

## RESEARCH PAPER

# K<sub>Ca</sub>3.1 channels maintain endothelium-dependent vasodilatation in isolated perfused kidneys of spontaneously hypertensive rats after chronic inhibition of NOS

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### BACKGROUND AND PURPOSE

The purpose of the study was to investigate renal endothelium-dependent vasodilatation in a model of severe hypertension associated with kidney injury.

### EXPERIMENTAL APPROACH

Changes in perfusion pressure were measured in isolated, perfused kidneys taken from 18-week-old Wistar–Kyoto rat (WKY), spontaneously hypertensive rats (SHR) and SHR treated for 2 weeks with N<sup>o</sup>-nitro-L-arginine methyl ester in the drinking water (L-NAME-treated SHR, 6 mg·kg<sup>-1</sup>·day<sup>-1</sup>).

### KEY RESULTS

Acetylcholine caused similar dose-dependent renal dilatation in the three groups. *In vitro* administration of indomethacin did not alter the vasodilatation, while the addition of N<sup>o</sup>-nitro-L-arginine (L-NA) produced a differential inhibition of the vasodilatation, (inhibition in WKY > SHR > L-NAME-treated SHR). Further addition of ODQ, an inhibitor of soluble guanylyl cyclase, abolished the responses to sodium nitroprusside but did not affect the vasodilatation to acetylcholine. However, the addition of TRAM-34 (or charybdotoxin) inhibitors of Ca<sup>2+</sup>-activated K<sup>+</sup> channels of intermediate conductance (K<sub>Ca</sub>3.1), blocked the vasodilatation to acetylcholine, while apamin, an inhibitor of Ca<sup>2+</sup>-activated K<sup>+</sup> channels of small conductance (K<sub>Ca</sub>2.3), was ineffective. Dilatation induced by an opener of K<sub>Ca</sub>3.1/K<sub>Ca</sub>2.3 channels, NS-309, was also blocked by TRAM-34, but not by apamin. The magnitude and duration of NS-309-induced vasodilatation and the renal expression of mRNA for K<sub>Ca</sub>3.1, but not K<sub>Ca</sub>2.3, channels followed the same ranking order (WKY < SHR < L-NAME-treated SHR).

### CONCLUSIONS AND IMPLICATIONS

In SHR kidneys, an EDHF-mediated response, involving activation of K<sub>Ca</sub>3.1 channels, contributed to the mechanism of endothelium-dependent vasodilatation. In kidneys from L-NAME-treated SHR, up-regulation of this pathway fully compensated for the decrease in NO availability.

### Abbreviations

EDHF, endothelium-derived hyperpolarizing factor; HbNO, iron-nitroxyl-haemoglobin; L-NA, N<sup>o</sup>-nitro-L-arginine; L-NAME, N<sup>o</sup>-nitro-L-arginine methyl ester; NS-309, 6–7-dichloro-1 *H*-indole-2,3-dione 3-oxime; ODQ, 1H-(1,2,4)-oxadiazolo(4,2-a)quinoxalin-1-one; SHR, spontaneously hypertensive rat; TRAM-34, 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole; WKY rat, Wistar–Kyoto rat

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### Keywords

SHR; L-NAME chronic treatment; EDHF-mediated responses; K<sub>Ca</sub>3.1; K<sub>Ca</sub>2.3; perfused rat kidney

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## Introduction

Hypertension is a major pathological factor in the development of cardiovascular disease and in the progression towards end-stage renal disease, the incidence of both being increased during aging (Zhou and Frohlich, 2007). The aging spontaneously hypertensive rat (SHR) is an animal model exhibiting end-stage renal disease that shows some similarities with those observed in clinical hypertension (i.e. elevated renal vascular resistance, impaired renal functions and increased renal fibrosis). However, this interesting model is difficult to manage since SHR require careful husbandry and up to the age of 12–16 months when these renal dysfunctions become manifest (Zhou and Frohlich, 2007). However, SHR treated with a moderate dose of a NOS inhibitor show impaired kidney function and altered renal structures similar to those observed in untreated, ageing SHR, and hence, represents an accelerated model of hypertension associated with kidney damage (Zhou and Frohlich, 2007). This experimental model of severe hypertension and nephrosclerosis is also associated with cardiac fibrosis and arterial stiffening (Ono *et al.*, 2001; Terata *et al.*, 2003; Zhou and Frohlich, 2007; Vayssettes-Courchay *et al.*, 2008; 2011; Isabelle *et al.*, 2012).

Endothelial cells control vascular tone not only by releasing NO but also by generating other vasoactive mediators such as metabolites of arachidonic acid. In addition, they also elicit endothelium-dependent hyperpolarizations (EDHF-mediated responses; Félétou *et al.*, 2011). In the renal vasculature, endothelium-dependent vasodilatation is an important regulatory mechanism and disruption of these processes, including the activity of various populations of potassium channels, can profoundly alter haemodynamic resistance, renal blood flow and, hence, affect glomerular filtration pressure and renal function (Benter *et al.*, 2005; Sorensen *et al.*, 2012). In hypertension, this endothelial dysfunction is characterized by an alteration of the balance between endothelial vasoconstrictor and vasodilator responses (Büssemaker *et al.*, 2003; Félétou and Vanhoutte, 2006a; Michel *et al.*, 2008). The purpose of the present work was, in the accelerated model of hypertension associated with kidney damage (i.e. SHR treated with a moderate dose of a NOS inhibitor), to study renal function and to characterise endothelial function. In isolated perfused kidneys from these SHR, the role of the various endothelial mechanisms involved in acetylcholine-induced vasodilatation was assessed, with a particular emphasis on EDHF-mediated responses.

## Methods

All studies reported were performed in accordance with the European Community Guidelines for the use of experimental animals and were approved by the ethical committee on Animal Experiments of the Servier Research Institute. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 123 animals were used in the experiments described here.

## Experimental procedures

Male spontaneously hypertensive rats (SHR) were purchased from Janvier (Le Genest St Isle, France). All animals were fed a standard laboratory diet and water *ad libitum* and were maintained in a humidity- and temperature-controlled room. Four groups of 4-month-old Wistar–Kyoto rat (WKY) and SHR were treated for 2 weeks. Group 1: control WKY rats drinking regular water; group 2: L-NAME-treated WKY rats drinking water containing 50 mg·L<sup>-1</sup> of N<sup>o</sup>-nitro-L-arginine methyl ester (L-NAME); group 3: control SHR drinking regular water; group 4: L-NAME-treated SHR drinking water containing 50 mg·L<sup>-1</sup> of L-NAME (approximately 6 mg·kg<sup>-1</sup>·day<sup>-1</sup>).

In WKY rats, this moderate dose of L-NAME administered for this period of time did not significantly affect arterial blood pressure (systolic blood pressure: 140 ± 6 mm Hg, *n* = 10) or body weight and did not influence either the renal parameters measured or the vascular reactivity of the isolated perfused kidney. Therefore, for the sake of clarity, the data from this group of animals will not be presented (see also Figures S1 and S2).

## Urine volume and protein determination

At the end of the 2 week treatment period, animals were placed in individual metabolic cages, with access to regular water or L-NAME containing water *ad libitum*, depending on the groups, but without food, for 24 h urine collection. The collected urine from each animal was centrifuged at 2500× *g* and at 4°C for 15 min, and then stored at –80°C until biochemical analysis (auto analyser Pentra 400 ABX). Urinary excretion of nitrite was measured by a colorimetric assay (Cayman Chemicals, Spibio, France).

## Blood pressure measurement and blood sampling

Animals were anaesthetized with sodium pentobarbital (55 mg·kg<sup>-1</sup>, i.p.) and then placed on a homoeothermic blanket to maintain rectal temperature at 37°C. After a tracheotomy, a catheter was inserted into the right carotid artery for blood pressure measurement and blood sampling. Systolic blood pressure and heart rate were recorded through a pressure transducer (P10EZ, Statham, France) connected to a data acquisition system (Biopac 02, BIOPAC Systems Inc., CEROM, Paris, France). At the end of the blood pressure measurement, blood samples were collected into two tubes containing lithium-heparin and EDTA respectively. Then the left kidney was cannulated for perfusion pressure experiments, and the right kidney was used for histological analysis.

## Iron-nitroxyl-haemoglobin (HbNO) measurement

Blood samples were collected in heparinised (50 U·mL<sup>-1</sup>) tubes and sodium dithionite (20 mg·mL<sup>-1</sup>) was added to prevent excessive oxygenation. Red blood cells were obtained by centrifugation (2500× *g*, 10 min), and HbNO levels were assayed by electron spin resonance and recorded in a finger Dewar refrigerated with liquid nitrogen and with a Magnetech MS200. System settings were the following: BO field (3340 G), BO sweep (300 G), Sweep-time (30 s), Pass (4),

Modulation (7000), MW attenuation (4 db). Spectra were analysed on the Analysis 2.02 software provided with the instrument.

### RT-qPCR analysis

Freshly collected aortae and kidneys were snap-frozen in liquid nitrogen and the latter homogenized on ice in 300  $\mu\text{L}$  of RLT lysis buffer for mRNA extraction (RNeasy<sup>®</sup> mini kit; Qiagen, Hilden, Germany). Samples with a RQI >7 (RNA quality integrity) were converted to cDNA (1  $\mu\text{g}$  of starting material) with the Superscript<sup>™</sup> III first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). Expression of  $K_{\text{Ca}}3.1$  and  $K_{\text{Ca}}2.3$  protein was quantified by quantitative RT-qPCR using an iCycler iQ Detection System (Bio-Rad Laboratories, Hercules, CA). Each reaction was performed using the IQ<sup>™</sup> SYBR<sup>®</sup> Green supermix (containing 3 mM  $\text{MgCl}_2$  and the iTaq DNA polymerase at 25 units·mL<sup>-1</sup>; Bio-Rad), 2  $\mu\text{L}$  of DNA samples and 150 nM of each specific primer in a final volume of 25  $\mu\text{L}$ . Samples were denatured for 300 s at 95°C and amplified for 40 cycles as follows: denaturation for 20 s at 95°C and annealing for 1 min at the specific oligonucleotide optimal temperature (Table S1). Each real-time PCR run included cDNAs in duplicate in parallel with serial dilutions of a cDNA mix tested for each primer pair to generate a linear standard curve with the mean cycle thresholds. This curve was then used to estimate the relative quantity of the relevant mRNA in each sample. The values obtained with our samples were normalized to the geometric mean of the values obtained with three internal controls: hypoxanthine-guanine phosphoribosyltransferase (HPRT),  $\beta$ -actin and GAPDH. The specificity of PCR products was confirmed by a melting curve analysis. All the primers were designed with the Beacon Designer Software (Premier Biosoft, Palo Alto, CA, USA).

### Isolated, perfused rat kidney

Isolated, perfused kidneys were prepared as previously described (Collis and Vanhoutte, 1977). Briefly, after anaesthesia, the left kidney was exposed by midline ventral laparotomy. The left renal artery was cannulated via an incision made in the aorta. Then the kidney was perfused at constant flow by a peristaltic pump (Gilson Minipuls 2), with a warmed (37°C) and oxygenated (95%  $\text{O}_2$ –5%  $\text{CO}_2$ ) Tyrode solution of the following composition (mM): NaCl 137; KCl 2.7;  $\text{CaCl}_2$  1.8;  $\text{MgCl}_2$  1.1;  $\text{NaHCO}_3$  12.0;  $\text{NaH}_2\text{PO}_4$  0.42;  $\text{Na}_2\text{-Ca-EDTA}$  0.026 and glucose 5.6. The perfused kidney was removed from the surrounding adipose tissue and placed in a perfusion chamber pre-warmed at 37°C. The perfusate was not re-circulated. Flow rate was adjusted to optimum flow rate corresponding to 5 mL·min<sup>-1</sup> per g of kidney weight (Collis and Vanhoutte, 1977; Heuzé-Joubert *et al.*, 1992). The changes in renal vascular resistance were recorded as changes in perfusion pressure measured downstream from the pump via a pressure transducer (P10EZ, Statham, France), which was connected to a data acquisition system IOX2 (EMKA Technologies, Paris, France). Once the perfusion pressure reached its steady state, after a 60 min equilibration period, experiments were started.

Pharmacological agents were administered in three different ways: (i) 20  $\mu\text{L}$  bolus could be injected into the perfusion circuit (doses were expressed in moles); (ii) the

inhibitors or antagonists could be infused into the perfusion circuit by an infusion pump placed upstream to the perfusion pump; or (iii) compounds could be directly added into the perfusing Tyrode solution (concentrations were expressed in mol·L<sup>-1</sup>).

### Experimental design

**Part 1.** Under the present experimental conditions, papaverine (10 nmol) did not affect basal perfusion pressure, indicating that the perfused rat kidney lacks intrinsic tone. A concentration–response curve to noradrenaline (0.001–10 nmol) was performed. The maximal perfusion pressure achieved (basal perfusion pressure + the increase in perfusion pressure produced by the highest dose of noradrenaline tested) was not significantly different between the various groups tested. However, kidneys from L-NAME-treated SHR were slightly but significantly more sensitive to the  $\alpha$ -adrenoceptor agonist than kidneys from other groups of rats (Figure S1). In order to study endothelium-dependent and -independent vasodilator compounds, a stable level of tension was produced by the infusion of the metabolically stable  $\alpha$ -adrenoceptor agonist, methoxamine. The rate of infusion was slightly different between groups and between kidneys from different animals within the same group in order to achieve the same stable level of perfusion pressure (70% to 80% of the maximal perfusion pressure), either in control conditions or in the presence of the various pharmacological agents studied. Then vasodilator substances were injected as a bolus into the circuit at intervals of at least 10 min: acetylcholine (from 1 pmol to 10 nmol), sodium nitroprusside (from 10 pmol to 10 nmol) and forskolin (6 nmol).

**Part 2.** In the vasodilator responses to acetylcholine, the contributions of cyclooxygenase derivatives and/or NO were studied. The experiments were performed in the presence of the cyclooxygenase inhibitor, indomethacin (3  $\mu\text{mol}\cdot\text{L}^{-1}$ ) added to the Tyrode solution or/and of the NOS inhibitor, N<sup>o</sup>-nitro-L-arginine (L-NA) perfused at 100  $\mu\text{mol}\cdot\text{L}^{-1}$ , 20 min before the initiation of the perfusion with methoxamine (Heuzé-Joubert *et al.*, 1992).

**Part 3.** To assess the role of calcium-activated potassium channels involved in the EDHF-mediated vasodilator responses, a third series of experiments was performed in the presence of indomethacin and L-NA combined. The selective small-conductance ( $K_{\text{Ca}}2.3$ ) channel blocker, apamin (0.5  $\mu\text{mol}\cdot\text{L}^{-1}$ ) and the selective intermediate-conductance ( $K_{\text{Ca}}3.1$ ) channel blocker, TRAM-34 (0.5, 1 and 5  $\mu\text{mol}\cdot\text{L}^{-1}$ ) were perfused 30 min after the stabilization of the methoxamine-induced constriction. In some experiments, charybdotoxin (10 nmol·L<sup>-1</sup>), a non-selective blocker of  $K_{\text{Ca}}3.1$  channels was also studied. All channel nomenclature follows Alexander *et al.*, (2011).

### Data analysis

Perfusion pressure was measured in mmHg and changes in renal vascular pressure were expressed as the difference from basal perfusion pressure. Values are expressed as mean  $\pm$  SEM, and *n* indicates the number of animals from which the kidneys were taken. Vasodilator responses to each agonist

Table 1

Physiological and biochemical parameters in normotensive WKY, and SHR with or without treatment with L-NAME

	WKY (untreated)	SHR (untreated)	SHR (L-NAME)
SBP (mmHg)	123 ± 5 (11)	212 ± 10 (21)*	246 ± 6 (22)*\$
Heart rate (beats·min <sup>-1</sup> )	387 ± 9 (11)	380 ± 10 (21)	383 ± 8 (22)
Body weight (g)	391 ± 9 (30)	333 ± 7 (33)*	282 ± 8 (43)*\$
Kidney weight (g)	1.46 ± 0.03 (30)	1.32 ± 0.02 (33)*	1.10 ± 0.01 (43)*\$
Kidney/body weight (10 <sup>3</sup> )	3.63 ± 0.13 (30)	4.03 ± 0.09 (33)	4.16 ± 0.16 (43)*
Micro-albuminuria (mg·24 h <sup>-1</sup> )	<0.01 (9)	0.09 ± 0.01 (12)*	2.7 ± 0.68 (13)*\$
Urinary proteins (mg·24 h <sup>-1</sup> )	19.6 ± 2.6 (15)	11.7 ± 0.8 (15)*	91.1 ± 7.8 (13)*\$
Creatinine (mL·min <sup>-1</sup> ·kg <sup>-1</sup> )	0.61 ± 0.05 (8)	0.38 ± 0.02 (11)*	0.17 ± 0.02 (10)*\$
HbNO (arbitrary units)	2171 ± 365 (6)	1582 ± 255 (6)	399 ± 75 (7)*\$
Nitrite/nitrate (μmol·24 h <sup>-1</sup> )	4.02 ± 0.81 (10)	1.38 ± 0.22 (14)*	0.66 ± 0.18 (17)*

Values are shown as mean ± SEM with the number of animal from which kidneys were taken, shown in brackets.

\* $P < 0.05$  significantly different from WKY, \$ $P < 0.05$ , significantly different from untreated SHR; one-way ANOVA, followed by a Bonferroni *post hoc* test, three multiple comparisons.

SBP, systolic blood pressure; HbNO, iron nitroxyl-haemoglobin.

were measured at the peak of maximum change and were expressed as a percentage of the constriction induced by methoxamine, just before the injection of agonist. As NS-309 induced a long-lasting vasodilatation in kidneys from the SHR-L-NAME group, the area under each individual curve (AUC) expressed in arbitrary units was also used to compute the vasodilator responses induced by NS 309 alone or in the presence of potassium channel inhibitors. In order to evaluate significant differences between groups, two-sided one- and two-way ANOVA, followed by Bonferroni complementary tests, were performed. In all cases,  $P < 0.05$  was considered to be significant.

## Materials

Acetylcholine chloride, forskolin (from *Coleus forskohli*), indomethacin, L-NA, L-NAME, methoxamine hydrochloride, NS-309 (6-7-dichloro-1H-indole-2,3-dione 3-oxime), ODQ (1H-(1,2,4)-oxadiazolo(4,2-a)quinoxalin-1-one) and TRAM-34 (1-[(2-chlorophenyl) diphenylmethyl]-1H-pyrazole) were obtained from Sigma-Aldrich (St Quentin-Fallavier, France); sodium nitroprusside was purchased from Prolabo (France), apamin and charybdotoxin from Latoxan (Valence, France). Forskolin, indomethacin, NS-309, ODQ and TRAM-34 were prepared as stock solutions in DMSO and subsequently diluted in physiological salt solution. All other drugs were dissolved in deionized water.

## Results

### Physiological and biochemical parameters

The systolic blood pressure was significantly higher in SHR than in WKY and in L-NAME-treated SHR than in SHR (without changes in heart rate), whilst the body weight was significantly lower in SHR than in WKY, and in L-NAME-treated SHR than in SHR. Blood HbNO was significantly less

in L-NAME-treated SHR than in the two other groups, and urine levels of nitrite/nitrate were significantly less in SHR and L-NAME-treated SHR than in WKY (Table 1).

The weight of the kidneys was significantly lower in L-NAME-treated SHR than in the other groups and a profound renal dysfunction was observed, characterized by an increased micro-albuminuria and urinary protein excretion and a decreased creatinine clearance (Table 1). In SHR, the renal histological sections did not show any major structural alterations when compared with normotensive WKY of the same age, while, in L-NAME-treated SHR, major changes were observed, including tubulopathies and, in small to medium-size arteries, wall hyperplasia and fibrinoid necrosis (Figure S2).

### Basal perfusion pressure of isolated perfused kidneys

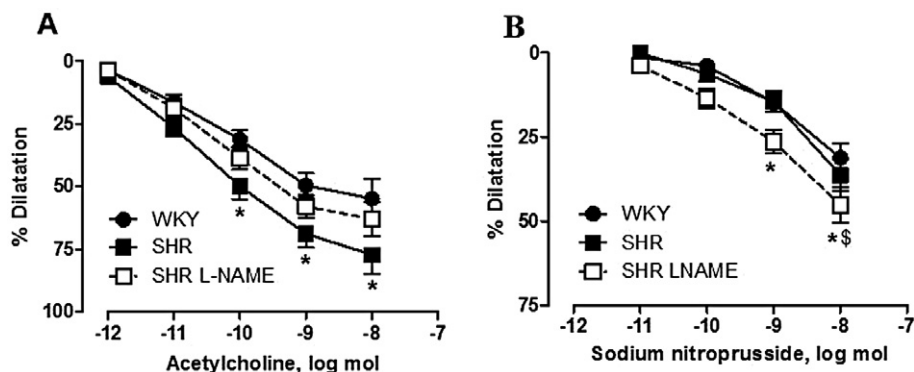
The basal perfusion pressures were comparable in WKY and SHR: 62 ± 2 ( $n = 19$ ) and 65 ± 5 mmHg ( $n = 11$ ), respectively, but significantly enhanced in those of L-NAME-treated SHR, 90 ± 6 mmHg ( $n = 19$ ) ( $P < 0.05$  vs. SHR, one-way ANOVA).

### Vasodilator responses

Infusion of methoxamine (2–5 μM), an α<sub>1</sub>-adrenoceptor agonist, increased the renal perfusion pressure in WKY, SHR and L-NAME-treated SHR to 144 ± 8 ( $n = 12$ ), 165 ± 8 ( $n = 20$ ) and 147 ± 6 mmHg ( $n = 33$ ) respectively. In all the groups, the increase in perfusion pressure in responses to methoxamine remained stable during the duration of the experiments. The level of perfusion achieved (basal pressure plus methoxamine-induced pressure) was similar in each group of rats.

In WKY kidneys, acetylcholine (1 pmol to 10 nmol) caused a dose-dependent dilatation with a maximal amplitude of 54.8 ± 7.7 mmHg ( $n = 9$ ). In SHR, the dilator responses to acetylcholine were slightly but significantly





**Figure 1**

Bolus injections of increasing doses of (A) acetylcholine and (B) sodium nitroprusside during vasoconstrictions caused by methoxamine in isolated perfused kidneys from WKY, SHR and SHR treated with L-NAME (SHR L-NAME). The results are shown as % dilatation of the increase in perfusion pressure induced by methoxamine, and values are expressed as means  $\pm$  SEM. ( $n = 9-10$ ). \* $P < 0.05$ , significantly different from the WKY group; \$ $P < 0.05$ , significant difference between the SHR and L-NAME-treated SHR groups; two-way ANOVA, followed by a Bonferroni *post hoc* test, 15 and 12 multiple comparisons for acetylcholine and sodium nitroprusside experiments, respectively.

increased when compared with those observed in WKY (Figure 1A). In L-NAME-treated SHR, the dilator responses to acetylcholine were similar to those observed in WKY and SHR (Figure 1A). In contrast, when compared with the two other groups, the dilatation induced by the endothelium-independent NO donor, sodium nitroprusside (10 pmol to 10 nmol) was significantly increased in the kidneys of L-NAME-treated SHR (Figure 1B). Forskolin (6 nmol), an adenylate cyclase activator, produced similar dilatation in the three groups [WKY:  $67.0 \pm 4.7\%$  ( $n = 6$ ); SHR:  $68.6 \pm 8.4\%$  ( $n = 8$ ); L-NAME-treated SHR:  $70.8 \pm 4.9\%$  ( $n = 8$ )].

### Acetylcholine-induced vasodilatation: NO and prostacyclin

Indomethacin ( $3 \mu\text{mol}\cdot\text{L}^{-1}$ ), an inhibitor of cyclooxygenases, did not significantly modify either the basal tone or the amplitude of the constriction induced by methoxamine (WKY:  $151 \pm 15$  mmHg, SHR:  $161 \pm 6$  mmHg,  $n = 7$ ; L-NAME-treated SHR:  $144 \pm 18$  mmHg,  $n = 6$ ). The presence of indomethacin did not affect the dilator responses to acetylcholine or sodium nitroprusside (Figure 2).

In the presence of indomethacin, the additional infusion of another NOS inhibitor, L-NA ( $100 \mu\text{mol}\cdot\text{L}^{-1}$ ), induced only a small increase in the basal perfusion pressure of WKY and L-NAME-treated SHR kidneys ( $7.5 \pm 1.5$  mmHg,  $n = 13$ , and  $10.7 \pm 1.5$  mmHg,  $n = 16$ , respectively), but a larger increase in that of SHR ( $33.7 \pm 7.1$  mmHg,  $n = 14$ ). In the presence of the combination of indomethacin plus L-NA, the perfusion pressure reached after methoxamine perfusion was  $136 \pm 10$  mmHg ( $n = 12$ ),  $171 \pm 10$  mmHg ( $n = 12$ ) and  $157 \pm 8$  mmHg ( $n = 22$ ) for WKY, SHR and L-NAME-treated SHR respectively ( $P = \text{NS}$ ).

L-NA produced a substantial inhibition of the dilatation to acetylcholine in WKY and SHR kidneys, but only a modest inhibition in those from L-NAME-treated SHR (Figure 2A). Thus, the response to acetylcholine at the highest dose studied of 10 nmol was significantly diminished by 59% and 61% in WKY and SHR kidneys, respectively; while in the

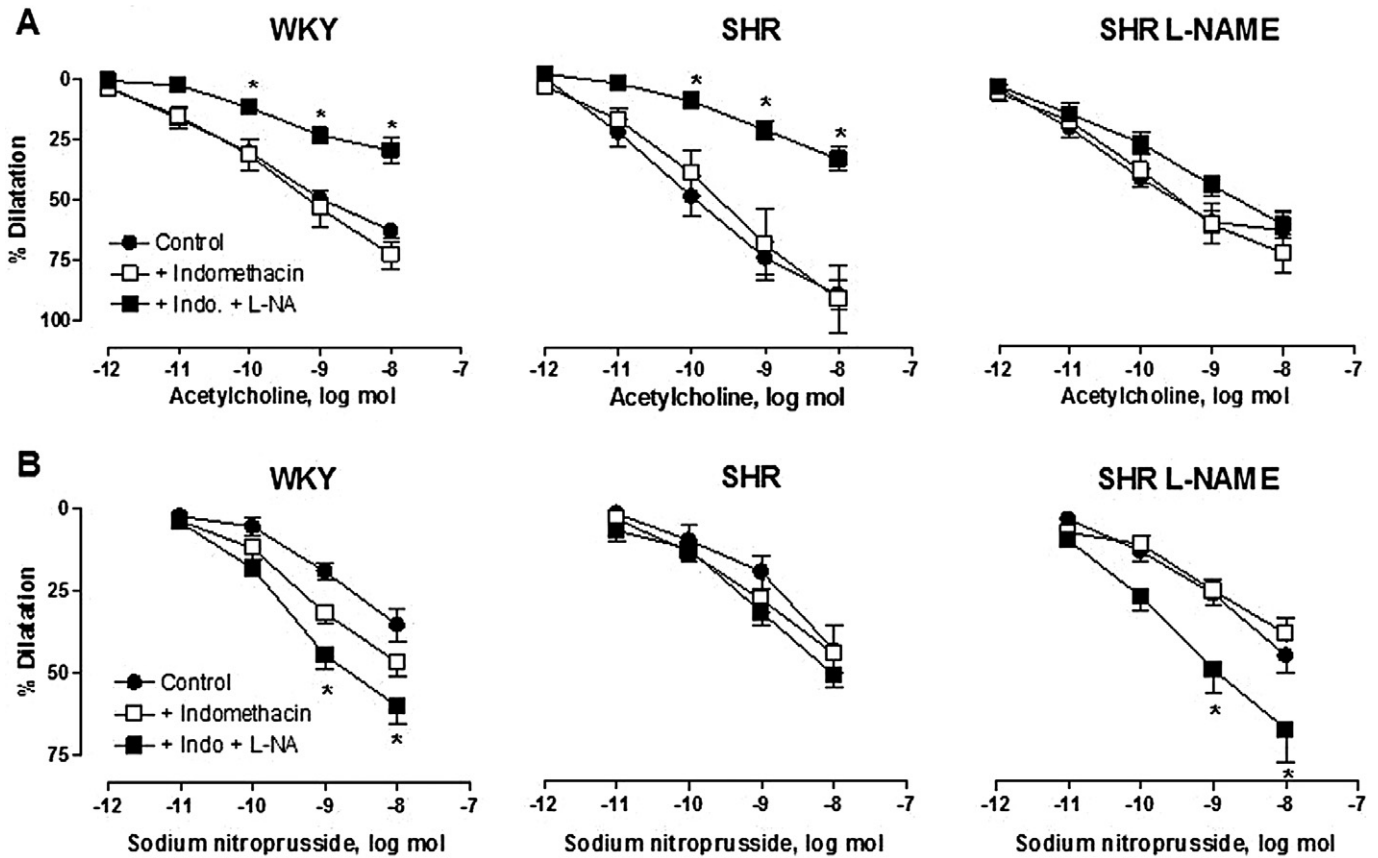
L-NAME-treated SHR, the response was only diminished by 16%. In contrast, the responses to sodium nitroprusside (10 nmol) were increased by 22% and 12% in WKY and SHR, respectively, and by 43% in the L-NAME-treated SHR groups (Figure 2B).

In L-NAME-treated SHR kidneys, in the presence of indomethacin plus L-NA, the dilator responses to acetylcholine were not impaired any further by the additional presence of the inhibitor of soluble guanylyl cyclase, ODQ ( $3 \mu\text{mol}\cdot\text{L}^{-1}$ ). In contrast, this concentration of ODQ abolished sodium nitroprusside-induced vasodilatation (Figure 3).

### Acetylcholine-induced vasodilatation: potassium channels

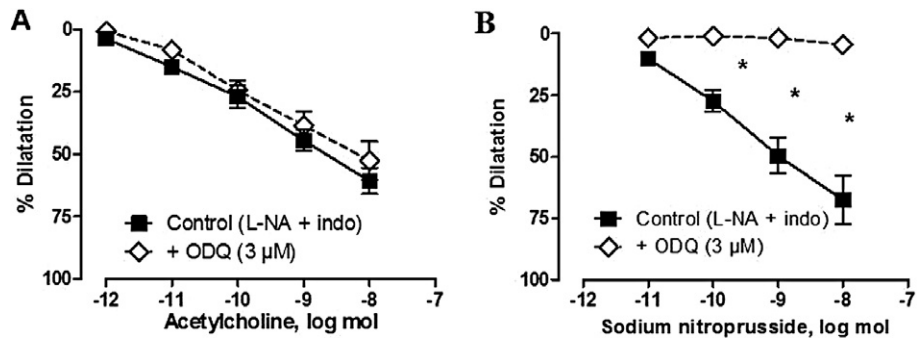
In the presence of indomethacin and L-NA, TRAM-34 (0.5 and  $1 \mu\text{M}$ ), a potent and selective inhibitor of  $\text{K}_{\text{Ca}3.1}$  channels did not affect the renal vasoconstriction to methoxamine. However, at the higher concentration of  $5 \mu\text{M}$ , it significantly reduced the constrictions to methoxamine in kidneys from the three groups of rats [WKY: from  $152 \pm 14$  to  $122 \pm 12$  mmHg ( $n = 6$ ); SHR: from  $192 \pm 3$  to  $159 \pm 25$  mmHg ( $n = 7$ ) and L-NAME-treated SHR: from  $168 \pm 7$  to  $123 \pm 11$  mmHg ( $n = 6$ )].

TRAM-34 (1 and  $5 \mu\text{M}$ ) inhibited the response to acetylcholine in both WKY and SHR kidneys (Figure 4) without altering those to sodium nitroprusside (10 nmol) and forskolin (6 nmol) (Table 2) In kidneys from L-NAME-treated SHR, TRAM-34 (0.5, 1 and  $5 \mu\text{mol}\cdot\text{L}^{-1}$ ) caused a significant and dose-dependent inhibition of the vasodilator responses to acetylcholine (Figure 4 and Table 2). Similarly, in L-NAME-treated SHR kidneys, charybdotoxin ( $10 \text{ nmol}\cdot\text{L}^{-1}$ ) [a non-selective inhibitor of  $\text{K}_{\text{Ca}3.1}$  channels and of large conductance calcium-activated potassium channels ( $\text{K}_{\text{Ca}1.1}$ ) and some voltage-activated potassium channels ( $\text{K}_{\text{v}}$ )], significantly inhibited the vasodilator responses to 10 nmol acetylcholine ( $59.6 \pm 4.1\%$ ,  $n = 9$  and  $27.1 \pm 7.2\%$ ,  $n = 3$ ; in control condition or in the presence of charybdotoxin, respectively;



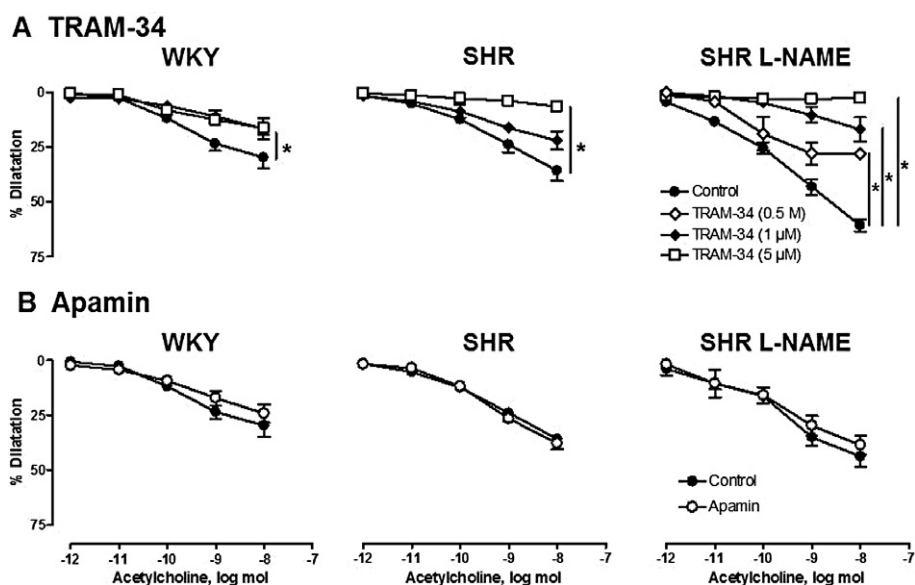
**Figure 2**

Effects of indomethacin (INDO) and L-NA on the dilatation due to acetylcholine (A) and sodium nitroprusside (B) of isolated perfused kidney from WKY, SHR and SHR treated with L-NAME (SHR L-NAME). The results are shown as % dilatation of the increase in perfusion pressure induced by methoxamine, and values are expressed as means  $\pm$  SEM. ( $n = 5-10$ ).  $*P < 0.05$ , significant difference between the indomethacin and the indomethacin + L-NA groups; two-way ANOVA followed by a Bonferroni *post hoc* test, 15 and 12 multiple comparisons for acetylcholine and sodium nitroprusside experiments respectively.



**Figure 3**

Effects of ODQ on the dilatation evoked by acetylcholine and sodium nitroprusside in isolated perfused kidneys from L-NAME-treated SHR (in the presence of L-NA plus indomethacin). The results are shown as % dilatation of the increase in perfusion pressure induced by methoxamine and values are expressed as mean  $\pm$  SEM ( $n = 5-8$ ).  $*P < 0.05$ , significantly different from control; two-way ANOVA, followed by a Bonferroni *post hoc* test, five and four multiple comparisons for acetylcholine and sodium nitroprusside experiments respectively.



**Figure 4**

Effects of TRAM-34 (0.5, 1 and 5  $\mu\text{mol}\cdot\text{L}^{-1}$ , top) and apamin (0.5  $\mu\text{mol}\cdot\text{L}^{-1}$ , bottom) on the dilatation due to acetylcholine in isolated perfused kidneys from WKY, SHR and L-NAME-treated SHR ( $n = 3-7$ ). The experiments were performed in the presence of indomethacin (3  $\mu\text{M}$ ) and L-NA (100  $\mu\text{M}$ ). The results are shown as % dilatation of the increase in perfusion pressure induced by methoxamine and values are expressed as means  $\pm$  SEM ( $n \geq 3$ ). \* $P < 0.05$ , significantly different from control; two-way ANOVA, followed by a Bonferroni *post hoc* test, 10–15 and 5 multiple comparisons for TRAM-34 and apamin experiments respectively.

**Table 2**

Maximal decreases in perfusion pressure caused by sodium nitroprusside (SNP: 10 nmol) and forskolin (6 nmol) in kidneys from WKY, SHR and L-NAME-treated SHR (in the presence of indomethacin and L-NA). Effects of the potassium channel blockers: TRAM-34, apamin and charybdotoxin

		WKY (untreated)	SHR (untreated)	SHR (L-NAME)
Control	SNP	60.3 $\pm$ 5.4 (5)	51.0 $\pm$ 3.7 (6)	64.6 $\pm$ 4.4 (14)
	Forskolin	62.1 $\pm$ 6.9 (6)	57.3 $\pm$ 3.2 (7)	67.0 $\pm$ 4.6 (14)
TRAM-34 (0.5 $\mu\text{mol}\cdot\text{L}^{-1}$ )	SNP	–	–	53.2 $\pm$ 5.7 (5)
	Forskolin	–	–	56.3 $\pm$ 4.7 (5)
TRAM-34 (1 $\mu\text{mol}\cdot\text{L}^{-1}$ )	SNP	64.9 $\pm$ 5.3 (4)	42.9 $\pm$ 1.5 (3)	58.6 $\pm$ 6.3 (5)
	Forskolin	45.3 $\pm$ 6.4 (5)	49.2 $\pm$ 5.2 (3)	58.9 $\pm$ 7.0 (5)
TRAM-34 (5 $\mu\text{mol}\cdot\text{L}^{-1}$ )	SNP	66.8 $\pm$ 4.6 (5)	58.4 $\pm$ 4.7 (5)	46.3 $\pm$ 5.3 (5)
	Forskolin	65.9 $\pm$ 7.7 (6)	55.3 $\pm$ 6.7 (5)	54.0 $\pm$ 2.9 (5)
Apamin (0.5 $\mu\text{mol}\cdot\text{L}^{-1}$ )	SNP	46.6 $\pm$ 9.1 (3)	48.4 $\pm$ 4.3 (3)	52.1 $\pm$ 6.8 (4)
	Forskolin	55.5 $\pm$ 6.9 (3)	59.4 $\pm$ 3.8 (3)	52.4 $\pm$ 4.5 (6)
Apamin (0.5 $\mu\text{mol}\cdot\text{L}^{-1}$ ) + TRAM-34 (5 $\mu\text{mol}\cdot\text{L}^{-1}$ )	SNP	–	–	43.4 $\pm$ 7.8 (5)
	Forskolin	–	–	71.8 $\pm$ 9.4 (5)
Charybdotoxin (10 nmol $\cdot\text{L}^{-1}$ )	SNP	–	–	65.5 $\pm$ 9.8 (3)
	Forskolin	–	–	68.2 $\pm$ 9.8 (3)

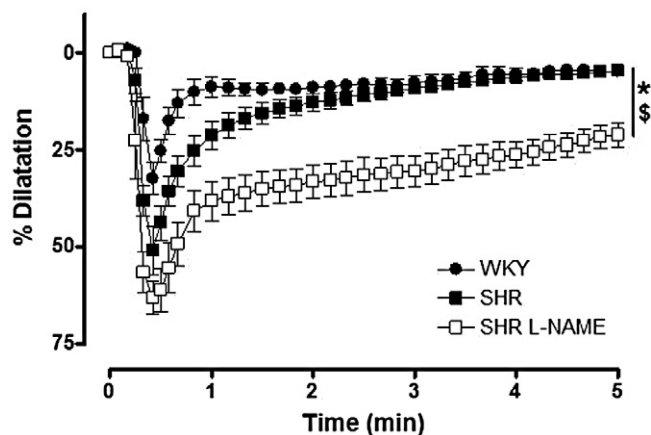
Values are expressed in mmHg and shown as mean  $\pm$  SEM with the number of animal from which kidneys were taken, shown in brackets.

$P < 0.05$ ), without altering the vasodilatation to sodium nitroprusside or forskolin (Table 2).

In contrast, the perfusion of apamin ( $0.5 \mu\text{mol}\cdot\text{L}^{-1}$ ), a selective inhibitor of  $K_{Ca}2.3$  channels did not modify the renal vasoconstriction to methoxamine, did not affect the vasodilator responses to acetylcholine, sodium nitroprusside or forskolin and did not influence the inhibitory effect of TRAM-34 (Figure 4, Table 2).

### NS-309-induced vasodilatation

In the presence of indomethacin and L-NA, and in the kidneys of the three groups of rats, the bolus administration of NS-309, a mixed activator of  $K_{Ca}3.1$  and  $K_{Ca}2.3$  channels ( $10 \text{ nmol}$ ), caused vasodilatation. The amplitude and the



**Figure 5**

Bolus injection of NS 309 ( $10 \text{ nmol}$ ) in isolated perfused kidneys from normotensive WKY, SHR and L-NAME-treated SHR ( $n = 5-7$ ). The experiments were performed in the presence of indomethacin plus L-NA. The results are shown as % dilatation of the increase in perfusion pressure induced by methoxamine and values are expressed as mean  $\pm$  SEM. \* $P < 0.05$ , AUC significantly different from control; \$ $P < 0.05$ , significant difference between SHR and L-NAME-treated SHR; one-way ANOVA, followed by a Bonferroni *post hoc* test, three multiple comparisons.

**Table 3**

Effects of TRAM-34 and apamin on NS-309 ( $10 \text{ nmol}$ )-induced vasodilatation (in the presence of indomethacin + L-NA)

	WKY (untreated)	SHR (untreated)	SHR (L-NAME)
Control	40.9 $\pm$ 7.6 (5)	65.0 $\pm$ 7.5 (7)	161.2 $\pm$ 14.7 (7)
TRAM-34 ( $0.5 \mu\text{mol}\cdot\text{L}^{-1}$ )			96.4 $\pm$ 15.4 (5)*
TRAM-34 ( $1 \mu\text{mol}\cdot\text{L}^{-1}$ )	43.8 $\pm$ 13.8 (5)	41.7 $\pm$ 8.5 (3)	48.6 $\pm$ 10.9 (3)*
TRAM-34 ( $5 \mu\text{mol}\cdot\text{L}^{-1}$ )	48.1 $\pm$ 11.9 (5)	18.4 $\pm$ 5.2 (5)*	38.6 $\pm$ 6.1 (3)*
Apamin ( $0.5 \mu\text{mol}\cdot\text{L}^{-1}$ )	24.3 $\pm$ 11.4 (2)	67.6 $\pm$ 5.6 (3)	164.4 $\pm$ 29.1 (6)
Apamin + TRAM-34 ( $5 \mu\text{mol}\cdot\text{L}^{-1}$ )			42.5 $\pm$ 8.3 (6)*

Values are expressed as AUC (arbitrary units) and are shown as mean  $\pm$  SEM with the number of animal from which kidneys were taken in brackets.

\* $P < 0.05$ , significantly different from control; one-way ANOVA.

time course of these responses were larger in SHR than in WKY and larger in L-NAME-treated SHR than in SHR (Figure 5 and Table 3).

TRAM-34 ( $1$  and  $5 \mu\text{M}$ ) did not affect the vasodilator responses to NS 309 in WKY kidneys but produced a significant concentration-dependent but partial inhibition in kidneys from both SHR and SHR L-NAME (Figure 6). Thus, in the presence of TRAM-34 ( $5 \mu\text{M}$ ), the residual responses to NS 309 observed in SHR and L-NAME-treated SHR were similar to the response observed in WKY kidneys. In the latter group, charybdotoxin ( $10 \text{ nmol}\cdot\text{L}^{-1}$ ) also significantly inhibited the vasodilator responses to NS-309 ( $63.5 \pm 4.1\%$ ,  $n = 7$  and  $30.9 \pm 7.1\%$ ,  $n = 3$ ; in control and charybdotoxin-treated kidneys,  $P < 0.05$ ). In contrast, in isolated kidneys from the three groups of rats, the presence of apamin ( $0.5 \mu\text{M}$ ), did not affect the vasodilatation to NS 309 (Figure 6). In addition, the combination of apamin ( $0.5 \mu\text{M}$ ) plus TRAM-34 ( $5 \mu\text{M}$ ) was no more effective in inhibiting NS 309-induced vasodilatation than the presence of TRAM-34 alone (Table 3).

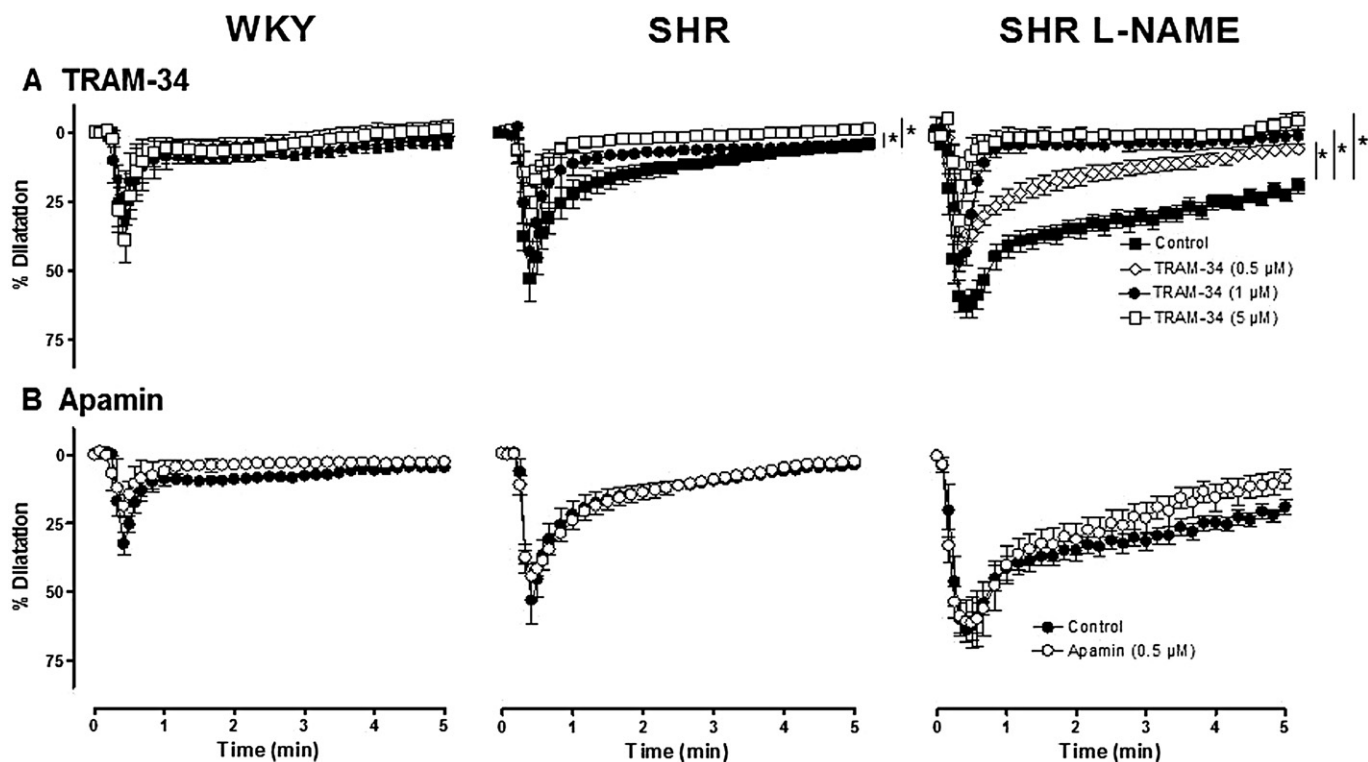
### $K_{Ca}2.3$ and $K_{Ca}3.1$ mRNA expression

The expression of mRNA for  $K_{Ca}2.3$  protein was not significantly different in kidneys of WKY, SHR and L-NAME-treated SHR. However, that for  $K_{Ca}3.1$  protein was significantly enhanced in L-NAME-treated SHR kidneys (Figure 7). In contrast, in aorta of the three groups of rats, the mRNA expression for these two potassium channel proteins was not significantly different (data not shown).

## Discussion

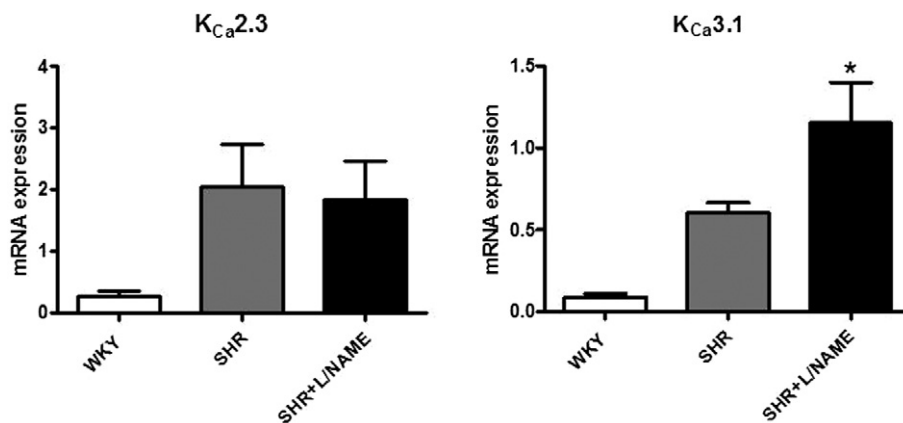
The present study confirmed that chronic administration of a moderate dose of L-NAME over a 2 week period in 4-month-old SHR induced severe hypertension and pronounced renal injury, characterised by increased proteinuria and reduced creatinine clearance. Interestingly, in L-NAME-treated SHR, the marked structural alterations in the renal microvasculature were associated with only minor changes in acetylcholine-induced renal vasodilatation, most likely due to the development of a compensatory mechanism involving the activation of the intermediate-conductance calcium-activated potassium channels ( $K_{Ca}3.1$ ).





**Figure 6**

Effects of TRAM-34 (0.5, 1 and 5  $\mu\text{M}$ ) on the dilatation due to NS 309 (10 nmol) in isolated perfused kidneys from WKY, SHR and L-NAME-treated SHR ( $n = 3-7$ ). The experiments were performed in the presence of indomethacin plus L-NA. The results are shown as % dilatation of the increase in perfusion pressure induced by methoxamine, and values are expressed as mean  $\pm$  SEM. \* $P < 0.05$ , AUC significantly different from control; one-way ANOVA followed by a Bonferroni *post hoc* test, 2-3 and 1 multiple comparisons for TRAM-34 and apamin experiments respectively.



**Figure 7**

Quantitative RT-PCR for mRNA for  $K_{Ca2.3}$  and  $K_{Ca3.1}$  protein in kidneys of WKY, SHR and L-NAME-treated SHR ( $n = 8-13$ ). The values are normalized to the geometric mean of the values obtained with three internal controls: HPRT,  $\beta$ -actin and GAPDH. Values are expressed as mean  $\pm$  SEM. \* $P < 0.05$ , significant difference between L-NAME-treated SHR and WKY kidneys; one-way ANOVA-1 followed by a Bonferroni *post hoc* test, three multiple comparisons.

NO is a major vasodilator and a regulator of renal haemodynamics and blood pressure (Baylis and Qiu, 1996). The dose of L-NAME chosen in this study ( $6 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ) was moderate and did not produce any significant alterations in

the normotensive WKY strain. In this strain, the L-NAME doses producing significant changes in blood pressure are above  $10 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  and, in order to induce substantial long-lasting changes in blood pressure, a dose of

100 mg·kg<sup>-1</sup>·day<sup>-1</sup> is routinely used (Arnal *et al.*, 1992). In contrast to the WKY, in the SHR, this same low dose of NOS inhibitor was sufficient to produce a substantial further increase in systolic blood pressure (Ono *et al.*, 1995; Benter *et al.*, 2005; Hilgers and Webb, 2007; Vayssettes-Courchay *et al.*, 2011; Isabelle *et al.*, 2012). In control SHR, the circulating iron-nitrosyl-haemoglobin, an index of blood NO levels, and the concentration of urinary nitrite/nitrate were lower than in corresponding WKY, confirming an overall reduction of NO bioavailability in this genetic model of hypertension (Benter *et al.*, 2005). These two indices of NO generation were further reduced in L-NAME-treated SHR, confirming NOS inhibition and a further reduction in NO bioavailability. Additionally, in the L-NAME-treated SHR, the inhibition of NO generation was associated with profound renal injuries as shown by structural alterations in renal arteries of small and medium size as well as tubular injuries. These alterations are similar to those observed in ageing SHR (Ono *et al.*, 1995; 2001; Hilgers and Webb, 2007; Zhou and Frohlich, 2007) and are comparable to those attributed to a reduced NO availability in human cardiovascular pathologies (Taddei *et al.*, 1993; Panza *et al.*, 1995; Virdis *et al.*, 2010).

Hypertension is associated with endothelial dysfunction, which is generally characterised by impaired endothelial dilator responses, observed both *in vivo* and *in vitro*, either in isolated arteries or in perfused organs. However, these endothelial dysfunctions present different characteristics depending on the vascular bed, the animal model used and/or the age of the animals (see Félétou and Vanhoutte, 2006a). In the aorta or in the mesenteric vascular bed of SHR, the impaired endothelium-dependent dilatation is associated with the generation of endothelium-derived and cyclooxygenase-derived contracting factors with no or little alteration in the production of NO (Lüscher *et al.*, 1990; Fujii *et al.*, 1992; Mantelli *et al.*, 1995; Hutri-Kahonen *et al.*, 1997; Félétou *et al.*, 2009) whereas, in the carotid artery, the attenuated endothelium-dependent relaxations to acetylcholine are probably related to a smooth muscle dysfunction in the responsiveness to NO, with no involvement of endothelium-derived contracting factors (Hongo *et al.*, 1988; Lüscher *et al.*, 1988). In perfused kidneys of young SHR, when compared with those of normotensive WKY rats, the endothelium-dependent dilatation to acetylcholine or to bradykinin is minimally inhibited (Hayakawa *et al.*, 1993), unaffected (Burton *et al.*, 1991) or even enhanced (Cachofeiro and Nasjletti, 1991; Chamorro *et al.*, 2004; Simonet *et al.*, 2009). In the present study, we observed a significant enhancement of acetylcholine-induced decrease in perfusion pressure, which could not be attributed to any change in smooth muscle sensitivity towards NO, as the endothelium-independent responses to the NO donor, sodium nitroprusside were unaffected.

Chronic L-NAME treatment also inhibited the renal NOS. In the kidneys of L-NAME-treated SHR, the vasodilator response to sodium nitroprusside, a donor of NO, was significantly enhanced, a result which is in agreement with previous reports showing that the inhibition of NOS increases the sensitivity of soluble guanylyl cyclase to NO (Moncada *et al.*, 1991; Fulton *et al.*, 1992; Desai *et al.*, 2006). Furthermore, both basal and acetylcholine-induced NO release were inhibited. Indeed, in isolated kidneys of L-NAME-treated SHR, the *in vitro* administration of a differ-

ent NOS inhibitor, L-NA, produced a reduced intrinsic vasoconstriction and induced virtually no inhibition of acetylcholine-induced vasodilatation.

In WKY and SHR kidneys, the endothelium-dependent vasodilatation to acetylcholine involves NO because the acute administration of L-NA produced a significant but partial inhibition of the responses. Indomethacin, an inhibitor of cyclooxygenase, was without effect, ruling out prostaglandins, and especially prostacyclin, as a component of this mechanism. In many peripheral arteries, EDHF-mediated responses are associated with the opening of endothelial calcium-activated potassium channels of small and/or intermediate conductance ( $K_{Ca}2.3$  and  $K_{Ca}3.1$ ; Eichler *et al.*, 2003; Félétou and Vanhoutte, 2006b; Grgic *et al.*, 2009a). Apamin, a toxin from bee venom, is a very selective inhibitor of  $K_{Ca}2.3$  channels while charybdotoxin, a scorpion toxin, is a non-selective inhibitor of  $K_{Ca}3.1$ ,  $K_{Ca}1.1$  and some  $K_V$  channels. The clotrimazole derivative TRAM-34 is a selective  $K_{Ca}3.1$  channel blocker with more than a 1000-fold selectivity ratio compared with  $K_{Ca}2.3$  and  $K_{Ca}1.1$  channels (Castle, 1999; Wulff *et al.*, 2000). In isolated kidneys from WKY, the COX-NOS-independent relaxations to acetylcholine were unaffected by the additional presence of apamin but were virtually abolished by TRAM-34 alone. These data are in agreement with earlier reports using kidneys from normotensive rats and showing that EDHF-mediated responses were attenuated by charybdotoxin, whilst apamin had no effect (Fulton *et al.*, 1992; Rapacon *et al.*, 1996; Mieyal *et al.*, 1998). Moreover, our data are in accord with results recently obtained *in vivo*, in an elegant study involving renal haemodynamics in anaesthetized rats (Edgley *et al.*, 2008). Thus, in isolated and perfused rat kidneys, the EDHF-mediated responses elicited by acetylcholine involve predominantly the activation of calcium-activated potassium channels and especially  $K_{Ca}3.1$  channels. Furthermore, the TRAM-34-sensitive response was greater in kidneys from SHR than in WKY, indicating an up-regulation of this pathway in the kidneys of the hypertensive strain.

In L-NAME-treated SHR kidneys, the vasodilatation to acetylcholine was virtually unaffected. Again, indomethacin did not modify the responses to acetylcholine, ruling out prostacyclin as a component of this vasodilatation. Moreover, in the presence of the cyclooxygenase inhibitor and after acute administration of L-NA, no further inhibition of the responses to acetylcholine was observed. Furthermore, the addition of both L-NA and the selective inhibitor of soluble guanylyl cyclase, ODQ, used at a concentration that totally blocked the vasodilator responses to sodium nitroprusside, did not affect the vasodilatation to acetylcholine. Thus, a large component of this response is NO- and prostacyclin-independent and therefore could be attributed to a compensatory EDHF-like mechanism. These data are in agreement with previous results in normotensive rats, showing that, after chronic, but not acute treatment, with L-NAME, a proper hypotensive response to acetylcholine is restored and that compensatory mechanism(s) develop in resistant vascular beds, including the mesenteric (Ruiz-Marcos *et al.*, 2001; Hilgers *et al.*, 2010) and the renal circulatory beds (Bryant *et al.*, 1995; Vargas *et al.*, 1996; Maeso *et al.*, 1999; Strøbæk *et al.*, 2004; Desai *et al.*, 2006). Very similar observations have been reported in mice with genetic deletion of eNOS (Brandes *et al.*, 2000; Ding *et al.*, 2000; Huang *et al.*, 2000) or in patients with essential hyper-

tension or primary hyperparathyroidism (Taddei *et al.*, 2006; Sainsbury *et al.*, 2007; Viridis *et al.*, 2010). However, the mechanisms underlying the compensatory effect of EDHF-mediated responses, when the endothelial production of NO is impaired, have not been fully characterized.

In L-NAME-treated SHR, the EDHF-mediated response is significantly and markedly enhanced when compared with control WKY or SHR. The predominant role of  $K_{Ca}3.1$  has been demonstrated by its sensitivity to TRAM-34 and further confirmed by the inhibition elicited by charybdotoxin. In contrast to TRAM-34, this toxin did not totally abolish the vasodilator effect of acetylcholine, possibly because TRAM-34 can also block non-selective cationic channels involved in calcium homeostasis (Schilling and Eder, 2007), which could be associated to EDHF-mediated responses (Félétou and Vanhoutte, 2006b).

NS-309 has been described as a potent opener of both  $K_{Ca}3.1$  and  $K_{Ca}2.3$  channels, with a preferential selectivity for the former (Strøbæk *et al.*, 2004; Leuranguer *et al.*, 2008). In kidneys from WKY rats, NS 309 produced a transient vasodilatation that was not affected by the presence of either TRAM-34 or apamin. This vasodilatation in WKY and, in the kidneys of SHR and L-NAME-treated SHR, the residual vasodilatation to NS-309, which was insensitive to blockers of  $K_{Ca}3.1$  channels, may involve a non-selective effect of NS 309, the inhibition of L-type voltage-operated calcium channels (Morimura *et al.*, 2006). Nevertheless, in kidneys of SHR and L-NAME-treated SHR, NS-309 induced dilatation inhibited by either TRAM-34 or charybdotoxin but not by apamin alone, indicating the predominant activation of  $K_{Ca}3.1$  channels. In L-NAME-treated SHR, the amplitude of the dilator responses to NS-309 was increased and the duration was markedly prolonged. Because the combination of TRAM-34 plus apamin was not more effective than TRAM-34 alone, the involvement of  $K_{Ca}2.3$  channels in this dilator response could again be excluded. Again, the TRAM-34-sensitive response was larger in SHR than in WKY kidneys and, significantly and markedly enhanced in those from L-NAME-treated SHR, compared with kidneys from control SHR. Altogether, these findings suggest that an enhanced contribution of  $K_{Ca}3.1$  channel activation compensated for the decrease in NO bioavailability. In SHR kidneys, a minor up-regulation of the TRAM-34-sensitive response (to both acetylcholine and NS 309) was associated with a moderate decrease in NO bioavailability, whereas in kidneys from L-NAME-treated SHR, a pronounced up-regulation of the TRAM-34-sensitive response was associated with a major decrease in NO bio-availability. Interestingly, in the kidneys of these rats, these functional responses were accompanied by similar changes in expression of the mRNA for  $K_{Ca}3.1$ , but not in that of  $K_{Ca}2.3$  channels (which do not contribute to the vasodilator response). Furthermore, this up-regulation of  $K_{Ca}3.1$  channels was observed in the kidney but not in the aorta, a blood vessel that does not exhibit EDHF-mediated responses (Félétou and Vanhoutte, 2006b). Unfortunately, the present quantitative RT-PCR experiments performed on the whole kidney does not allow us to determine whether or not the  $K_{Ca}3.1$  channel overexpression involves endothelial and/or other cell subtypes, for instance renal fibroblasts (Grgic *et al.*, 2009b).

In various pathological rat models of hypertension (SHR, SHR-SP and angiotensin-II infused) and type-II diabetes

(Zucker and cafeteria diet-induced obesity), a maintenance or an up-regulation of the activity of  $K_{Ca}3.1$  channels, in some instance associated with an increased expression of  $K_{Ca}3.1$  protein has been reported; whilst the reverse was observed for the  $K_{Ca}2.3$  channel (Burnham *et al.*, 2006; Hilgers and Webb, 2007; Giachini *et al.*, 2009; Chadha *et al.*, 2010; Weston *et al.*, 2010), suggesting that  $K_{Ca}3.1$  channels are the last line of defence to maintain endothelium-dependent vasodilatation. Expression of  $K_{Ca}3.1$  protein is negatively regulated by the repressor element-1 silencing transcription factor (REST) (Cheong *et al.*, 2005; Tharp *et al.*, 2008). In SHR-SP rats, it has been suggested that up-regulation of  $K_{Ca}3.1$  channels via a decreased expression of REST, compensates for the decreased NO bioavailability (Giachini *et al.*, 2009). This regulating mechanism may occur in other models including the renal resistance vessels of L-NAME-treated SHR rats studied in the present work.

The physiopathological role of EDHF-mediated responses in the kidney is basically unknown. In rats with low NO activity, EDHF-mediated responses might compensate for the NO deficiency and act as a dilator system in attenuating endothelial dysfunction. However, the EDHF contribution, in contrast to the NO contribution, is positively related to the development of proteinuria (Gschwend *et al.*, 2002), suggesting that, although EDHF-mediated responses seem to attenuate endothelial dysfunction in the kidney, they could be involved in the detrimental mechanisms associated with these renal alterations. Indeed, the  $K_{Ca}3.1$  channel can also play a pivotal role in promoting mitogenesis, not only in endothelial and vascular smooth muscle cells (Köhler *et al.*, 2003; Grgic *et al.*, 2005), but also in renal fibroblasts (Grgic *et al.*, 2009b). In a model of balloon catheter injury and in a model of renal fibrosis, TRAM-34 treatment prevented restenosis in the former and reduced the chronic tubulointerstitial damage in the latter (Köhler *et al.*, 2003; Grgic *et al.*, 2009b).

## Conclusions and perspectives

In conclusion, this study confirmed that, in SHR, chronic L-NAME treatment increased blood pressure and markedly altered renal structure and function. In these animals, the renal COX-NOS-independent vasodilatation in response to either acetylcholine or to NS-309, a preferential  $K_{Ca}3.1$  channel opener, was increased and was associated with an enhanced expression of mRNA for  $K_{Ca}3.1$  protein, suggesting that these EDHF-mediated responses involve an up-regulation of the  $K_{Ca}3.1$  channels. The pathophysiological significance of the compensatory contribution of  $K_{Ca}3.1$  in the context of renal failure remains to be clarified. This would require a chronic *in vivo* study. However, NS 309 remains a poorly selective tool that, besides activating both  $K_{Ca}3.1$  and  $K_{Ca}2.3$  channels, interacts with other targets for instance, hERG ( $K_V11.1$ ) and L-type  $Ca_v$  channels and adenosine  $A_{2A}$  receptors. Furthermore, the pharmacokinetic properties of this compound are not compatible with chronic *in vivo* administration (Félétou *et al.*, 2011). Performing experiments with the more selective and recently described compound SKA-31 (not commercially available when our studies were initiated) would be more appropriate, although this com-

pound is not orally active (Sankaranarayanan *et al.*, 2009; Damkjaer *et al.*, 2012). Such experiments would answer this fundamental question: 'Does the compensating renal vasodilator response by an EDHF mechanism influence kidney function or delay functional and/or structural alterations in the kidneys?'

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## Conflict of interests

All the authors were employees of Servier at the time of the experiments described here. None of the authors has a conflict of interest with the material presented in the submitted manuscript.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Noradrenaline-induced increases in perfusion pressure in rat isolated and perfused kidneys. The maximal perfusion pressure achieved (basal tone + the increase in perfusion pressure produced by the highest dose of noradrenaline tested) is not significantly different. However, kidneys from L-NAME-treated SHR were significantly more sensitive to the  $\alpha$ -adrenoceptor agonist than kidneys from other groups of rats. pD<sub>2</sub> values are shown in the inset. \**P* < 0.05, significant difference between the L-NAME-treated SHR and the other groups; one-way ANOVA, followed by a Bonferroni *post hoc* test, six multiple comparisons.

**Figure S2** Renal histology in WKY, L-NAME-treated WKY, SHR and L-NAME-treated SHR. Black arrows in the section from kidneys of L-NAME-treated SHR show tubulopathy, arterial wall hyperplasia, fibrinoid necrosis and red blood cell infiltration. A, arteriole; G, glomerulus; T, tubule. A 100  $\mu$ m scale is shown on each section.

**Table S1** RT-qPCR: Primer sequences and annealing temperature

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