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Effect of chronic tetrahydrobiopterin supplementation on blood pressure and proteinuria in 5/6 nephrectomized rats

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Abstract

Background. Tetrahydrobiopterin (BH4) is a key cofactor of nitric oxide (NO) synthase. Reduced BH4 levels may mediate endothelial NO synthase uncoupling, resulting in reduced NO synthesis and enhanced oxidative stress. In rats after 5/6 nephrectomy (Nx), administration of BH4 prevents the onset of hypertension, typically observed 10 days after Nx. This effect is associated with an increased synthesis of NO. The aim of the present study was to evaluate the effect of chronic BH4 therapy on blood pressure and renal morphology.

Methods. During an 8 week period, five groups of rats were studied: untreated 5/6 Nx rats, BH4-treated Nx rats (BH4, 10 mg/kg body weight/day administered intraperitoneally), L-arginine treated Nx rats (LA, 130 mg/kg/day), diltiazem-treated Nx rats (DILT, 30 mg/kg/day) and sham-operated rats. Treatments were commenced 24 h after surgery. Systolic blood pressure values (SBP), 24 h proteinuria (UP) and creatinine clearance rate (CCR) were assessed before and at weeks 4 and 8 of the study period. Histological changes in the kidney were evaluated at the end of the study (week 8).

Results. Compared with baseline, in Nx rats both SBP and UP increased significantly (112 ± 1 to 136 ± 1.4 mmHg, $P < 0.01$ and 23 ± 2 to 127 ± 26 mg/day, $P < 0.01$, respectively). Treatment with BH4 normalized SBP values as did treatment with LA and DILT (109 ± 3, 115 ± 2 and 114 ± 2 mmHg, respectively). UP was markedly reduced by BH4, the reduction being similar to that obtained by LA and significantly more marked than that of DILT rats (20 ± 2, 28 ± 3 and 62 ± 14 mg/day, respectively). CCR was equally decreased in all Nx groups. Histological evaluation showed the development of mesangial expansion in Nx rats, an effect that was significantly blunted by all treatments.

Conclusions. In rats after 5/6 nephrectomy, BH4 supplementation initiated 24 h after surgery and maintained for 8 weeks preserved SBP, reduced UP and prevented the development of glomerular mesangial expansion.

Keywords: blood pressure; nitric oxide; proteinuria; rats; renal failure; tetrahydrobiopterin

Introduction

In the experimental model of 5/6 nephrectomy in rats, the development of hypertension and renal failure is associated with a relative lack of nitric oxide (NO) bioavailability. Treatment with L-arginine (a NO precursor) normalizes blood pressure and improves renal function [1]. Tetrahydrobiopterin (BH4) is a ubiquitous essential cofactor for various enzymatic processes present in every cell [2]. In endothelial cells, BH4 is a key cofactor of NO synthase (NOS) [2]. Reduced BH4 levels result in endothelial NOS (eNOS) uncoupling and, thus, may be an important contributor to decreased NO production [2]. In clinical conditions characterized by endothelial dysfunction, such as smoking or hypercholesterolaemia [3,4], the acute administration of BH4 ameliorates endothelial dysfunction. In spontaneously hypertensive rats and in those with insulin resistance [5,6], administration of BH4 normalizes blood pressure. We have demonstrated recently that BH4 supplementation prevents the onset of hypertension seen after 10 days in 5/6 nephrectomized rats. This effect was associated with an increased expression of eNOS protein in small arteries [7].

The aim of the present study was to (i) evaluate whether this antihypertensive action can be maintained

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on long-term treatment and (ii) examine the effect of BH4 therapy on renal morphology and proteinuria.

Subjects and methods

Protocol

Forty male Wistar rats, mean weight 384 ± 24 g, were fed a normal chow diet and allowed distilled water *ad libitum*. The 5/6 nephrectomy was performed according to standard protocol. In brief, the animals were anaesthetized with pentobarbital [35 mg/kg body weight (BW) intraperitoneally] and then underwent a right nephrectomy and ligature of two of the major branches of the left main renal artery (Nx) at day 0. All the Nx were carried out by one operator (G.H.). The animals were then randomly assigned to one of five groups, as follows.

Group 1. Nx ($n = 11$); untreated 5/6 Nx rats.
Group 2. BH4 ($n = 8$); Nx rats administered BH4 (Alexis Biochemical, Lausen), 10 mg/kg BW/day intraperitoneally [5].
Group 3. LA ($n = 7$); Nx rats to which L-arginine (Sigma) was added daily to the drinking water at a dose of 1 g/l, delivering ~130 mg/kg BW/day [1].
Group 4. DILT ($n = 7$); rats to which diltiazem was given daily at a dose of 30 mg/kg BW/day within the drinking water [8].
Group 5. SHAM ($n = 7$); sham-operated rats.

Drug administration was begun 24 h after surgery. Systolic blood pressure (SBP) was measured in trained unstressed rats 1 day before surgery and on weeks 4 and 8, by tail cuff manometry using an automated sphygmomanometer (Narco Bio Systems, Austin, TX, USA). The Animal Care and Use Committee of Meir Hospital approved all procedures.

Clinical and laboratory evaluations

Serum albumin and cholesterol (as nutritional indices) were assayed at the end of the study. Serum creatinine was determined 1 day before and at weeks 4 and 8 of the study. Twenty-four hour urine collections were obtained during two consecutive days before surgery and 4 and 8 weeks after surgery for the measurement of creatinine clearance and proteinuria. Rats were kept in metabolic cages 3 days before each urine collection and were fed a diet containing 0.35 g NaCl, 20 g protein and 1.17 g arginine per 100 g. Gentamicin (6 mg/tube) was added to the urine collection tubes to avoid bacterial growth. Serum creatinine, albumin, cholesterol and urinary proteinuria were assayed by standard methods.

Histological examinations

Kidneys were fixed in neutral buffered formalin and embedded in paraffin for light microscopic study. Histological sections were stained with haemotoxylin–eosin, periodic acid–Schiff (PAS) and Masson. The histological sections were coded so that the pathologist was unaware of the source of each sample. A minimum of 40 glomeruli from each kidney was examined. A semi-quantitative score was used to assess the severity of glomerular lesions [8].

Tubulointerstitial and vascular damage were assessed on PAS-stained paraffin sections at a magnification of ×100. Score evaluation was in accordance with Adamczak *et al.* [9].

Statistical analysis

Results are expressed as means ± SEM. A one-way analysis of variance with Bonferroni correction was performed in the statistical analysis of data. For paired and unpaired groups, *t*-tests were used as appropriate. A *P*-value of <0.05 was considered significant. As the semi-quantitative pathology data is not parametrically distributed, it is presented as the median and range and was evaluated using non-parametric tests (Kruskal–Wallis).

Results

Clinical evaluation

In group 1, three animals were excluded from the final analysis (27%). Two died during the last month of the study and one developed severe renal failure. All these rats developed a marked weight loss. One rat from group 2 (severe renal failure at week 4, death a week later) and one rat from group 3 (died 3 weeks after surgery) were also excluded. BW at the end of the study as well as the percentage increase in BW throughout the study were similar in all groups (Table 1). Daily water and food intake were equivalent between groups (data not shown). Compared with baseline, SBP increased significantly in Nx rats, from 112 ± 1 to

Table 1. Clinical and laboratory data at the end of the study

	Nx ($n = 8$)	BH4 ($n = 7$)	LA ($n = 6$)	DILT ($n = 7$)	SHAM ($n = 7$)
BW (g)	420 ± 7	430 ± 6	424 ± 15	427 ± 6	411 ± 11
BW increase (%)	15 ± 1.7	9.4 ± 2	9.5 ± 5	8.7 ± 3	10 ± 5
Urine output (ml/day)	21 ± 1.4	20 ± 1	22 ± 1.6	18 ± 0.6	18 ± 1.7
Serum creatinine (mg/dl)	0.95 ± 0.05	0.95 ± 0.03	0.94 ± 0.05	0.98 ± 0.04	0.65 ± 0.02[a]
Blood urea (mg/dl)	80 ± 11	83 ± 4	69 ± 6	78 ± 4	44 ± 3.5[a]
Serum Na (mEq/l)	142 ± 0.4	141 ± 0.4	141 ± 0.6	141 ± 1	140 ± 0.3
Serum cholesterol (mg/dl)	124 ± 18	60 ± 2.5[b]	71 ± 7[b]	83 ± 12[b]	52 ± 5[b]
Serum albumin (g/dl)	2.9 ± 0.05	3.5 ± 0.1[b]	3.3 ± 0.13	3.1 ± 0.07	3.8 ± 0.05[b]

Data are means ± SEM.
[a]$P < 0.05$ *vs* all groups; [b]$P < 0.01$ *vs* Nx.

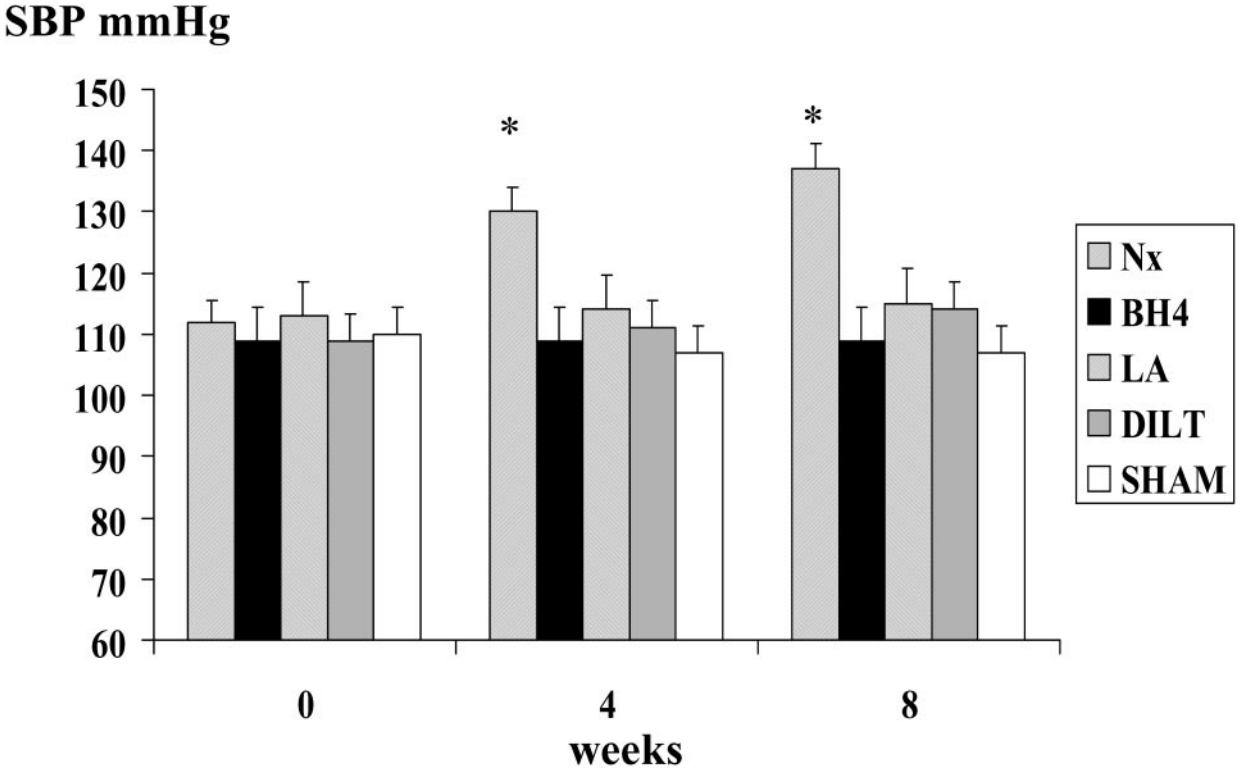

Fig. 1. Systolic blood pressure (mmHg) throughout the study. $*P < 0.05$ *vs* the other groups.

Table 2. Creatinine clearance (ml/min/100 g) throughout the study

	Nx	BH4	LA	DILT	SHAM
Before 5/6 Nx	0.51 ± 0.06	0.48 ± 0.03	0.47 ± 0.05	0.49 ± 0.05	0.52 ± 0.02
Week 4	0.27 ± 0.01	0.25 ± 0.01	0.26 ± 0.02	0.28 ± 0.02	0.46 ± 0.01[a]
Week 8	0.26 ± 0.01	0.28 ± 0.02	0.24 ± 0.01	0.26 ± 0.01	0.49 ± 0.03[a]

[a] $P < 0.001$ *vs* all groups.

136 ± 1.4 mmHg ($P < 0.01$). In all treated groups (BH4, LA and DILT), SBP values remained stable and were similar to those found in SHAM rats (Figure 1).

Laboratory data

Serum creatinine levels were increased and 24 h creatinine clearances markedly reduced following 5/6 nephrectomy (Table 2). The decrease in creatinine clearance was of equal magnitude in all nephrectomized groups. Treatment with BH4 and LA induced a significant decrease in 24 h urine protein excretion (Figure 2). In DILT rats the 24 h urine protein excretion also decreased significantly compared with Nx rats, but remained significantly elevated in comparison with BH4 and LA rats.

Histological examination

No hyalinized glomeruli were found in any group. In group 1, prominent mesangial expansion and chronic mild tubulointerstitial changes were universal findings (Table 3). Only the excluded rat that developed severe renal failure had prominent signs of glomerulosclerosis and hyalinosis. All treatments improved the mesangial histological score, which approached that of SHAM rats. Tubulointerstitial changes were ameliorated by treatment with diltiazem and BH4, but not with L-arginine. Vascular changes were minor and non-significant.

Discussion

A decrease in endothelial NO bioavailability is commonly found after subtotal nephrectomy and has been considered to play a pathogenic role in the development of hypertension and renal failure in this animal model [1,10–14]. Decreased activity of eNOS may be due to a reduction of substrate (L-arginine), an impaired function of the enzyme, a down-regulation of eNOS activity or an accumulation of eNOS inhibitors. Depleted stores of L-arginine lead to both a decrease in NO synthesis and an overproduction of free oxygen

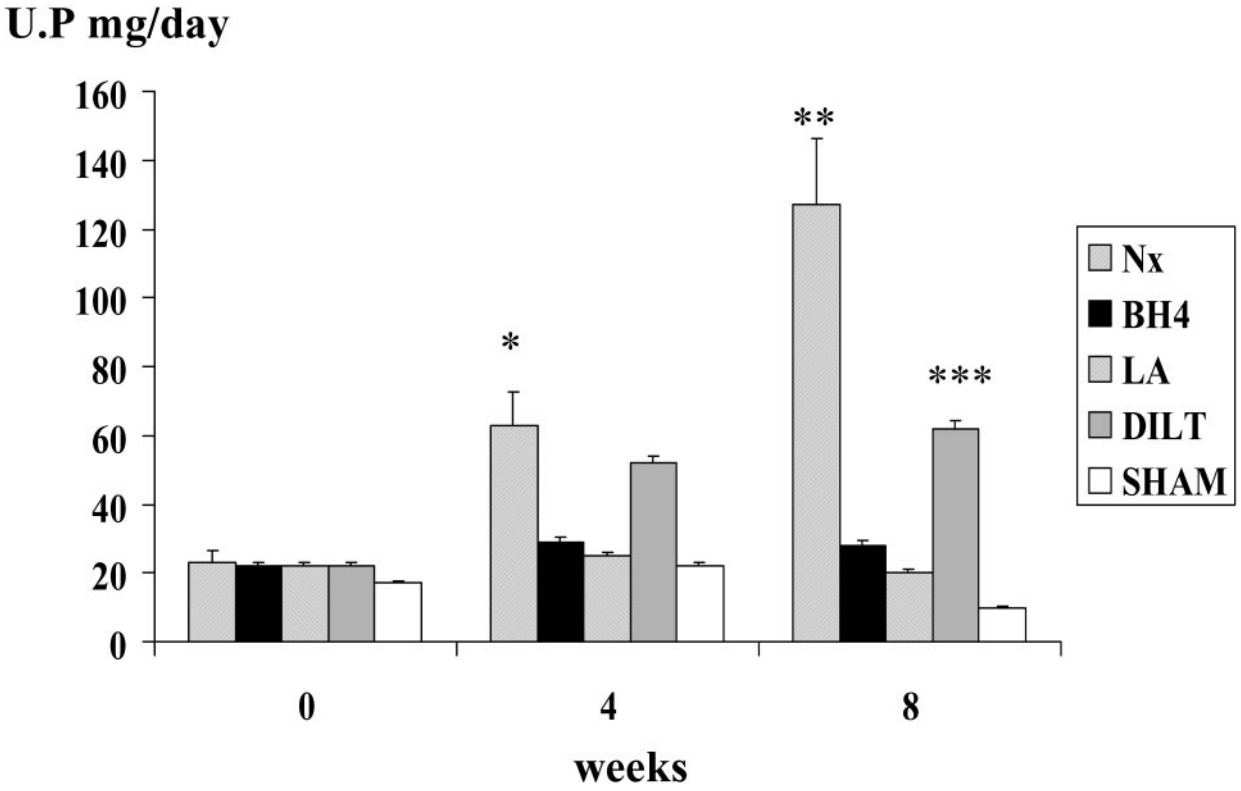

Fig. 2. 24 h urine protein excretion (mg/day) throughout the study. $*P<0.05$ *vs* all other groups; $**P<0.01$ *vs* BH4, LA and SHAM and $P<0.05$ *vs* DILT; $***P<0.05$ *vs* BH4, LA and SHAM.

Table 3. Semi-quantitative score of pathological changes expressed as the median and range (in parentheses)

	Nx	BH4	LA	DILT	SHAM
Mesangial expansion	25 (5-132)[a]	0 (0-2.5)	0 (0-32.5)	0 (0-0)	0 (0-5)
Tubulointerstitium	2 (0-3)[b]	0 (0-0)	1 (0-2)	0 (0-0)	0 (0-0)
Vascular changes	1 (0-1)	0 (0-1)	0 (0-1)	0 (0-1)	0 (0-0)

[a]$P<0.001$ *vs* all groups; [b]$P<0.05$ *vs* BH4, DILT and SHAM.

radicals [2]. The beneficial effect of treatment with L-arginine on blood pressure and renal function in rats after 5/6 nephrectomy has been documented [1].

Apart from substrate availability, NO formation is dependent on eNOS function. In this context, BH4 is of prime importance. BH4 is an essential cofactor for eNOS activity. In its absence, eNOS will produce mainly superoxide and hydrogen peroxide, a process referred to as uncoupled catalysis [2].

We have recently reported that BH4 supplementation in 5/6 nephrectomized rats blunts the early increase in blood pressure (after the first 10 days), associated with an increase in eNOS protein expression [7]. In the present study we have extended our observations administering BH4 for 8 weeks and comparing its effects with those of L-arginine and conventional antihypertensive treatment. The non-dihydropyridine calcium channel blocker (CCB) diltiazem was chosen because, in contrast to dihydropyridine CCB, there are no data to suggest that it affects NO production *in vivo* [15– 17].

After 8 weeks of treatment, BH4 reduced SBP to an equivalent degree to that obtained by L-arginine and diltiazem. This antihypertensive effect of BH4 has been described previously in hypertensive models [5,6] with normal renal function. In a recent publication, we reported the antihypertensive effect of BH4 occurring in the first 10 days after subtotal nephrectomy. The blood pressure-lowering effect of BH4 was accompanied by decreased proteinuria and a lower degree of mesangial proliferation. The decrease in urine protein excretion was equal to that seen with L-arginine and better than that found with diltiazem in the presence of a similar reduction of blood pressure values. This anti-proteinuric action of BH4 has not been described to date. Histologically, mesangial expansion, a forerunner of glomerular sclerosis, was found to be significantly diminished in BH4 rats compared with the untreated group. The scores did not differ between the treated groups. In this particular strain of rats, a more prolonged period of time may be necessary to either develop major pathological changes after 5/6 nephrectomy and/or to show the possible renoprotective effect of different drug treatments. Tubulointerstitial changes (another marker of severity of renal disease), although mild, were also improved by BH4 treatment. The creatinine clearance rate was decreased in all nephrectomized rats throughout the study period with no difference between groups.

The results observed with the addition of BH4 raise the question whether BH4 bioactivity is reduced in rats with chronic renal failure. Although this point is beyond the scope of the present work, the conjecture is quite plausible. Chronic renal failure is associated with depressed superoxide dismutase (SOD) production and elevated NAD(P)H oxidase expression, factors conducive to oxidative stress by increasing superoxide. This is supported by a favourable response to the administration of tempol, a SOD-mimetic drug, and by the increased tissue expression of nitrotyrosine (a footprint of NO interaction with superoxide) [18,19]. Increased NADPH oxidase expression leads to oxidation of BH4 and uncoupling of eNOS. NO production decreases whereas the formation of reactive oxygen species (ROS) is augmented. Treatment with oral BH4 reduces vascular ROS production, increases NO production and blunts the increase in blood pressure [20].

In summary, we have shown that in rats with chronic renal failure due to 5/6 nephrectomy, an 8 week treatment with BH4 initiated 24 h after surgery maintained blood pressure below normal values, reduced proteinuria and prevented the development of glomerular mesangial expansion. Treatment strategies aimed at increasing BH4 or preventing its oxidation may prove useful in the treatment of hypertensive chronic kidney disease, at least when initiated in its earlier stages.

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Conflict of interest statement. None declared.

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