Additive renoprotective effect of candesartan and tetrahydrobiopterin in rats after 5/6 nephrectomy

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Abstract

Background. Chronic treatment with candesartan cilexetil (C) improves the outcome of rats after 5/6 nephrectomy (Nx). Tetrahydrobiopterin (BH4), an essential cofactor for appropriate endothelial nitric oxide synthase (eNOS) activity, prevents an increase in blood pressure (BP) in Nx rats when given immediately after surgery. In the present study, we evaluated the renoprotective effect of a combined treatment.

Methods. Five groups of rats were studied: SHAM (sham-operated rats, n = 12); SNx (untreated 5/6 nephrectomized rats, n = 15); C (SNx rats treated with candesartan cilexetil, 5 mg/kg/day per os, n = 11); C + BH4 (SNx rats treated with candesartan cilexetil and BH4, 10 mg/kg/day intraperitoneally, n = 11); and BH4 (SNx rats treated with BH4, 10 mg/kg/day intraperitoneally, n = 11). Treatment began 30 days after surgery, when hypertension and renal insufficiency have developed. This day was considered as day 1 of treatment for statistical comparisons. The study was continued until 50% mortality was achieved in the SNx rats (4 months after surgery).

Results. The survival rates were 100% for SHAM, 47% for SNx, 50% for BH4, 64% for C and 80% for C + BH4 (P < 0.05 vs all). Untreated Nx rats developed hypertension, proteinuria (UP) and severe renal insufficiency. Mortality was associated with a lower renal function and increased urine protein excretion. In C and C + BH4 rats, systolic blood pressure (SBP) decreased significantly. BH4 alone had a mild non-significant effect on SBP. C and C + BH4 treatments attenuated significantly the increase in proteinuria found in SNx animals. The weight of the remnant kidneys as well as the severity of glomerulosclerosis were significantly lower in the C + BH4 rats.

Conclusion. This study shows that in subnephrectomized rats, addition of BH4 to a treatment with candesartan had an additive renoprotective effect. The mechanism of such action may include a better control of BP associated with a blockade of actions of angiotensin II (Ag II), an improvement in nitric oxide synthesis and a balanced redox.

Keywords: angiotensin receptor antagonist; hypertension; nitric oxide; proteinuria; renal failure; tetrahydrobiopterin

Introduction

Long-term management of chronic renal failure remains a major challenge in medicine. In rats after 5/6 nephrectomy (SNx), early treatment with angiotensin II receptor blockers (ARB) may prevent the progression of renal failure [1]. Control of blood pressure (BP) levels and efficient inhibition of the effects of angiotensin II (Ag II) are the main mechanisms through which these drugs exert their influence. When treatment with ARB begins after the development of the renal lesions, they may delay but do not prevent the progressive aggravation of renal disease [2]. Therefore, a combination of ARB with another renoprotective drug may be a relevant therapeutic option. In a recent article, Madaddu has mentioned that the ‘Achilles' heel' of the current anti-hypertensive drugs is that inhibition/blockade of single vasoconstrictor pathways may leave the cardiovascular system exposed to the damaging effects of unrelated mechanisms. An alternative strategy, which is gaining therapeutic relevance, consists of interfering with post-receptor signalling shared by different pathological stimuli [3]. It was proposed that the correction of endothelial dysfunction with tetrahydrobiopterin (BH4) might improve the treatment of hypertension [3].
In a recent study we could show that in SNx rats, early treatment with BH4 normalizes blood pressure (BP) values and reduces proteinuria after 8 weeks [4]. BH4 can restore the proper redox state in vascular cells by modulating the relative expression of constitutive nitric oxide synthase (NOS). Thus, nitric oxide (NO) production increases whilst there is a reduction in the synthesis of peroxinitrite, a radical cytotoxic agent [5].

The purpose of the present study was to evaluate whether a combined treatment with the angiotensin receptor blocker candesartan and BH4 may have an additive renoprotective effect in SNx rats with an established renal insufficiency.

**Materials and methods**

**Protocol of intervention**

Adult male Wistar rats, mean weight 320 ± 5 g, were housed under standard conditions and fed a normal chow diet and distilled water *ad libitum.*

Baseline clinical and laboratory data were obtained in all rats. Thereafter, the rats were randomly subdivided into five different groups and underwent 5/6 nephrectomy [4]. In brief, the animals were anaesthetized with pentobarbital [35 mg/kg body weight (BW) i.p] and then underwent a right nephrectomy and ligature of two of the major branches of the left main renal artery (SNx). Some rats underwent sham operations by laparotomy and mobilization of the renal vessels (sham rats). The same operator (GH) carried out all the SNx. Thirty days after surgery, when rats have already developed hypertension and renal insufficiency, all the SNx. Thirty days after surgery, when rats have already developed hypertension and renal insufficiency, clinical and laboratory data were obtained from all groups and the intervention programme treatment began as follows:

- **Group 1:** (SHAM, n = 12) sham operated rats
- **Group 2:** (SNx, n = 15) untreated 5/6 SNx rats
- **Group 3:** (C, n = 11) SNx rats received candesartan cilexetil (Astra Zeneca)
- **Group 4:** (C + BH4, n = 12) SNx rats treated with candesartan + BH4
- **Group 5:** (BH4, n = 11) SNx rats treated with BH4 (Almog Diagnostics, Israel).

Beginning of treatment was considered as day 1 for statistical comparisons. Candesartan cilexetil was prepared according to the manufacturer’s instructions to achieve water solubility. It was given daily, at a dose of 5 mg/kg, added to fresh drinking water. This dose was selected after a previous short evaluation in our laboratory. BH4, 10 mg/kg, was administered intraperitoneally (IP) by daily injection [4].

The study was continued until 50% mortality was achieved in the untreated Nx rats (4 months after surgery). The date of death of each rat was noted for further evaluations. At the end of the study the surviving rats were sacrificed and heart and kidney were weighed. Half of the remnant kidney of each rat was immediately frozen at −70°C and the second half was fixed in neutral buffered formalin.

**Clinical and laboratory evaluations**

Clinical data included BW and tail-measured systolic blood pressure (SBP), 24-h water intake, food intake and urine volume. The Animal Care and Use Committee of Meir Hospital approved all procedures. Laboratory data included serum albumin and creatinine, 24-h creatinine clearance (CCR), serum Na⁺, daily proteinuria (UP) and 24-hour urinary nitrate excretion (UNO3). SBP was measured in trained unstressed rats 1 day before surgery and each 4 weeks by tail-cuff manometry using an automated sphygmomanometer (Narco Bio Systems, Austin, TX). Serum albumin (as nutritional index) was assayed at the end of the study. Serum creatinine, CCR, UP and UNO3 were determined before surgery and each 4 weeks till the end of the study. For the 24-h urine collections (two consecutive days) the rats were housed in metabolic cages 3 days before each urine collection and were fed a low nitrate diet containing 0.35 g of NaCl, 20 g of protein and 1.17 g of arginine per 100 g. Gentamicin (6 mg/tube) was added to the urine collection tubes to avoid bacterial growth. Serum creatinine, albumin and Na⁺, urinary creatinine Na⁺ and protein were assayed by standard methods. Urine nitrate was determined as previously described [6].

**Histological examinations**

Kidneys were fixed in neutral buffered formalin and embedded in paraffin for light microscopic study. Histological sections were stained with H&E, PAS and Masson. The histological sections were coded so that the pathologist (J.B.) was unaware of the source of each sample. Sixty glomeruli from each kidney were examined.

A semi-quantitative score was used to assess the severity of glomerular lesions [4]. In brief, the degrees of glomerulosclerosis (GS) and mesangial expansion (ME) lesions were classified from zero to four according to the percentage of injury in the glomeruli (0 = 0%, 1 = 25%, 2 = 50%, 3 = 75% and 4 = 100% injury, respectively). Thereafter, the number of glomeruli in each degree was assessed and the mean ± SD values were calculated and compared between the different groups.

Tubulointerstitial and vascular damage were assessed on PAS-stained paraffin sections at a magnification of ×100, score evaluation in accordance with Adamczak et al. [7].

**Isolation of cortex renal vascular trees and immunobLOTS for endothelial nitric oxide synthase (eNOS)**

Half of the kidneys of SNX rats were snap frozen on liquid nitrogen and conserved at −70°C temperature.

For isolation of cortical renal vascular trees, the cortex was bisected from the medulla and pressed against stainless steel grids of 300 microns open (50 mesh) size. Renal vascular trees were gently rubbed against the grids and washed with ice-cooled PBS. With this procedure, the renal parenchyma passes through the mesh, whereas the vascular tree is retained on the grid and is collected after washing. The purity of the preparation was ~80–90% as observed under birefringence microscopy.

**Endothelial nitric oxide synthase protein expression [8]**

The vascular cortical vessels were homogenized using a lysis buffer containing 50 mM Tris–HCL, 0.1 mM EGTA, 0.1 EDTA, 5 mM sodium fluoride, 1 mM sodium
pyrophosphate, 1 mM sodium vanadate, 1 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride, protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany), 1% (vol/vol) Igepal ca360, 0.1% SDS and 0.1% deoxycholate, pH 7.5.

Protein purification of tissue samples was performed using the Pierce Micro BCA Protein assay reagent kit. For eNOS examination, 30 µg of protein were used for electrophoresis and separated by SDS-PAGE on a 7.5% acrylamide gel. Proteins were electroblotted on nitrocellular membranes. The membranes were blocked in 5% milk, washed in Tris-buffered saline with 0.1% Tween and incubated with eNOS monoclonal antibody (1:2000; BD Transduction Laboratories, Los Angeles, CA, USA). The immunoblots were visualized using horseradish peroxidase (HRP)-linked secondary antibody and ECL reagents, on Fuji Super RX film. The expression of eNOS protein was detected as a single band at 136 kDa. The autoradiograms were imaged with UMAX vitasC software, and the relative band densities were quantified using Adobe PhotoShop. Because this analysis involved comparison of many membranes, we factored the integrated density for each eNOS by internal standards (to correct between membranes).

Statistical analysis

Results are expressed as mean±SD. ANOVA was used to compare between groups, and Bonferroni as post hoc test. For small samples, a non-parametric test (Kruskal–Wallis test) was performed.

A t-test for paired groups or Wilcoxon signed ranks test was used as appropriate. The GLM repeated measures was used to compare between groups and between times of follow-up, with Bonferroni test. The chi-square test procedure was performed to compare nominal variables between groups. The Spearman correlation was used to check significance correlations between clinical data. The SPSS software (version 14) was used for statistical evaluations.

Results

Survival rate

Eighteen rats died during the study. Mortality was associated with lower BW, high SBP values and marked renal insufficiency.

Mortality rate was null in SHAM rats. By definition, 52% of the untreated SNx rats died during the study, mainly in the 3rd and 4th month of follow-up. In the C+ BH4 group, two rats that remained alive were excluded from the final evaluation due to the finding of neuroblastoma in the remnant kidney in one rat and transitional cell carcinoma of the bladder in the other. At the end of the study, the percentage of living animals and the mean survival time (days) were lower in the SNx, C and BH4 groups compared with SHAM rats. In contrast, there was no difference between SHAM and C + BH4 animals (Table 1, Figure 1). Mortality was associated with a decreased BW, CCR and increased serum creatinine and UP (data not shown).

Clinical and laboratory follow-up throughout the study

As previously described, the rats were subdivided into different groups before 5/6 nephrectomy. Unexpectedly, 30 days after surgery (before the beginning of treatment), SBP and UP were higher in the C + BH4 group compared with the other SNx groups. Due to this variability, we use the GLM repeated measures to evaluate the results of the different treatments.

BW and food intake in all SNx groups were similar at the end of the study. Water intake and urinary output increased in C + BH4 and BH4 rats. The level of serum albumin was significantly decreased in SNx rats compared with SHAM. In C and C + BH4 rats, serum albumin levels increased significantly compared with SNx and BH4 rats (Table 2). Serum Na⁺ was similar in all rats (Table 2).

As expected, SBP increased significantly in all SNx rats, from 114±7 to 152±22 mmHg 30 days after surgery (P<0.01 for all groups, Table 3). Thereafter, in SNx rats, there was an additional moderate increase of SBP until the end of the
Combined treatment with candesartan and tetrahydrobiopterin after renal mass reduction

Table 2. Clinical data of survival rats at the end of the study Mean ± SD (no. of rats)

<table>
<thead>
<tr>
<th></th>
<th>SHAM (12)</th>
<th>SNx (7)</th>
<th>C (7)</th>
<th>C + BH4 (8)</th>
<th>BH4 (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>433 ± 32</td>
<td>402 ± 63</td>
<td>469 ± 61</td>
<td>413 ± 26$^c$</td>
<td>468 ± 25</td>
</tr>
<tr>
<td>BW (% change since day 30 after Nx)</td>
<td>21 ± 4$^d$</td>
<td>17 ± 14</td>
<td>25 ± 6$^a$</td>
<td>7 ± 10</td>
<td>12 ± 5</td>
</tr>
<tr>
<td>Food intake, 24 h (g)</td>
<td>15 ± 2.8</td>
<td>13 ± 3.5</td>
<td>13 ± 3.1</td>
<td>17 ± 3.3</td>
<td>18 ± 1.5</td>
</tr>
<tr>
<td>Water intake, 24 h (ml)</td>
<td>23 ± 4.4$^b$</td>
<td>39 ± 12$^b$</td>
<td>34 ± 12</td>
<td>49 ± 15$^c$</td>
<td>52 ± 4.5$^d$</td>
</tr>
<tr>
<td>Urinary output, 24 h (ml)</td>
<td>13 ± 7</td>
<td>32 ± 11</td>
<td>20 ± 8</td>
<td>41 ± 6.6</td>
<td>33 ± 3.3</td>
</tr>
<tr>
<td>Serum albumin (g/l)</td>
<td>3.5 ± 0.2$^c$</td>
<td>2.2 ± 0.8$^c$</td>
<td>3.1 ± 0.25</td>
<td>3.2 ± 0.42</td>
<td>2.7 ± 0.64</td>
</tr>
<tr>
<td>Plasma Na$^+$ (mEq/l)</td>
<td>144 ± 2</td>
<td>146 ± 3.8</td>
<td>142 ± 1.7</td>
<td>143 ± 3.4</td>
<td>145 ± 2.2</td>
</tr>
<tr>
<td>Urinary NO3 (µmol/mg creatinine)</td>
<td>4.7 ± 2.2</td>
<td>2.6 ± 2.6</td>
<td>5.0 ± 2.1</td>
<td>6.7 ± 3.4</td>
<td>6.9 ± 4.9</td>
</tr>
</tbody>
</table>

SHAM, sham-treated rats; SNx, untreated 5/6 nephrectomized rats; C, SNx rats treated with candesartan cilexetil, 5 mg/kg per os; C + BH4, SNx rats treated with candesartan cilexetil and BH4; BH4, SNx rats treated with BH4, 10 mg kg/BW intraperitoneally.

*P < 0.01 vs all.
$^bP < 0.05$ vs BH4.
$^cP < 0.05$ vs C.
$^dP < 0.05$ vs SNx, C + BH4 and BH4.
$^eP < 0.05$ vs C and BH4.
$^fP < 0.05$ vs C and C + BH4.

Table 3. Clinical data before (B) and 30 (30) days after subtotal nephrectomy

<table>
<thead>
<tr>
<th></th>
<th>SHAM (12)</th>
<th>SNx (15)</th>
<th>C (11)</th>
<th>C + BH4 (10)</th>
<th>BH4 (10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW - B</td>
<td>312 ± 20</td>
<td>320 ± 13</td>
<td>331 ± 25</td>
<td>336 ± 26</td>
<td>329 ± 20</td>
</tr>
<tr>
<td>30</td>
<td>340 ± 42</td>
<td>350 ± 40</td>
<td>340 ± 33</td>
<td>375 ± 40</td>
<td>384 ± 34$^d$</td>
</tr>
<tr>
<td>SBP - B</td>
<td>113 ± 9</td>
<td>114 ± 3.5</td>
<td>112 ± 7</td>
<td>115 ± 9</td>
<td>113 ± 8</td>
</tr>
<tr>
<td>30</td>
<td>112 ± 6</td>
<td>148 ± 26</td>
<td>140 ± 18</td>
<td>161 ± 21</td>
<td>163 ± 16*</td>
</tr>
<tr>
<td>CCR - B</td>
<td>0.50 ± 0.08</td>
<td>0.50 ± 0.07</td>
<td>0.49 ± 0.06</td>
<td>0.44 ± 0.13</td>
<td>0.48 ± 0.06</td>
</tr>
<tr>
<td>30</td>
<td>0.52 ± 0.09</td>
<td>0.31 ± 0.05</td>
<td>0.30 ± 0.09</td>
<td>0.24 ± 0.1**</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td>U. P. - B</td>
<td>15 ± 3</td>
<td>10 ± 4</td>
<td>7 ± 4</td>
<td>12 ± 9</td>
<td>12 ± 9</td>
</tr>
<tr>
<td>30</td>
<td>14 ± 5</td>
<td>34 ± 21</td>
<td>42 ± 22</td>
<td>88 ± 47***</td>
<td>74 ± 47**</td>
</tr>
<tr>
<td>U.NOX - B</td>
<td>7.5 ± 3.7</td>
<td>7.9 ± 1.3</td>
<td>7.2 ± 2.3</td>
<td>7.6 ± 1.8</td>
<td>6.7 ± 2.9</td>
</tr>
<tr>
<td>30</td>
<td>5.8 ± 4.4</td>
<td>7.1 ± 2.2</td>
<td>6.8 ± 1.9</td>
<td>6.7 ± 2.6</td>
<td>6.4 ± 2.3</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD (no. of rats).

SHAM, sham-treated rats; SNx, untreated 5/6 nephrectomized rats; C, rats that will receive candesartan cilexetil; C + BH4, rats that will receive candesartan cilexetil and BH4; BH4, rats that will receive BH4.

Body weight (BW, g), systolic blood pressure (SBP, mmHg); creatinine clearance (CCR, ml/min/100 g-BW), 24-h urinary protein excretion (U.P., mg/mg creatinine) and urinary NO3 (U.NOx, µmol/mg creatinine) before (B) and 30 days (30) after 5/6 nephrectomy.

*P < 0.05 vs C.
**P < 0.05 vs SNx.
***P < 0.01 vs SNx and C.
$^dP < 0.05$ vs SHAM, SNx and C.

Fig. 2. Systolic blood pressure during treatment. SHAM, sham-treated rats; SNx, untreated 5/6 nephrectomized rats; C, SNx rats treated with candesartan cilexetil 5 mg/kg per os; C + BH4, SNx rats treated with candesartan cilexetil and BH4; BH4, SNx rats treated with BH4, 10 mg kg/BW intraperitoneally. *P < 0.01 vs all in the same month, **P < 0.01 vs SHAM, C and C + BH4 in the same month.

study (from 148 ± 26 to 157 ± 21 mmHg). In C and C + BH4 rats, SBP decreased significantly. Treatment with BH4 alone had a non-significant effect on SBP (Figure 2).

Renal function

(i) Creatinine clearance (CCR, ml/min/100 g, Tables 3 and 4)

The CCR decreased significantly 30 days after 5/6 SNx (from 0.47 ± 0.08 to 0.28 ± 0.07 in all rats, P < 0.01 vs before surgery). This decrease was more pronounced in the C + BH4 than in the C group. During the next 3 months of follow-up, the CCR in the untreated SNx rats continued to decrease until it reached –56 ± 33% at the end of the study. At each point studied, the CCR value was lower than...
Table 4. Effect of treatment on creatinine clearance rate (ml/min/100 g BW), before treatment (TRT) and after the 1st, 2nd and 3rd month of treatment

<table>
<thead>
<tr>
<th></th>
<th>Bef TRT (30 d after SNx)</th>
<th>1st month TRT</th>
<th>2nd month TRT</th>
<th>3rd month TRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>0.53 ± 0.08* (12)</td>
<td>0.46 ± 0.08* (12)</td>
<td>0.41 ± 0.07* (12)</td>
<td>0.41 ± 0.09* (12)</td>
</tr>
<tr>
<td>SNx</td>
<td>0.31 ± 0.05** (15)</td>
<td>0.25 ± 0.09* (11)</td>
<td>0.21 ± 0.06* (9)</td>
<td>0.14 ± 0.09* (7)</td>
</tr>
<tr>
<td>C</td>
<td>0.30 ± 0.09 (11)</td>
<td>0.28 ± 0.09 (9)</td>
<td>0.22 ± 0.13* (9)</td>
<td>0.22 ± 0.12 (7)</td>
</tr>
<tr>
<td>C + BH4</td>
<td>0.24 ± 0.09 (10)</td>
<td>0.20 ± 0.03 (9)</td>
<td>0.19 ± 0.05 (8)</td>
<td>0.13 ± 0.07* (8)</td>
</tr>
<tr>
<td>BH4</td>
<td>0.25 ± 0.04 (10)</td>
<td>0.26 ± 0.14 (10)</td>
<td>0.22 ± 0.09 (7)</td>
<td>0.19 ± 0.07* (5)</td>
</tr>
</tbody>
</table>

Mean ± SD (number of animals).
SHAM, sham-treated rats; SNx, untreated 5/6 nephrectomized rats; C, SNx rats treated with candesartan cilexetil, 5 mg/kg per os; C + BH4, SNx rats treated with candesartan cilexetil and BH4; BH4, SNx rats treated with BH4, 10 mg/kg/BW intraperitoneally.

*P < 0.05 vs all.
**P < 0.05 vs C + BH4 and BH4.

Both C and C + BH4 treatments attenuated significantly this increase in urinary protein excretion, compared with SNx rats. BH4 treatment alone had no effect on urinary protein excretion UP (Figure 3). A difference was found between C and C + BH4 groups. In the former, treatment blunted the increase in UP. In fact, after 1 month of treatment, UP values remained unchanged and thereafter there was a slow increase till the end of the study (P = 0.06 between the 1st and 2nd month and between the 2nd and 3rd month of follow-up). In contrast, in the C + BH4 group, treatment significantly reduced UP during the 1st month of treatment. Thereafter, UP remained stable with no significant change (P = 0.12 between the 1st and 2nd month and between the 2nd and 3rd month of follow-up).

(iii) Urine nitrate excretion (Tables 2, 3)

Thirty days after surgery, UNO3 was similar in SHAM and SNx animals. Thereafter, in SNx rats, UNO3 excretion decreased by 65 ± 8% at the end of the study, (P < 0.01 vs baseline). In C rats, UNO3 excretion diminished by 48 ± 15% throughout the study, (P = NS vs SNx). In contrast, in C + BH4 rats, a 22 ± 26% increase in UNO3 excretion was found (P < 0.05 vs SNx rats). In BH4 rats, a non-significant increase in UNO3 excretion was found. No correlation between UNO3 excretion, SBP, UP and CCR could be detected.

The eNOS protein expression in the isolated small arteries of the renal cortex was significantly higher in the BH4 group compared with the SNx animals.

Pathology (Tables 5–7, Figure 4)

Histological examination was carried out only in the surviving rats that were sacrificed at the end of the study. The weight of the remnant kidney in all SNx rats was markedly higher compared with sham-treated rats. This may be due to hypertrophy during the 1st month but also oedema and fibrosis 4 months later.
All treatments significantly reduced the weight of the remnant kidney, compared with SNx rats. This finding may indicate a significant effect of treatment in the prevention of renal hypertrophy and further fibrosis. Kidney weight in C + BH4 rats was significantly lower than that of C rats (Tables 6 and 7). In C + BH4 group, heart weight was significantly reduced, whilst in C and BH4 rats no change was noted (Tables 6 and 7).

At histological examination, the kidneys showed marked GS and ME in SNx rats (Tables 6 and 7). In the C + BH4 rats, the degree of GS was markedly reduced, compared with all the other SNx rats, being zero in five of eight surviving rats, minimal in two others and moderate in one rat. Treatment with candesartan alone also induces significant improvement in the GS and ME score (Tables 6 and 7). The tubulointerstitial and vascular changes were unaffected by the different treatments (data not shown). A direct correlation was found between GS and SBP ($r = 0.55$, $P = 0.01$) and UP ($r = 0.692$, $P = 0.0001$).

### Discussion

In normal wistar rats, 5/6 nephrectomy is associated with hypertension, proteinuria, ME and GS in the

### Table 5. Kidney and heart weight in surviving rats at the end of the study

<table>
<thead>
<tr>
<th></th>
<th>Kidney weight (mg)</th>
<th>Heart weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>167 ± 16</td>
<td>150 ± 26</td>
</tr>
<tr>
<td>SNx</td>
<td>402 ± 57</td>
<td>160 ± 32</td>
</tr>
<tr>
<td>C</td>
<td>321 ± 80**</td>
<td>140 ± 22</td>
</tr>
<tr>
<td>C + BH4</td>
<td>264 ± 51**</td>
<td>133 ± 11**</td>
</tr>
<tr>
<td>BH4</td>
<td>305 ± 61**</td>
<td>147 ± 18</td>
</tr>
</tbody>
</table>

Mean ± SD.

SHAM, sham-treated rats; SNx, untreated 5/6 nephrectomized rats; C, SNx rats treated with candesartan cilexetil, 5 mg/kg per os; C + BH4, SNx rats treated with candesartan cilexetil and BH4; BH4, SNx rats treated with BH4, 10 mg kg/BW intraperitoneally.

* $P < 0.001$ vs all. ** $P < 0.05$ vs SNx.

### Table 6. Semi-quantitative evaluation of the severity of glomerular sclerosis (GS) in the glomeruli of surviving rats

<table>
<thead>
<tr>
<th></th>
<th>0 (no GS)</th>
<th>1 (25% glomerular involvement)</th>
<th>2 (50% glomerular involvement)</th>
<th>3 (75% glomerular involvement)</th>
<th>4 (100% glomerular involvement)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>100 ± 0$^{ab}$</td>
<td>0 ± 0$^a$</td>
<td>0 ± 0$^{ab}$</td>
<td>0 ± 0$^a$</td>
<td>0 ± 0$^a$</td>
</tr>
<tr>
<td>SNx</td>
<td>49 ± 23$^a$</td>
<td>15 ± 6$^d$</td>
<td>10 ± 8$^d$</td>
<td>14 ± 11$^d$</td>
<td>9 ± 12$^d$</td>
</tr>
<tr>
<td>C</td>
<td>81 ± 13</td>
<td>10 ± 9$^e$</td>
<td>4 ± 4$^e$</td>
<td>3 ± 2.2$^e$</td>
<td>1.4 ± 2.4</td>
</tr>
<tr>
<td>C + BH4</td>
<td>95 ± 8</td>
<td>3 ± 7</td>
<td>0.8 ± 1.5</td>
<td>0.4 ± 1.3$^e$</td>
<td>0 ± 0$^b$</td>
</tr>
<tr>
<td>BH4</td>
<td>67 ± 14</td>
<td>13 ± 17</td>
<td>7 ± 13</td>
<td>8 ± 9</td>
<td>5 ± 7</td>
</tr>
</tbody>
</table>

Mean ± SD.

SHAM, sham-treated rats; SNx, untreated 5/6 nephrectomized rats; C, SNx rats treated with candesartan cilexetil, 5 mg/kg per os; C + BH4, SNx rats treated with candesartan cilexetil and BH4; BH4, SNx rats treated with BH4, 10 mg kg/BW intraperitoneally.

$^a$ $P < 0.01$ vs SNx, C and BH4.  
$^b$ $P = 0.07$ vs C + BH4.  
$^c$ $P = 0.01$ vs C and BH4.  
$^d$ $P < 0.05$ vs C + BH4.  
$^e$ $P = 0.07$ vs C + BH4.  
$^f$ $P = 0.01$ vs C + BH4.  
$^g$ $P = 0.03$ vs B.  
$^h$ $P = 0.08$ vs B.

### Table 7. Semi-quantitative evaluation of the severity of mesangial expansion (ME) in the glomeruli of surviving rats

<table>
<thead>
<tr>
<th></th>
<th>0 (no ME)</th>
<th>1 (25% glomerular involvement)</th>
<th>2 (50% glomerular involvement)</th>
<th>3 (75% glomerular involvement)</th>
<th>4 (100% glomerular involvement)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>100 ± 0$^a$</td>
<td>0 ± 0$^a$</td>
<td>0 ± 0$^a$</td>
<td>0 ± 0$^b$</td>
<td>0 ± 0$^b$</td>
</tr>
<tr>
<td>SNx</td>
<td>26 ± 18</td>
<td>18 ± 9$^e$</td>
<td>13 ± 8</td>
<td>15 ± 4$^d$</td>
<td>27 ± 20$^e$</td>
</tr>
<tr>
<td>C</td>
<td>49 ± 25</td>
<td>29 ± 16</td>
<td>10 ± 7</td>
<td>9 ± 6</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>C + BH4</td>
<td>25 ± 19</td>
<td>45 ± 22</td>
<td>17 ± 12</td>
<td>8 ± 7</td>
<td>6 ± 8</td>
</tr>
<tr>
<td>BH4</td>
<td>27 ± 41</td>
<td>33 ± 28</td>
<td>16 ± 16</td>
<td>11 ± 14</td>
<td>13 ± 17</td>
</tr>
</tbody>
</table>

Mean ± SD.

SHAM, sham-treated rats; SNx, untreated 5/6 nephrectomized rats; C, SNx rats treated with candesartan cilexetil, 5 mg/kg BW per os; C + BH4, SNx rats treated with candesartan cilexetil and BH4; BH4, SNx rats treated with BH4, 10 mg kg/BW intraperitoneally.

$^a$ $P < 0.001$ vs all.  
$^b$ $P = 0.02$ vs C and C + BH4.  
$^c$ $P = 0.01$ vs C + BH4.  
$^d$ $P = 0.04$ vs C and C + BH4.  
$^e$ $P < 0.01$ vs C and C + BH4.
remnant kidney, progressive aggravation of renal insufficiency and death. BP control, low protein diet and use of drugs acting on the renin-angiotensin system, like ACEI and ARB, have been shown to be beneficial in blunting the aggravation of renal insufficiency, mainly if started early after surgery [1]. Treatment in a late phase, when renal lesions are well established, may induce a delay but cannot prevent the progressive aggravation of renal disease [2].

The working hypothesis of the present study was that the association of candesartan with BH4, an essential cofactor for eNOS activation, could exert a better renoprotective effect than ARB alone.

Recent evidence in the literature supports the concept that treatment with BH4 has beneficial actions in animals with renal disease. The renal tissue levels of BH4 are decreased in diabetic rats [9,10]. It is associated with the development of focal segmental sclerosis, proteinuria and lower creatinine clearance [10]. Treatment with BH4 in 5/6 nephrectomized rats blunts the early increase in BP (after the first 10 days), associated with an increase in mesenteric artery eNOS protein expression [8]. Furthermore, treatment with BH4 during 8 weeks after SNx blunts the development of hypertension, proteinuria and glomerular ME compared with untreated SNx rats [4].

These findings suggest the possibility of a renoprotective effect of treatment with BH4. BH4 is known to bind to the oxidase domain of NO synthase (NOS), and is an essential cofactor for the synthesis of NO. The presence of suboptimal levels of BH4 causes uncoupling of NOS with generation of both NO and superoxide anions, resulting in the formation of peroxinitrite, a potent cytotoxic agent [11,12]. Peroxynitrite, thus formed, in turn releases zinc from the zinc-thiolate cluster of endothelial NOS leading to a further decrease in NO formation and an increase in superoxide anion production [12]. The result is a vicious cycle, increasing cellular damage and apoptosis. Experimental studies have shown that the administration of BH4 in vitro and/or in vivo decreases oxidative stress, improves endothelial dysfunction and...
lowers BP [13–15]. Based on this background, we have decided to evaluate the possible renoprotective effect of BH4 in the presence of established chronic renal failure. Fifty-two percent of the untreated SNx rats died after a follow-up of 4 months. This was the period chosen to compare the possible impact of the different treatments. As mortality was directly correlated with a decrease in renal function, the effect of treatment on the mortality rate could be used as an index of renal protection. Drug administration was initiated 30 days after surgery. After 3 months of treatment the survival rate in the C + BH4 group was 80%, vs 63% in C, 47% in BH4 and 50% in SNx group (P < 0.05 vs all).

C and C + BH4 treatment, but not BH4 alone, normalized BP values and attenuated the increase in urine protein excretion. CCR values were similar in all treated groups. Treatment with BH4 alone had no effect on urinary protein excretion. In our previous study, BH4, given within 24 h after surgery, diminished SBP and UP. These results suggest that the timing of administration of this drug plays a role in its efficacy. The improvement in survival found in the C + BH4 group was confirmed by the lower indexes of GS found in these animals, as compared with all the others (Tables 6 and 7). Candesartan cilexetil alone also significantly reduced the incidence of GS, but to a lesser degree. As a whole, the clinical, laboratory and pathological data strongly suggest that the addition of BH4 to candesartan could further improve the ARB renoprotective action in this model.

Our results show the addition of BH4 to candesartan results in a marked increase in NO excretion and in the eNOS protein expression. This may be due to a decrease in BP values, an improvement in NO synthesis and in the redox state [3].

The urinary excretion of NO3 decreased in untreated SNx rats, as previously reported [16]. In the C + BH4 treated rats, UNO3 excretion as well as the eNOS protein expression were significantly augmented, but no correlation was found between UNO3 excretion, CCR and UP. As the number of animals studied was relatively small, a lack of correlation does not exclude that the renoprotective effect of BH4 was related to an increased renal NO synthesis.

The lower renal and cardiac weight found in the C + BH4 group may correspond to a lesser degree of hypertrophy. This could be due to better BP control, but also reflects a more specific metabolic and antioxidant effect of the combined treatment.

The study has certain limitations that must be noted:

(i) The survival rats at the end of the study were the best rats of each group. Therefore, contrasting with mortality that is unquestionable data, the evaluation of the survival rats may be biased.

(ii) There is a variable response to the surgical technique between SNx groups with some significant differences in creatinine clearance and proteinuria that could affect the results. A month after surgery, the group with the worst clinical data was the C + BH4. However, this group had the best results regarding survival as well as pathological data. This finding strengthens the positive effect of combined treatment compared with candesartan alone.

(iii) Evaluation of proteinuria instead of albuminuria has certain limitations. In male rats, a partial amount of proteinuria may be of sexual organ origin.

(iv) The scoring systems used in this article are valuable, yet subjective. Use of morphological data, like glomerular volume and other morphometric parameters (which are possible only after collection of tissue with the use of pressure-controlled retrograde aortal perfusion) are more objective and increase the quality of data.

In summary, the addition of BH4 to candesartan cilexetil in the 5/6 nephrectomy model of chronic renal failure has an additive renoprotective effect. The mechanism of action may include a better control of BP, an efficient blockade of the deleterious actions of Ag II, an appropriate NO synthesis and a balanced redox status. The main decline in renal function in candesartan cilexetil rats occurred during the 2nd month of treatment, whilst in the combined treatment it was noted during the last month of follow-up, probably explaining the better survival rate. This suggests that the combined treatment, while being able to delay the deterioration of the renal function, could not blunt it completely. It will be relevant to evaluate whether higher doses of candesartan and/or BH4 may further improve this renoprotective effect. The finding of two different malignancies in the urinary tract in rats of the C + BH4 group will need particular attention in further studies.

Conflict of interest statement. This study was supported by a grant from Astra-Zeneca.

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*Received for publication: 15.4.06*  
*Accepted in revised form: 14.2.07*