular arteries), which reflects a glomerulus with ischaemic appearance, were observed. (C) L-NAME + ZOFE + HCTZ-treated rats: a mild effect was observed, exclusively in afferent arterioles (arrows) of some glomeruli. (D) L-NAME + ENAL + HCTZ-treated rats: a mild-to-moderate effect on afferent arterioles (arrows) of many glomeruli was observed. Sections were stained by PAS. Magnification, ×10.

with either ZOFE or ENAL in combination with the diuretic (0.85 ± 0.30 and 0.69 ± 0.23 nmol/mg of protein in the ZOFE + HCTZ and ENAL + HCTZ groups respectively).

Histopathology results
Lesions in control rats were negligible, no glomerular or vascular lesions were present in renal parenchyma and only weak and scattered tubular cast (three out of nine rats) and tubular atrophy (one out of nine rats) were observed. The injury in the L-NAME group was severe, with more than 75% of rats presenting prominent glomerular lesions (glomerulosclerosis or capsular fibrosis, an indicator of hypertension). In this group, weak/moderate tubulointerstitial lesions were also observed in almost all animals. L-NAME-treated rats also had severe vascular alterations. HA was present in almost all afferent arterioles of glomeruli and PAS-stain-positive deposits were identified in the vascular wall of most interlobular arteries; HA was absent only in the renal artery and subsegmentary vessels (Figure 3). All of the kidneys examined in the L-NAME-treated group had developed proliferative arteriopathy (MH) with thrombosis and severe luminal obliteration. In the groups that received both L-NAME and either ACEI in combination with diuretic, glomerular and tubulointerstitial lesions were substantially reduced (P < 0.05) and the intensity, number and size of vessels affected with the presence of HA and MH was dramatically reduced (P < 0.05; mean intensity of HA: 1.62 ± 0.74 in the ENAL + HCTZ-treated group, 1.00 ± 0.0 in ZOFE + HCTZ-treated group and 2.37 ± 0.56 in the L-NAME group; and mean intensity of MH: 0.12 ± 0.35 in the ENAL + HCTZ-treated group, 0.36 ± 0.50 in the ZOFE + HCTZ-treated group and 2.68 ± 0.79 in L-NAME group). Not only was the intensity of HA lower in the ZOFE + HCTZ-treated group, but the number of vessels affected by HA was also significantly lower (24.27 ± 5.74; P < 0.05) compared with the ENAL + HCTZ-treated (40.75 ± 18.39) and the L-NAME-treated groups (42.75 ± 13.97).

DISCUSSION
Previous studies have shown that chronic inhibition of NOS by L-NAME administration produced a dose-dependent form of arterial hypertension [1–3] that is mainly dependent on an increased activity of the renin–angiotensin system [5–7] and is prevented by treatment with ACEIs or angiotensin receptor antagonists [7]. The findings of the present study indicate, for the first time, that the combination of an ACEI with a diuretic, which is widely used to treat moderate-to-severe hypertension in humans, is very effective in reducing the increase in BP...
induced by NOS inhibition and in preventing the renal injury associated with this form of arterial hypertension.

Monotherapy with ZOFE has been reported to normalize the inflammatory and prothrombotic status of vascular walls subjected to NO deficiency [21,22]. The present study shows that ZOFE in combination with HCTZ effectively prevented arterial hypertension and almost all associated alterations at the kidney level. In terms of hypotensive effects and protection from the mortality induced by l-NAME administration, the effects of the combination of a diuretic with the sulphydryl ACEI ZOFE or the carboxylic ACEI ENAL were superimposable.

Regarding renal function, the present results confirm previous data with ACEIs alone [6–8] showing that, with the combination treatments, there was a decrease in the glomerular filtration rate and a maintenance of the water and sodium excretion levels. Thiazides are known to affect the distal renal tubular mechanism of electrolyte reabsorption, directly increasing excretion of sodium and chloride. In addition, the reduction of vasoconstrictor tone induced by the ACEI and the decrease in plasma volume induced by the diuretic co-operate to enhance the antihypertensive action and the effects on kidney function. In fact, the BP/diuresis and BP/natriuresis ratios changed by l-NAME intake were normalized in the groups receiving either ACEI in combination with the diuretic. It is interesting to note that, in a previous and similar study performed in our laboratory [7], captopril alone did not completely normalize the lower pressure diuretic and natriuretic responses of the l-NAME-treated animals, in spite of complete normalization of the BP levels. It is possible that the presence of HCTZ in the present experiments may have helped the normalization of these renal parameters; however, the present data also indicate that, in spite of the normalization of these excretory variables, the increased proteinuria associated with the administration of l-NAME was not completely restored by the treatments and this is likely to be a consequence of scant remaining HA observed in the afferent arterioles and glomeruli (see Figure 3). In any case, the renal protection afforded by either ACEI in combination with the diuretic was evident from the histological point of view, indicating the beneficial role of this class of antihypertensive drugs in the prevention of organ damage. In this context, the efficacy of treatment with ZOFE + HCTZ was significantly greater than that of ENAL + HCTZ to reduce the extent and presence of renal HA and the number of vessels affected and to normalize the diuresis. This might be due to the marked difference in lipophilia [23] and tissue uptake [24] between ZOFE and ENAL as, although they have similar BP-lowering effects, the protection of target organ damage is different.

eNOS expression, which was reduced by the chronic treatment with l-NAME, was similarly restored by the combination therapies in the present study. A similar behaviour was observed for nitrotyrosine, the other protein analysed, whose expression was also decreased in the l-NAME-treated group, probably due to the lower production of NO from the kidney. In this regard, it is important to note that TBARS, an index of lipid peroxidation, have been found to be increased by l-NAME treatment. It is likely that the reduction in TBARS, associated with the beneficial effect of both ZOFE and ENAL, is related to the local inhibition of angiotensin II production, due to ACE inhibition and to the normalization of BP.

In summary, the combined treatment with an ACEI and a diuretic not only reduced the hypertension induced by NO deficiency, but was able to prevent kidney damage that usually develops during a sustained hypertensive state.

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