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ARGININE AND TETRAHYDROBIOPTERIN SUPPLEMENTATION IN RATS WITH SALT-INDUCED BLOOD PRESSURE INCREASE: MINOR HYPOTENSIVE EFFECT BUT IMPROVEMENT OF RENAL HAEMODYNAMICS

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High salt (HS) intake can lead to hypertension, probably the result of the predominance of vasoconstrictor reactive oxygen species over vasodilator nitric oxide (NO). We aimed to examine if the supposed NO deficiency and the resultant blood pressure increase could be corrected by supplementation of L-arginine, the substrate, and tetrahydrobiopterin (BH_4), a co-factor of NO synthases. Wistar rats without known genetic background of salt sensitivity were exposed to HS diet (4%Na) for 10 or 26 days, without or with supplementation with oral L-arginine, 1.4 mg/kg b.w. daily, alone or together with intraperitoneal BH_4, 10 mg/kg daily. Systolic blood pressure (SBP, tail-cuff method) was measured repeatedly and found to increase ~40 mmHg after 26 days; L-arginine and BH4 did not significantly attenuate this increase. At the end of chronic studies, in anaesthetized rats the diet- and treatment-induced changes in renal haemodynamics were assessed. HS diet selectively decreased (–30%, $P < 0.03$) the inner medullary blood flow (IMBF, laser-Doppler flux) without changing total or cortical renal perfusion. Arginine supplementation tended to raise all renal circulatory parameters, and distinctly increased IMBF, to 61% above the HS diet level ($P < 0.05$). In conclusion, unlike in confirmed genetically determined salt-dependent hypertension, L-arginine and BH_4 supplementation failed to attenuate the SBP increase observed after exposure to HS diet. On the other hand, arginine increased total and regional renal perfusion, especially IMBF. This suggests that the delivery of arginine increased intrarenal NO synthesis, an action of renoprotective potential which presumably countered the harmful influence of the local tissue oxidative stress.

Key words: *nitric oxide, renal circulation, oxidative stress, renal microvessel reactivity, high salt diet, L-arginine, tetrahydrobiopterin*

INTRODUCTION

High dietary salt intake can lead to hypertension in some individuals; while the obvious reason is sodium retention, the exact mechanisms of blood pressure elevation have been debated over past decades and repeatedly reviewed (1, 2). As in any form of hypertension, the background may involve increased tone of the wall of peripheral vessel due to vascular endothelial dysfunction and imbalance between the vasoconstrictor action of reactive oxygen species (ROS) and vasodilator influence of nitric oxide (NO). Since excessive salt intake is known to induce oxidative stress (3, 4) the above mechanism might be of particular importance in salt-induced hypertension. One reason for the harmful domination of ROS over NO actions could be deficient NO synthesis.

L-arginine is the substrate for NO synthesis and its rate was shown to depend on extracellular arginine concentration (5-7) and also on the availability of co-factors of the process, such as tetrahydrobiopterin (BH_4) (8). Its deficit may result in "uncoupling" of NO synthase subunits, which results in generation of superoxide instead of NO. Moreover, in oxidative stress BH_4 is degraded to dihydrobiopterin (BH_2) and biopterin, the molecules devoid of the cofactor activity (9, 10). In agreement with all this knowledge, supplementation of L-arginine and BH_4 was indeed reported to reduce oxidative stress and at least attenuate blood pressure elevation in genetically determined rat hypertension: in Dahl salt-sensitive rats (11, 12), and in spontaneously hypertensive rats (SHR) (13) and also in 5/6 nephrectomized rats (14, 15).

We reasoned that the deficiency of L-arginine and/or BH_4 could be a more important pro-hypertensive factor in the early phase of salt-dependent progressing blood pressure increase than in models of established genetically determined hypertension. Therefore, in this study we first confirmed our earlier observation that in our colony of normotensive Wistar rats without confirmed genetic background of salt sensitivity, exposure to high salt intake results in an appreciable increase in arterial blood pressure (16). Second, we aimed to find out if the expected development of mild hypertension, would be attenuated by supplementation with L-arginine, alone or together with BH_4, which would point to the deficiency of these agents as the pathogenetic background of the

salt-dependent pressure increase. Since the vascular ROS/NO imbalance in salt-induced hypertension might be more conspicuous in the kidney than in systemic extrarenal vasculature, we examined the influence of the supplementation on renal total and regional (especially medullary) perfusion, as well as on the reactivity of intrarenal vessels to the vasoactive agents: acetylcholine (ACh) and noradrenalin (NA).

MATERIALS AND METHODS

Animals

Every effort was taken to minimize the number of animals used. The study was designed and is reported in compliance with the ARRIVE by NC3R guidelines. The experimental procedures were approved by the First Extramural Ethical Committee for Animal Care, Warsaw, Poland. Male Wistar rats (body weight 290 – 320 g) had free access to food and tap water. They were maintained on standard diet (STD, 0.25% Na w/w) or placed on high-sodium diet (HS, 4% Na, SSNIFF GmbH, Soest, Germany) for 10 or 26 days. Over these periods, at midday, blood pressure (BP, tail cuff method, CODA, Kent Scientific, USA) was measured and blood samples for plasma osmolality (P_{osm}) and plasma sodium concentration (P_{Na}) were taken (without anaesthesia): this was done on the days: 0, 2, 5, 9 and 10 in the 10-day diet exposure and on the days: 0, 2, 5, 9, 12, 16, 19, 23 and 26 in the 26-day exposure groups. During 7 – 10 days before the start of experimental measurements, the rats were accustomed to the necessary restrainment.

Chronic experiments (Fig. 1)

The rats fed HS diet for 10 or 26 days (HS10, HS26) were either untreated (control groups) or, beginning from day 3 of exposure to the diet, received L-arginine (L-arg, Sigma-Aldrich, St. Louis, MO, USA), delivered in drinking water (3 g/L). Taking account of the amount of water drunk daily, the dose of L-arg was 1.1 to 1.7 mg/kg/24 h *i.e.* comparable to that employed by others (17).

Another group of HS10 rats received, in addition to L-arg, tetrahydrobiopterin, an NO synthesis cofactor (BH_4, Enzo, Life Science, New York, USA), at 10 mg/kg, given daily as an intraperitoneal injection. The effectiveness of this dosage was tested by others (13-15). Because there was no effect of BH_4 addition to L-arg in HS10 group, such combined treatment was not applied in HS26 group, to obey the principle (3R) of possibly reducing the number of animals used. Ultimately, the following rat groups were studied:

1) Untreated, standard diet (STD, n = 5);
2) Untreated, HS diet: 10 days (HS10, n = 7);
3) Untreated, HS diet: 26 days (HS26, n = 8);
4) L-arg HS10 rats (HS10 + L-arg, n = 10);
5) L-arg + BH4;: HS10 rats (HS10 + L-arg + BH4, n = 10);
6) L-arg HS26 rats (HS26 + L-arg, n = 10).

Acute experiments

After completing the chronic part of the study, an acute experiment was performed under anaesthesia to measure renal haemodynamics and excretion, and to examine the responses of renal total and regional perfusion parameters to short renal artery infusion of acetylcholine and noradrenalin. All the measurements were performed between 10:00 and 13:30.

Surgical preparations. The rats were anaesthetized with intraperitoneal sodium thiopental (Sandoz GmbH, Kundl, Austria), 100 mg/kg, which provided stable anaesthesia for at least 4 hours. In HS rats the initial dose of thiopental was reduced by one-third, because of increased sensitivity of these animals to the drug. The rats were placed on a heated surgery table to maintain rectal temperature at about 37ºC. A polyethylene tube was placed in the trachea to ensure free airways.

The jugular vein was cannulated for fluid infusions, the femoral artery for blood sampling and the carotid artery for blood pressure (MABP) measurement (Stoelting blood pressure meter and transducers, Wood Dale, Illinois, USA). During surgery, 3% bovine serum albumin solution was infused intravenously at 3 ml/h to preserve plasma volume. The left kidney was exposed from a subcostal flank incision and placed in a plastic holder. The ureter was cannulated for timed urine collection. For measurement of total renal blood flow (RBF) a noncannulating ultrasound probe, 1 mm in diameter, connected with a transonic flowmeter (type T106; Transonic System, Ithaca, NY, USA) was placed on the renal artery. Blood perfusion rates of the renal cortex and medulla were measured separately as laser-Doppler fluxes, using the laser-Doppler Periflux 4001 system (Perimed AB, Jarfalla, Sweden). The cortical blood flow (CBF) was measured using a PF 408 probe placed on the kidney surface. The outer (OMBF) and

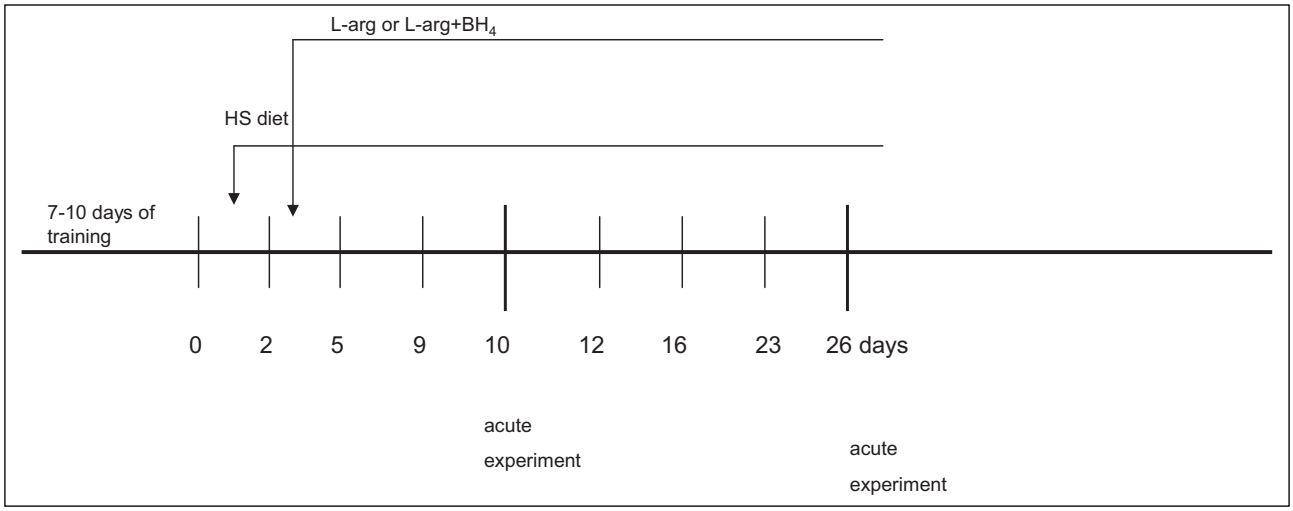

Fig. 1. The protocols of chronic studies showing starting points of high sodium (HS) diet and L-arginine (L-arg) and/or tetrahydrobiopterin (BH4) treatment (arrows). On the days denoted by numbers blood pressure was measured, urine was collected and blood was sampled.

inner medullary blood flow (IMBF) was measured, using a needle probe (PF 411) inserted into the kidney to the depth of about 3 or 5 mm, respectively. Since in rats fed high-salt diet the kidney was larger than in their standard diet counterparts, the depth of probe insertion was increased by 0.5 – 1.0 mm.

For intrarenal infusion of acetylcholine (ACh, Sigma Chemical, Co., St Louis, MO, USA) or noradrenalin (NA, Sigma-Aldrich, Basel, Switzerland) an L-shaped needle (0.45 mm diameter) was inserted *via* the aorta into the left renal artery. The changes in RBF, CBF, OMBF and IMBF in response to ACh were used as a measure of intrarenal microvascular reactivity in individual kidney zones. Since an associated MABP increase could *per se* influence blood flow responses and preclude interpretation of the effect, analyzed were only the data from the experiments in which the change in MABP was below 10% (in fact, it never exceeded 8%).

Protocols. At the end of surgical preparations and overall functional stabilization, a 20-min period was allowed for baseline haemodynamic measurements and control urine collection, subsequently, ACh (5 μg/kg/h;) or NA (10 μg/kg/h) was infused during 10 minutes at the volume infusion rate of 16 μl/min.

Morphologic studies and analytical procedures

In separate parallel subsets of anaesthetized rats, the kidneys were removed and fixed in 4% paraformaldehyde solution (PFA; BGD Eurochem, Tarnow, Poland), and formalin-fixed paraffin-embedded slices were stained with hematoxylin-eosin (HE) for

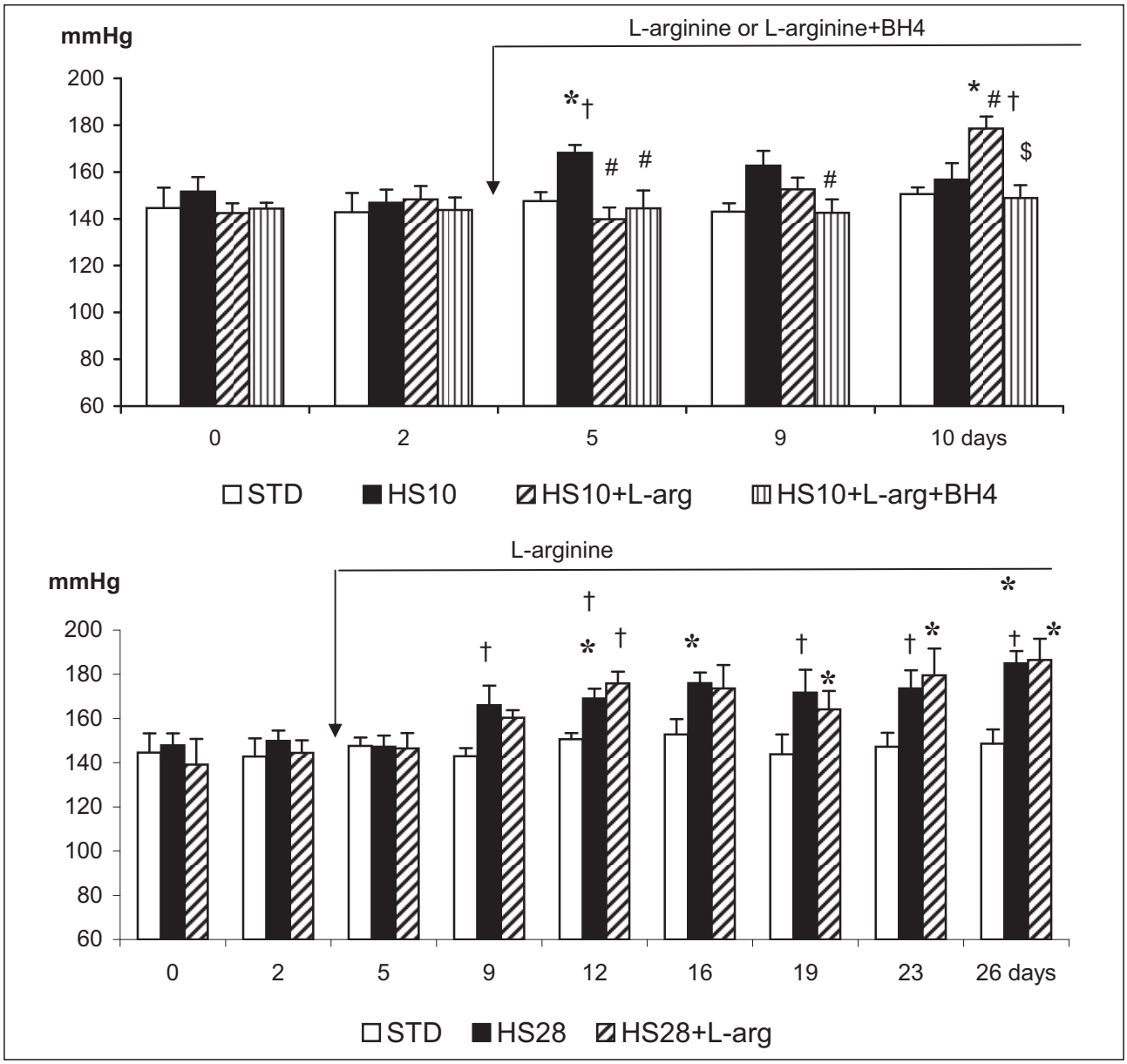

Fig. 2. Systolic blood pressure (SBP) in conscious Wistar male rats maintained on standard diet or on high sodium diet for 10 days (HS10, upper panel) or 26 days (HS26, lower panel). The rats were, untreated or received L-arginine, alone (L-arg) or together with tetrahydrobiopterine (BH4, HS10 rats only). Means ± SEM. N values: STD - 5; HS10 - 7; HS26 - 8; HS10 + L-arg - 10; HS26 + Larg - 6; HS + L-arg + BH4 - 10.
* - significantly different from day 0 (control); † - significantly different from STD diet; # - significantly different from HS 10 diet; $ - significantly different from L-arginine alone (by ANOVA and *post hoc* Student's or Duncan test).

general morphologic studies or the studies using Van Gieson (VG) staining to estimate the level of fibrosis.

Plasma and urine osmolality were measured by freezing-point depression using a cryoscopic osmometer (Osmomat 030, Gonotec GmbH, Berlin, Germany) and sodium concentration with the flame photometer (Jenway PFP7, Essex, UK).

Statistical analysis

Data are expressed as means ± SEM. Significance of changes within one group over time was first evaluated by repeated measures analysis of variance (ANOVA; STATISTICA, version 10, StatSoft Inc.), followed by Student t-test for dependent variables. Differences between groups were first analyzed by the classical one-way ANOVA followed by Student t-test for independent variables or by Duncan test, as appropriate. Bonferroni correction was applied for multiple comparisons; $P \leq 0.05$ was taken to indicate significance of differences.

RESULTS

Effects of chronic exposure to HS diet and chronic treatment with L-arg or L-arg + BH_4 in conscious rats on systolic blood pressure (SBP) are presented in *Fig. 2*. In the rats on standard diet (STD), SBP remained stable throughout the study.

In untreated short-exposure rats (HS10), SBP increased transiently; the change to 168 ± 3 mmHg on day 5 of observation was significant ($P < 0.05$). Under L-arg treatment such elevation did not occur whereas an aberrant SBP increase was seen on day 10. Notably, no such arginine dependent early increase could be reproduced in the prolonged-exposure (HS26) group (see below). Addition of BH_4, did not generally alter the SPB level obtained with L-arginine given alone, however, it seemed to abolish the puzzling increase in SBP observed in the HS + L-arg group on day 10 (*Fig. 2*).

In untreated rats kept on HS diet for 26 days (HS26), SBP was seen to increase progressively, which was very clear on days 9 and 12 of observation, and on the day 26 it reached the level of 185 ± 6 mmHg ($P < 0.006$ versus control). Supplementation of L-arg did not consistently alter the SBP levels or dynamics, however, the increase did not become significant until day 19 of observation *i.e.* with one week delay compared to untreated rats. Apart from occasional changes in plasma osmolality and sodium concentration on single days, there were no consistent alterations related to the 10-day or 26-day exposure of rats to HS diet or to the treatment regimes (*Table 1*).

Effects of HS diet and chronic treatments (HS + L-arg or HS + L-arg + BH_4) on mean arterial blood pressure, renal haemodynamics and renal excretion in anaesthetized rats are shown in *Table 2*. It presents the data from control periods of acute experiments performed at the end of chronic studies. MABP was the lowest on standard diet, but no between-group differences were significant. Short and long term exposure to high salt intake (HS10, HS26) did not significantly change RBF when compared to STD rats. In HS10 rats L-arg treatment tended to increase RBF (+52%, NS), and with L-arg + BH_4 treatment RBF was significantly higher (+67%, $P < 0.007$) than in untreated animals. Interestingly, IMBF (but not RBF, CBF or OMBF) was significantly lower in untreated HS10 compared to

Table 1. Plasma sodium concentration (PNa) and osmolality (Posm) in all experimental groups. There were no significant inter-group or time-dependent differences in either value.
STD, untreated rats on standard diet; HS10 or HS26, untreated rats maintained on high-sodium diet for 10 or 26 days; HS10 + L-arg and HS26 + L-arg, the rats of the two diet protocols treated with L-arginine (L-arg); HS10 + L-arg + BH_4, HS10 rats and treated with L-arginine (L-arg) and tetrahydrobiopterin (BH_4).

PNa (mmol/l)									
	0	**2**	**5**	**9**	**10 days**				
HS10, n = 6	146 ± 3	151 ± 4	146 ± 4	147 ± 3	146 ± 4				
HS10+L-arg, n = 10	147 ± 3	147 ± 3	144 ± 3	146 ± 3	150 ± 3				
HS10+L-arg+BH4, n = 10	143 ± 1	144±1	142 ± 1	143 ± 1	145 ± 1				
	0	**2**	**5**	**9**	**12**	**16**	**19**	**23**	**26 days**
HS26, n = 8	142 ± 3	147±3	141 ± 2	–	143 ± 4	142 ± 3	141 ± 4	145 ± 4	149 ± 4
HS26+L-arg, n = 6	136 ± 5	137±4	136 ± 5	138 ± 4	137 ± 6	–	142 ± 2	144 ± 2	141 ± 3
STD, n = 5	148 ± 3	146±2	144 ± 3	147 ± 3	145 ± 4	147±3	147 ± 3	144 ± 2	145 ± 2
Posm (mosmol/l)									
	0	**2**	**5**	**9**	**10 days**				
HS10, n = 6	298 ± 5	307±5	300 ± 10	303 ± 5	304 ± 4				
HS10+L-arg, n = 10	295 ± 2	293±2	298 ± 5	280 ± 4	292 ± 3				
HS10+L-arg+BH4, n = 10	300 ± 11	309±2	308 ± 5	308 ± 4	302 ± 2				
	0	**2**	**5**	**9**	**12**	**16**	**19**	**23**	**26 days**
HS26, n = 8	300 ± 5	315 ± 3	303 ± 6	300 ± 4	293 ± 3	297 ± 2	307 ± 4	303 ± 3	304 ± 3
HS26+L-arg, n = 6	290 ± 3	307±3	309 ± 1	309 ± 3	298 ± 5	301 ± 2	301 ± 3	293 ± 2	299 ± 3
STD, n = 5	295 ± 3	299 ± 4	290 ± 5	317±6	309±1	304 ± 3	301 ± 4	292 ± 2	294 ± 4

STD rats ($P < 0.03$). Addition of L-arg abolished this difference whereas under combined treatment (L-arg + BH_4) this effect was reversed and IMBF was even lower than in untreated HS10 rats. Briefly, the data show that IMBF was the only renal haemodynamic parameter significantly responsive to HS diet (a clear decrease was seen), and also most sensitive to arginine treatment (a significant 61% increase). RBF, CBF and OMBF increased in parallel after arginine but the changes were not significant.

Under long term exposure to HS diet (HS26), RBF tended to be higher than in HS10 rats (+19%) and even higher (+26%) after L-arg treatment. A similar pattern of changes was also observed for CBF. IMBF was higher than in the HS10 group (+63%, $P < 0.003$); unexpectedly, addition of L-arg tended to decrease it.

As expected, V, UosmV and $U_{Na}V$ were the lowest in STD group rats, otherwise there were no significant differences between diet or treatment groups (*Table 2*).

Effects of chronic exposure to HS diet and chronic treatment with L-arg or L-arg + BH_4 on the vascular responses to intrarenal acetylcholine (ACh) are shown in *Fig. 3*. As expected, ACh increased or tended to increase RBF, CBF, OMBF and IMBF, both in the STD and HS10 groups (*Fig. 3*). Interestingly, the

Table 2. Mean arterial blood pressure (MABP), total renal blood flow (RBF), renal regional perfusion of the cortex (CBF), outer- (OMBF) and inner medulla (IMBF), urine flow (V), and total osmole and sodium excretion ($U_{osm}V$, $U_{Na}V$) in anaesthetized rats. The data are baseline values measured in acute experiments. The values showing consistent elevation of renal perfusion parameters in arginine treated rats are given in bold font.
STD - untreated rats on standard diet; HS10 and HS26 - untreated rats maintained on high-sodium diet for 10 or 26 days; HS10 + L-arg and HS26 + L-arg - the rats of the two diet protocols treated with L-arginine (L-arg); HS10 + L-arg + BH_4 - HS10 rats and treated with L-arginine (L-arg) and tetrahydrobiopterin (BH_4).

	STD (n = 6)	**HS10** (n = 8)	**HS10 + L-arg** (n = 8)	**HS10 + L-arg + BH4** (n = 9)	**HS 26** (n = 5)	**HS 26 + L-arg** (n = 7)
MABP (mmHg)	103 ± 4	112 ± 4	110 ± 5	113 ± 3	114 ± 6	106 ± 4
RBF (ml/min)	**8.4 ± 1.6**	**8.1 ± 1.7**	**12.3 ± 1.4**	13.5 ± 0.9 †	9.6 ± 1.2	10.2 ± 1.4
CBF (PU)	**611 ± 58**	**619 ± 58**	**724 ± 40**	661 ± 31	719 ± 68	773 ± 85
OMBF (PU)	**165 ± 24**	**163 ± 24**	**212 ± 35**	173 ± 22	152 ± 21	191 ± 30
IMBF (PU)	**193 ± 15** †	**135 ± 16**	**217 ± 33** †	104 ± 15 *	220 ± 16 †	160 ± 29 #
V (µl/min)	3.6 ± 1	11.3 ± 4	9.6 ± 3	13.2 ± 2 *	9.0 ± 3	5.8 ± 1
UosmV (µosm/min)	3.4 ± 1	8.8 ± 4	10.4 ± 3	14.0 ± 3 *	7.6 ± 3	4.6 ± 1
UNaV (µmol/min)	0.09 ± 0.01	2.7 ± 1.2	2.3 ± 0.7	2.7 ± 0.7	2.0 ± 0.6	2.0 ± 0.7

* - significantly different from STD diet; † - significantly different from HS10 diet, # - siginificantly different from HS26 diet.

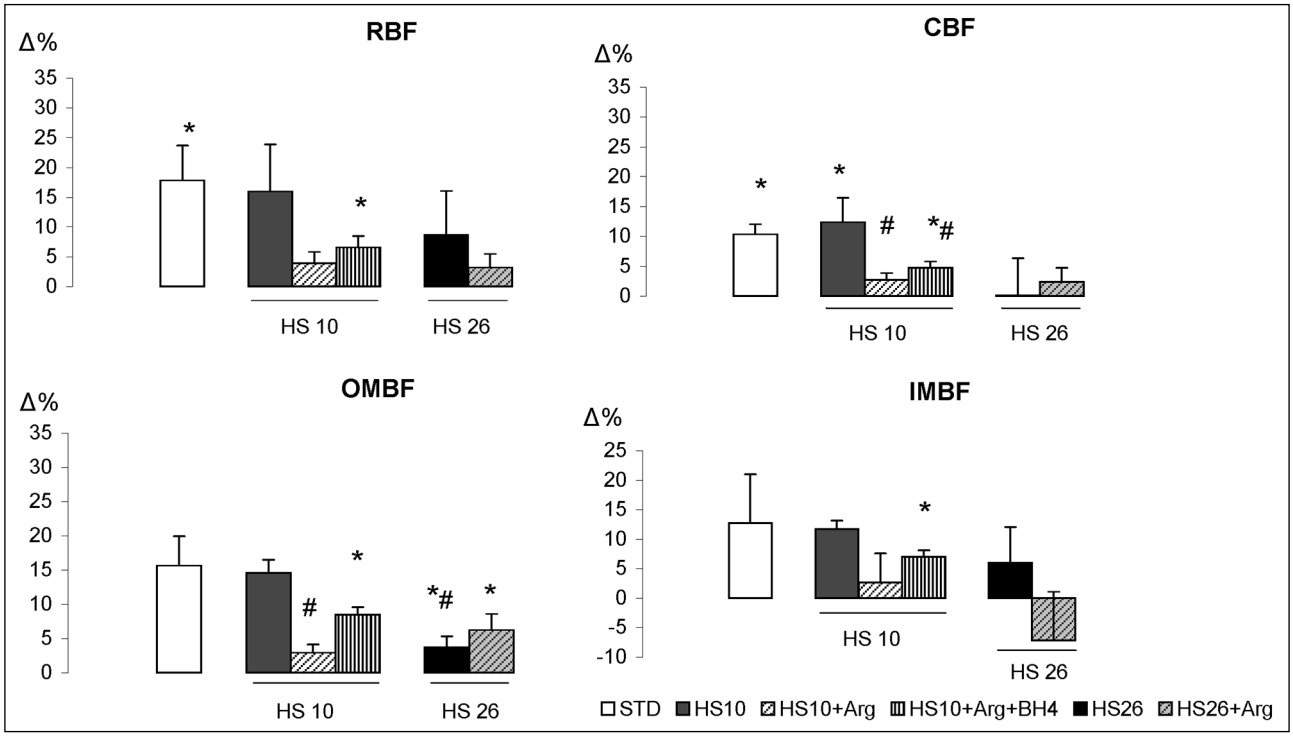

Fig. 3. Changes (%) in total renal blood flow (RBF) and cortical, outer- and inner medullary perfusions (CBF, OMBF, IMBF) in response to acetylcholine (ACh, 5 µg/kg/h) infused into the renal artery. Means ± SEM. * - significantly different from the pre-infusion control, # - significantly different from high sodium diet.

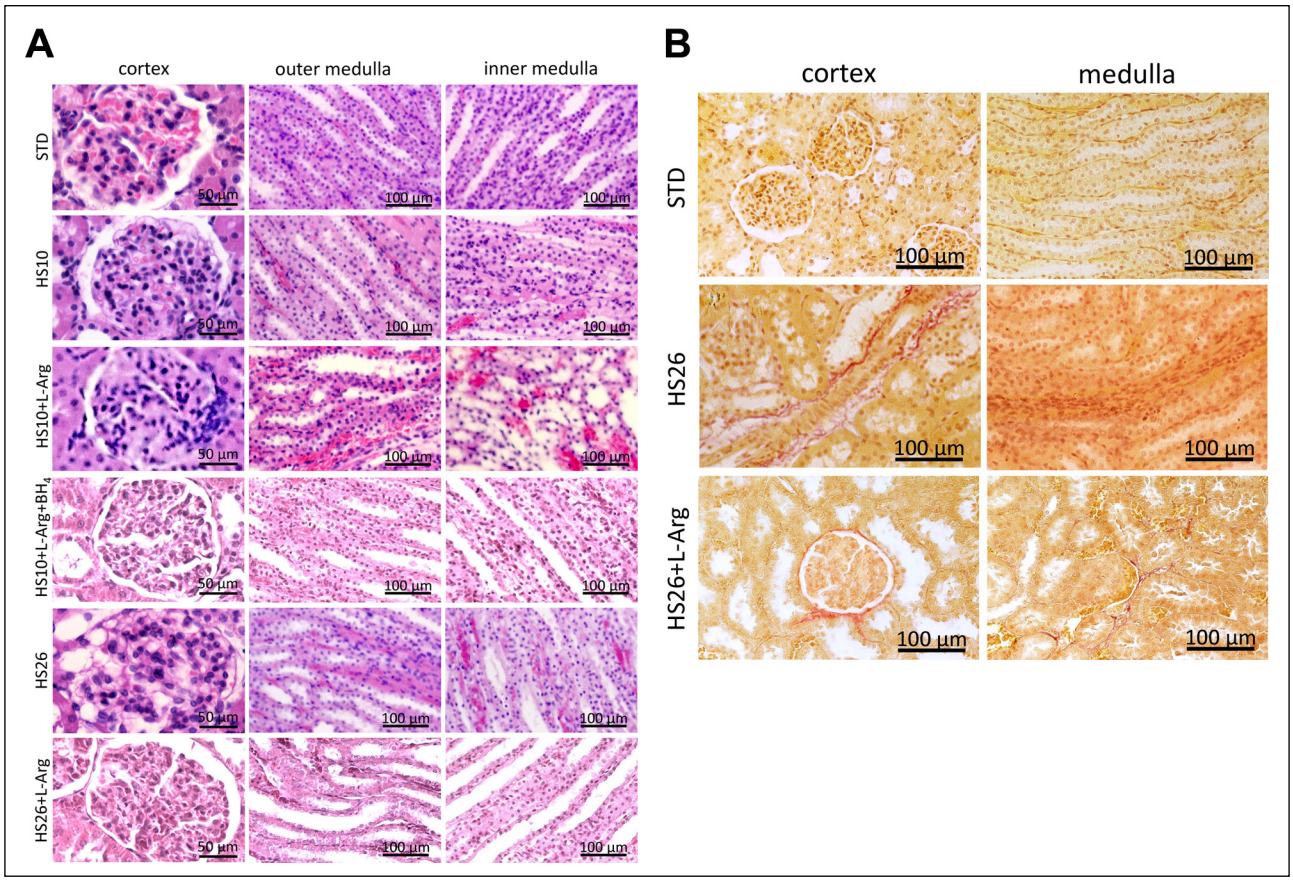

Fig. 4. Morphologic features of the kidney in individual experimental groups. (A): hematoxylin-eosin staining; magnification: × 200 (glomeruli) and ×100 (medulla). (B): Van Gieson staining; magnification × 100.
STD, standard diet; HS10, 10 days of high salt diet; HS10 + L-arg, 10 days of high salt diet with chronic L-arginine supplementation; HS10 + L-arg + BH4, 10 days of high salt diet with chronic L-arginine and tetrahydrobiopterin supplementation; HS26, 26 days of high salt diet; HS26 + L-arg, 6 days of high salt diet with chronic L-arginine supplementation.

responses were generally smaller (significantly so for OMBF) in rats exposed to HS diet for 26 days. L-arg treatment appeared to reduce the response to ACh in HS10 rats; the reduction was significant for CBF and OMBF. No consistent effects of L-arg were seen in HS26 rats. Addition of BH_4 tended to diminish the decreasing action of L-arg alone, however, the changes were not significant. In general, in HS10 rats L-arg reduced the post-ACh increases of parameters of renal perfusion but no further reduction was seen after addition of BH_4.

Renal artery infusion of noradrenalin (NA) invariably induced decreases in each of the renal haemodynamic parameters measured but the intergroup differences were inconsistent and not significant (data not shown).

In kidney morphological studies (*Fig. 4*) a slight vacuolization in some glomeruli and widening of medullary tubule segments were observed after 10 days of HS diet. Prolongation of HS diet to 26 days increased glomerular vacuolization and increased congestion within each of the three renal zones (cortex, outer and inner medulla). Van Gieson (VG) staining revealed tissue fibrosis in HS26 rats only. In HS10 + L-arg rats renal morphology did not differ from the untreated rats, however, in HS10 + L-arg + BH_4 group the changes caused by HS diet appeared less prominent than in HS10 + L-arg group, even though the medullary tubules seemed more dilated. Similar morphologic features were present in medullary tubule segments in HS26 + L-arg group. Moreover, supplementation of L-arg in HS26 rats did not reverse fibrosis, which was more distinct in the vicinity of the glomeruli and within the vascular bundles. All these alterations were in clear contrast to normal morphologic structure seen in rats on standard diet.

DISCUSSION

In agreement with our original report (16) we found that conscious male Wistar rats maintained on HS diet showed a progressing elevation of SBP, by about 40 mmHg when measured after 26 days. In the anaesthetized rats MABP tended to be higher by about 10 mmHg. Such findings are remarkable since Wistar rats are not usually regarded as salt-sensitive, even though consistent modest increase in systolic and diastolic BP after exposure to high salt diet was recently reported in this strain (18). This model may be thought to better mimic the situation in those human subjects in whom any genetic defect, if present, is rarely defined, and excessive salt intake appears to be the only detectable cause of blood pressure elevation. Remarkably, our Wistar rats displayed a modest elevation of blood pressure also on standard diet, which might be compared with not infrequent "borderline hypertension" of young human subjects, the state that may develop to overt hypertension after changing eating habits to meals containing more sodium.

While in our model the salt-dependent blood pressure elevation must have been related to sodium retention, no consistent increase of plasma sodium was demonstrable. The retention may have been compensated in part by the observed increased renal excretion, moreover, excessive sodium was

reported to be stored in body fluid compartments other than intravascular plasma (19, 20).

At least when measured under anaesthesia, 10-day exposure to high salt intake did not change renal haemodynamics, apart from a reduction of inner medullary perfusion (IMBF); the value re-increased after 26-day exposure. The reduction did not depend on impaired reactivity of intrarenal vessels to vasodilator acetylcholine (ACh): we showed that renal total and regional perfusion responses to ACh (including that for IMBF) were not altered. More likely, IMBF lowering depended on reduced local bioavailability of NO secondary to the oxidative stress, a known consequence of high salt intake.

It is known that all three NOS isoforms are constitutively present in the kidney but there is no consensus regarding the effect of salt intake on their expression: increased (21, 22), unchanged (23) or decreased expression (24) was reported. This depended on the experimental conditions in general, the rat strain used and on the actual method of NOS assessment (23). We showed previously that in normal rats NO biovailability is much higher in the renal medulla compared to the cortex (25) and it is lowered by exposure to high salt (16). nNOS is the most abundant isoform in the medulla (26). While NO synthase expression was not determined in the present study, NO bioavailability was likely to be lowered on HS diet due to increased generation of ROS, largely because nNOS (which prevails in the medulla) is especially liable to uncoupling under oxidative stress conditions (27) and produces superoxide instead of NO. Admittedly, appropriately focused studies would be needed to establish if arginine supplementation did increase NO synthesis.

The transiency of the IMBF decrease as opposed to progressing increase in blood pressure speaks against the proposed long term interrelationship of the two phenomena (28, 29) in the present model and experimental setting.

Microscopic examination of kidney samples confirmed the common high salt intake sequels, such as dilatation of medullary tubule segments, diffuse tissue congestion and, after prolonged exposure, tissue fibrosis.

The principal aim of this study was to examine if supplementation of HS rats with arginine, a substrate for NO synthesis, and BH_4, a co-factor needed for effective NO synthase action, would attenuate the development of salt-dependent blood pressure elevation and thus indicate that deficient NO synthesis was here the major culprit.

Almost all earlier studies exploring the role of arginine were performed in the model of Dahl salt-sensitive rats. In elegant experiments Miyata and Cowley found that chronic infusion of L-arginine into the renal medulla prevented the BP increase (and medullary blood flow decrease) that normally developed after exposure to high salt diet (11). The effect of L-arginine given in drinking water at widely differing doses (1.3 – 20 g/l) seemed to depend on the stage of development of hypertension and actual pressure levels attained. Full prevention of the pressure increase was observed in younger rats which, when untreated, reached the pressure of about 150 mmHg (30, 31). With the pressure values of about 200 mmHg or more only attenuation (12) or no effect on hypertension (32) were seen, which suggested that arginine supplementation would be at least less effective with more advanced cardiovascular and perhaps kidney damage associated with severe hypertension. Such a notion agrees with the finding that arginine treatment prevented the blood pressure increase in the model of moderate hypertension (~150 mmHg) induced by uninephrectomy followed by exposure to high salt diet (17). For comparison, however, arginine was found ineffective in our rats which, without treatment, attained SBP level of ~180 mmHg. Evidently, the severity of hypertension should be considered as a possible determinant of the effectiveness of arginine.

In our model the effect of arginine supplementation on the salt-induced blood pressure increase was negligible: an early transient pressure-decreasing influence was seen within the first week but was not maintained in the prolonged exposure group. When measured in anaesthetized rats, MABP only tended to be lower in L-arg treated compared to untreated animals and this was seen only after prolonged HS intake (*Table 2*). On the whole, these findings indicate that in Wistar rats in which high salt intake appeared to be the decisive causal factor of blood pressure elevation (in the absence of any history of genetic determination or morphologic changes in the kidney), arginine deficiency was not crucial for blood pressure elevation. Remarkably, 26-day arginine supplementation was followed by a decrease in IMBF, compared with the non-supplemented group, and did not prevent renal tissue fibrosis. The latter is compatible with the finding that arginine supplementation can accelerate the development of renal fibrosis, probably depending on the toxic effect of NO generated by iNOS (33).

Interestingly, in anaesthetized (HS10) rats L-arg at least tended to increase renal haemodynamic parameters; in the case of medullary perfusion (IMBF) a 61% significant increase was seen so that its decrease observed on HS diet was totally abolished. A similar response pattern was earlier observed in Dahl salt-sensitive rats: infusion of L-arginine into the renal medulla prevented the usual early decrease in medullary blood flow observed under high salt intake, and appeared to prevent an early increase in blood pressure (11). In our model, the arginine-induced increase in IMBF was not associated with any attenuation of the salt-induced blood pressure increase. Possibly, the vasodilator action was confined to the renal medulla, a tissue characterized by abundance of NO synthases: in the absence of systemic vasodilation the blood pressure was not affected.

It will be recalled that elevation of medullary blood flow has long been proposed to be a specific trigger for a decrease in blood pressure (28, 29) and such a mechanism was also suggested to be operative in Dahl salt sensitive hypertensive rats (34, 35). However, no such causal relationship could be demonstrated in our hypertensive rats with salt-induced hypertension but without genetic background of salt sensitivity (36).

Arginine supplementation substantially reduced the vasodilator responses of renal circulation parameters, also of IMBF, to ACh. At the first sight, this manoeuvre might be thought to increase the availability of NO in the intrarenal microvessel wall and thus act in the same direction as arginine supplementation. However, it is likely that at post-arginine high (perhaps close to maximal) rates of NO synthesis and action, there was little space for a further increase of NO effects after stimulation of its release with ACh, hence the observed reduced vasodilator response.

As observed with both conscious and anaesthetized rats, addition of BH_4 did not substantially alter the blood pressure levels observed with arginine treatment alone. Thus, the results do not confirm the beneficial hypotensive action of BH_4 reported from studies with spontaneously hypertensive rats (13). Apparently, the role of BH_4 is likely in genetically determined hypertension or in 5/6 nephrectomized rats (14, 15) but not in the model of salt-induced blood pressure elevation employed in the present study. Multiple other factors might be engaged as co-determinants of blood pressure increase in response to increased salt intake, such, for instance, as alterations of the expression and activity of mineralocorticoid receptors, as reviewed recently (37). There is no explanation why, when studied under anaesthesia, BH_4 tended to decrease the renal cortical and medullary perfusion below the rates measured during arginine supplementation.

In conclusion, in outbred male Wistar rats without apparent genetic predisposition for salt sensitivity, high salt intake induced a moderate increase in blood pressure which was only marginally attenuated by supplementation with L-arginine and

BH_4. Thus, even if under high salt intake the NO bioavailability in the vascular wall was reduced, which contributed to the pressure elevation, its deficient synthesis may not have been the decisive factor. Determination of expression levels of nNOS and eNOS would be needed to resolve this question. Even at normal NO synthesis rate, decreased NO activity could have been due to increased ROS generation, as commonly observed on HS diet and well-pronounced in the kidney. The major source of the ROS would be NO synthases (specially nNOS) which undergo uncoupling in HS animals and generate ROS instead of NO.

Arginine supplementation modestly increased renal total and cortical blood flow but it substantially increased perfusion of the inner medulla, which suggested correction of the prevalence of vasoconstrictor reactive oxygen species over vasodilator NO action in this region. Such beneficial action was probably confined to the medulla or perhaps the whole kidney and therefore was not translated into a decrease in blood pressure. Notably, the arginine-induced improvement of renal haemodynamics, potentially a nephroprotective action, occurred only in the early phase of the exposure to high salt diet.

We suggest that the arginine-induced enhancement of NO bioavailability in the kidney was so effective that further increasing NO delivery caused by intrarenal acetylcholine resulted in only minor vasodilation. BH_4 did not enhance the effects of arginine on renal circulation.

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REFERENCES

1. Boegehold MA. Microvascular structure and function in salt-sensitive hypertension. *Microcirculation* 2002; 9: 225-241.
2. Boegehold MA. The effect of high salt intake on endothelial function: reduced vascular nitric oxide in the absence of hypertension. *J Vasc Res* 2013; 50: 458-467.
3. Feng W, Ying WZ, Aaron KJ, Sanders PW. Transforming growth factor-β mediates endothelial dysfunction in rats during high salt intake. *Am J Physiol Renal Physiol* 2015; 309: F1018-F1025.
4. Edwards DG, Farquhar WB. Vascular effects of dietary salt. *Curr Opin Nephrol Hypertens*. 2015; 24: 8-13.
5. Deng X, Welch WJ, Wilcox CS. Renal vasodilation with L-arginine. Effects of dietary salt. *Hypertension* 1995; 26: 256-262.
6. Luiking YC, Ten Have GA, Wolfe RR, Deutz NE. Arginine de novo and nitric oxide production in disease states. *Am J Physiol Endocrinol Metab* 2012; 303: E1177-E1189.
7. Rajapakse NW, Mattson DL. Role of L-arginine in nitric oxide production in health and hypertension. *Clin Exp Pharmacol Physiol* 2009; 36: 249-255.
8. Yan J, Tie G, Messina LM. Tetrahydrobiopterin, L-arginine and vitamin C act synergistically to decrease oxidant stress and increase nitric oxide that increases blood flow recovery after hindlimb ischemia in the rat. *Mol Med* 2012; 18: 1221-1230.
9. Vasquez-Vivar J, Kalyanaraman B, Martasek P, *et al.* Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci USA* 1998; 95: 9220-9225.
10. Xia Y, Tsai AL, Berka V, Zweier JL. Superoxide generation from endothelial nitric-oxide synthase: a Ca2+/calmodulin-dependent and tetrahydrobiopterin regulatory process. *J Biol Chem* 1998; 273: 25804-25808.
11. Miyata N, Cowley AW Jr. Renal intramedullary infusion of L-arginine prevents reduction of medullary blood flow and hypertension in Dahl salt-sensitive rats. *Hypertension* 1999; 33: 446-450.
12. Fujii S, Zhang L, Igarashi J, Kosaka H. L-arginine reverses p47phox and gp91phox expression induced by high salt in Dahl rats. *Hypertension* 2003; 42: 1014-1020.
13. Hong HJ, Hsiao G, Cheng TH, Yen MH. Supplementation with tetrahydrobiopterin suppresses the development of hypertension in spontaneously hypertensive rats. *Hypertension* 2001; 38: 1044-1048.
14. Podjarny E, Hasdan G, Bernheim J, *et al.* Effect of chronic tetrahydrobiopterin on blood pressure and proteinuria in 5/6 nephrectomized rats. *Nephrol Dial Transplant* 2004; 19: 2223-2227.
15. Podjarny E, Bernheim J, Hasdan G, *et al.* Additive renoprotective effect of candesartan and tetrahydrobiopterin in rats after 5/6 nephrectomy. *Nephrol Dial Transplant* 2007; 22: 864-1872.
16. Walkowska A, Kuczeriszka M, Sadowski J, *et al.* High salt intake increases blood pressure in normal rats: putative role of 20-HETE and no evidence on changes in renal vascular reactivity. *Kidney Blood Press Res* 2015; 40: 323-334.
17. Carlstrom M, Brown RD, Yang T, *et al.* L-arginine or tempol supplementation improves renal and cardiovascular function in rats with reduced renal mass and chronic high salt intake. *Acta Physiologica* 2013; 207: 732-741.
18. Stepien M, Kujawska-Luczak M, Szulinska M, *et al.* Beneficial dose-independent influence of Camellia sinensis supplementation on lipid profile, glycemia, and insulin resistance in an NaCl-induced hypertensive rat model. *J Physiol Pharmacol* 2018; 69: 275-282.
19. Jantsch J, Schatz V, Friedrich D, *et al.* Cutaneous Na+ storage strengthens the antimicrobial barrier function of the skin and boosts macrophage-driven host defense. *Cell Metab* 2015; 21: 493-501.
20. Titze J. A different view on sodium balance. *Curr Opin Nephrol Hypertens* 2015; 24: 14-20.
21. Mattson DL, Higgins DJ. Influence of dietary sodium intake on renal medullary nitric oxide synthase. *Hypertension* 1996; 27: 688-692.
22. Wilcox CS, Deng X, Welch WJ. NO generation and action during changes in salt intake: roles of nNOS and macula densa. *Am J Physiol* 1998; 274: R1588-R1593.
23. Hyndman KA, Xue J, MacDonell A, Speed JS, Jin C, Pollock JS. Distinct regulation of inner medullary collecting duct NO production from mice and rats. *Clin Exp Pharmacol Physiol* 2013; 40: 1440-1681.
24. Ni Z, Vaziri ND. Effect of salt loading on nitric oxide synthase expression in normotensive rats. *Am J Hypertens* 2001; 14: 155-163.
25. Grzelec-Mojzesowicz M, Sadowski J. Renal tissue NO and intrarenal haemodynamics during experimental variations of NO content in anaesthetised rats. *J Physiol Pharmacol* 2007; 58: 149-163.
26. Martin PY, Bianchi M, Roger F, Niksic L, Feraille E. Arginine vasopressin modulates expression of neuronal NOS in rat renal medulla. *Am J Physiol Renal Physiol* 2002; 283: F559-F568.
27. Sun J, Druhan LJ, Zweiter JL. Dose dependent effects of reactive oxygen and nitrogen species on the function of neuronal nitric oxide synthase. *Arch Biochem Biophys* 2008; 471: 126-133.
28. Cowley AW Jr. Role of the renal medulla in volume and arterial pressure regulation. *Am J Physiol* 1997; 273: R1-R15.

29. Mattson DL. Importance of the renal medullary circulation in the control of sodium excretion and blood pressure. *Am J Physiol Regul Integr Comp Physiol* 2003; 284: R13-R27.
30. Chen PY, Sanders PW. L-arginine abrogates salt-sensitive hypertension in Dahl/Rapp rats. *J Clin Invest* 1991; 88: 1559-1567.
31. Zhou MS, Kosaka H, Tian RX, *et al.* L-arginine improves endothelial function in renal artery of hypertensive Dahl rats. *J Hypertens* 2001; 19: 421-429.
32. Artigues C, Richard V, Roussel C, Lallemand F, Henry JP, Thuillez C. Increased endothelium-monocyte interactions in salt-sensitive hypertension: effect of L-arginine. *J Cardiovasc Pharmacol* 2000; 35: 468-473.
33. Peters H, Border WA, Ruckert M, Kramer S, Neumayer HH, Noble NA. L-arginine supplementation accelerates renal fibrosis and shortens life span in experimental lupus nephritis. *Kidney Int* 2003; 63: 1382-1392.
34. Cowley AW Jr, Mori T, Mattson D, Zou AP. Role of renal NO production in the regulation of medullary blood flow. *Am J Physiol Regul Integr Comp Physiol* 2003; 284: R1355-R1369.
35. Cowley AW Jr, Abe M, Mori T, O'Connor PM, Ohsaki Y, Zheleznova NN. Reactive oxygen species as important determinants of medullary flow, sodium excretion, and hypertension. *Am J Physiol Renal Physiol* 2015; 308: F179-F197.
36. Badzynska B, Baranowska I, Gawrys O, Sadowski J. Evidence against a crucial role of renal medullary perfusion in blood pressure control of hypertensive rats. *J Physiol* 2019; 597: 211-223.
37. Sztechman D, Czarzasta K, Cudnoch-Jedrzejewska A, Szczepanska-Sadowska E, Zera T. Aldosterone and mineralortycoid receptors in regulation of the cardiovascular system and pathological remodeling of the heart and arteries. *J Physiol Pharmacol* 2018; 69: 829-845.

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