*Original Article*

# Prehypertensive Renin-Angiotensin-Aldosterone System Blockade in Spontaneously Hypertensive Rats Ameliorates the Loss of Long-Term Vascular Function

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**Arterial function after long-term hypertension is characterized by remodeling, endothelial dysfunction and reduction of previously enhanced contractile responses. We investigated whether transient prehypertensive renin-angiotensin-aldosterone system (RAAS) blockade modifies long-term arterial function. Wistar Kyoto rats (WKY) (i) and spontaneously hypertensive rats (SHR) (ii) were prehypertensively (week 4–8) treated with losartan (iii) or spironolactone (iv) (20 and 0.5 mg/kg/day, respectively) and investigated at 8 and 72 weeks of age. Systolic blood pressure (SBP) was measured intra-arterially. In isolated mesenteric arteries, active wall stress (AWS), relaxation in response to acetylcholine and wall-to-lumen ratio (*W/L*) were assessed. Western blotting and immunofluorescent staining of whole-mount arterial preparations and two photon laser scanning microscopy (TPLSM) were performed to quantify endothelial nitric oxide synthase (eNOS) and analyze its intracellular distribution. In 8-week-old SHR treatments were found to have reduced SBP. Relaxation, contractile responses and vascular morphology remained unaffected irrespective of treatment. At 72 weeks, SBP was similar in all SHR groups ((i) 129±6, (ii) 222±10, (iii) 210±16, (iv) 214±9 mmHg). Relaxation and maximum AWS were enhanced after treatments. *W/L* demonstrated hypertrophy ((i) 0.10±0.01, (ii) 0.16±0.02, (iii) 0.15±0.01, (iv) 0.17±0.01). Untreated SHR ($p<0.01$), SHR treated with losartan and SHR treated with spironolactone ($p<0.05$) showed less eNOS as compared to WKY. In treated SHR eNOS was concentrated in a perinuclear endothelial cell compartment. In conclusion, these findings demonstrate that transient prehypertensive blockade results in a long-lasting and blood pressure independent improvement of arterial contractility and endothelium-dependent vasodilatation that persists in aging SHR. This might be associated with an intracellular redistribution of eNOS in the endothelial cell layer. (*Hypertens Res* 2007; 30: 853–861)**

***Key Words*: prehypertensive treatment, vascular structure, endothelial function, endothelial nitric oxide synthase**

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## Introduction

It is well established that transient prehypertensive treatment of young spontaneously hypertensive rats (SHR) with various antihypertensive agents' results in prolonged reduction of mean arterial pressure (MAP). The reduction persists into adulthood after the withdrawal of treatment. This has in particular been investigated for agents that suppress components of the renin-angiotensin-aldosterone system (RAAS) (*1–4*). The factors responsible for altering the blood pressure development of SHR remain unknown. However, several authors have suggested that these drugs have long-lasting influences on the feedback regulation of the RAAS (*4*, *5*).

The mechanisms are a matter of controversy. It is generally believed that an increased responsiveness to vasoconstrictor stimuli and alterations of arterial structure are involved. Intraluminal pressure and constrictor stimuli are persistently increased in SHR. These effects in turn induce and maintain arterial remodelling and prolonged vasoconstriction (*1*). The components of the RAAS contribute to these contractile stimuli which show lifelong activity (*6*). Angiotensin II and aldosterone, the active components of the RAAS, in turn further complicate hypertension and cardiovascular pathologies by enhancing blood pressure and cardiovascular end-organ damage (*7–9*).

Endothelial dysfunction can be considered one of the long-term consequences of hypertension along with arterial remodelling and increased contractility (*6*, *10*). Pharmacological interventions targeting RAAS improve the endothelium-dependent relaxant responses in aorta and mesenteric arteries of SHR. This has been shown for an angiotensin-converting enzyme (ACE)-inhibitor, angiotensin II type 1 (AT1) receptor-antagonist and an aldosterone antagonist (*8*, *11*). These agents have also been shown to reduce blood pressure and end-organ damage (*11–13*).

Effects of transient prehypertensive treatment in this context are largely unknown. The blood pressure reduction induced in SHR by RAAS blockade is known to persist into adulthood (36 weeks of age). This is associated with cardiac and renal protection (*3*, *5*) while arterial hypertrophy remains unaffected (*10*). However, there are no data on the effects of transient prehypertensive treatment on vascular reactivity. In addition, there are no data on the long-term effects of this treatment after restoration of hypertension at an even older age (*e.g.*, 72 weeks of age). Therefore it remains unclear whether transient prehypertensive treatment has effects on arterial structure and reactivity and to what extent they are related. Theoretically, arterial effects could be 1) related to the direct antihypertensive action during prehypertensive treatment, 2) related to the prolonged antihypertensive action, or 3) blood pressure independent. The latter would imply that the effects of RAAS modulation during the early stages of life would have consequences for long-term mechanisms such as aging.

The purpose of the present study was to investigate: 1) whether prehypertensive RAAS blockade has blood pressure related or unrelated effects on arterial morphology or reactivity that persist up to advanced age and 2) whether effects differ between AT1 receptor blocker and aldosterone antagonism in SHR.

## Methods

### Experimental Set-Up

Male Wistar Kyoto rats (WKY) ($n=9$) and SHR ($n=45$) were obtained from Charles River (Maastricht, The Netherlands). The rats were fed a normal sodium diet and had free access to water. All experiments were approved by the Animal Ethics Committee of Maastricht University and were performed in accordance with institutional guidelines.

At 4 weeks of age, rats were randomly assigned to either the treatment (SHR-Los: losartan 20 mg/kg per day; SHR-Spiro: spironolactone 0.5 mg/kg per day; $n=15$ each group) or the control groups (SHR [$n=15$] or WKY [$n=9$]). Treatments were performed continuously from week 4 to 8 of age using subcutaneously implanted minipumps. At 8 weeks systolic blood pressure (SBP) was measured in SHR ($n=6$ per group), using an intra-arterial catheter under pentobarbital anaesthesia. Then, two first-order mesenteric artery branches were removed from each rat and subjected to *in vitro* investigation. The remaining rats ($n=9$ per group) were housed under controlled conditions of temperature (21°C) and light (12-h light/dark cycle, 7 AM to 7 PM) and maintained to 72 weeks of age. In aged rats blood pressure was measured and small mesenteric arteries were harvested using similar techniques as in 8-week-old rats.

### Arterial Reactivity

Two first order mesenteric artery segments were analyzed for each experimental animal. The segments were mounted using steel wires (diameter: 40 μm) in myograph organ bath (model 610M Danish Myotechnology by J.P. Trading, Denmark) for isometric tension measurements. Organ baths were filled with a Krebs-Ringer bicarbonate buffer, maintained at 37°C and aerated with 95% $O_2$ and 5% $CO_2$. The arterial segments were stretched until maximal contractile responses to 40 mmol/L potassium solution ($K^+$) were obtained; this was considered the optimal diameter for the remainder of the study. Contractile responses were further obtained with noradrenaline (NA, $10^{-8}$–$10^{-4}$ mol/L) and electrical field stimulation (EFS, 0.1–16 Hz) of the perivascular autonomic nerves. Effects were investigated during contraction with 40 mmol/L $K^+$ using acetylcholine (ACh, $10^{-8}$ to $10^{-5}$ mol/L, half-log units) and sodium nitroprusside (SNP, $10^{-8}$–$10^{-5}$ mol/L).

### Drugs and Solutions

Krebs-Ringer bicarbonate buffer contained the following (in

mmol/L): NaCl, 118.5; $MgSO_4 \cdot 7H_2O$, 1.2; $KH_2PO_4$, 1.2; $NaHCO_3$, 25.0; $CaCl_2$, 2.5; glucose, 5.5. Solutions containing different concentrations of $K^+$ were prepared by replacing part of the NaCl an equimolar amount of KCl. Noradrenaline was obtained from Sigma Chemical Co. (St. Louis, USA), ACh chloride from Janssen Chimica (Beersen, Belgium), and SNP from Acros (Geel, Belgium). All agents were dissolved in distilled water.

## Vascular Morphology of Mesenteric Arteries

After experimentation, arteries were fixed overnight at their optimal diameter by replacing the organ-bath solution with phosphate-buffered (pH 7.4) formaldehyde (4%). The next day, arteries were transferred to 70% ethanol and processed for histological examination. Arterial segments were embedded in paraffin and Lawson solution (Boom B.V. Meppel, The Netherlands) was used to stain the internal and external elastic laminae in 4-μm–thick cross-sections. To visualize the elastic laminae, a Zeiss Axioscope (Zeiss, Jena, Germany) and a standard CCD camera were used (Stemmer, Munich, Germany). Media cross-sectional area (CSA) was calculated by subtracting the area enclosed by the internal elastic lamina from the area enclosed by the media-adventitial border using commercial software (JAVA 1.21; Jandel Scientific, Corte Madera, USA). Lumen diameter (*D*) was calculated from the internal circumference assuming a circular cross-section. Media thickness (Mt) was derived from the CSA and *D* measurements.

## Endothelial Nitiric Oxide Synthase Staining/Two-Photon Laser Scanning Microscopy

Formalin fixed whole vessel segments were pretreated with Triton-PBS buffer. Thereafter the whole segment was incubated with monoclonal endothelial nitiric oxide synthase (eNOS) mouse antibody (N30020 Transduction Laboratories, Exeter, UK), followed by a fluorescein isothiocyanate (FITC) labeled rabbit anti-mouse antibody (Alexa 488 Molecular Probes, Leiden, The Netherlands).

After eNOS antibody treatment, vessels were imbedded in 1.5% agarose gel and co-stained with cell nuclei markers SYTO 41 (final concentration = 2.0 μmol/L) or propidium iodide (final concentration = 1.5 μmol/L). Both probes were obtained from Molecular Probes and dissolved in Hanks Buffered Saline Solution (HBSS, pH 7.4) containing (mmol/L): 137 NaCl, 14.9 HEPES, 5.5 glucose, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $KH_2PO_4$, and 1.2 $MgSO_4$.

Imaging was performed on a Nikon E600FN microscope (Nikon Corporation, Tokyo, Japan), coupled to a standard Biorad 2100 MP multiphoton system (BioRad, Hemel Hempstead, UK). The excitation source was a pulsed Ti:sapphire laser (Spectra Physics Tsunami, Mountain View, USA) tuned and mode-locked at 800 nm. Laser light reached the sample through the microscope objective (60×, water dipping, numerical aperture 1.0). Maximum field of view was 206 μm × 206 μm. Further magnification with higher resolution was achieved by optical zoom in the scan head.

**Table 1. Systolic Blood Pressure in 8 and 72 Weeks Old Rats**

| Age | WKY | SHR | SHR-Los | SHR-Spiro |
|---|---|---|---|---|
| 8 weeks | 123±4 | 164±2* | 155±4*,$ | 156±2*,$ |
| 72 weeks | 130±5 | 222±9* | 210±15* | 214±9* |

WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats; SHR-Los, prehypertensively losartan treated SHR; SHR-Spiro, prehypertensively spironolactone treated SHR. *$p<0.001$, all SHR groups *vs*. WKY. $^{\$}p<0.05$, prehypertensively treated SHR *vs*. SHR.

Three tubes (PMT) were used to detect the emitted fluorescent signals (*14*). For imaging of the fluorescent markers, PMTs were tuned corresponding to the emission spectra of the used fluorescent markers: SYTO 41 450–470-nm (PMT I); FITC labeled eNOS antibody, 500–550 nm (PMT II); propidium iodide (PI) 570–700 nm (PMT III). Laser power was kept as low as possible to prevent photochemical and thermal damage to the sample. Imaging speed was 0.1 Hz with a pixel dwell time of 39 μs, or 0.3 Hz with a pixel dwell time of 12 μs combined with Kalman filtering ($n=3$ cycles) for noise reduction. From all PMTs, separate images of 512 × 512 pixels were obtained (color coded blue, green and red) and combined into a single image. All images were recorded in the *xy*-plane and are shown without further image processing. Three-dimensional (3D)-reconstructions of the scanned volume were obtained from *z*-stacks (a series of *xy*-images at successive depths; *z*-step 0.45 μm or 0.6 μm). For the reconstruction of 3D images additional processing was performed using the Image-pro plus 6.0 and 3D-constructor 5.1 packages (Media Cybernetics, Silver Spring, USA).

The two-photon laser scanning microscopy (TPLSM) images were semiquantitatively analyzed. Intensity of staining was differentiated into poor (−), good (+) and very good (++). We determined the intensity for whole endothelial cells, their cytoplasmand their perinuclear region.

## Western Blotting

Aorta from adult untreated and treated SHR ($n=5$ per group) were prepared and analyzed by Western blotting. Briefly, membranes were incubated with rabbit monoclonal antibody against eNOS (~135 kD) diluted 1:2,500, in washing solution at room temperature for 1 h. The membranes were then washed, incubated with anti-rabbit horseradish peroxidase–conjugated second antibody 1:2,000 for 1 h at room temperature, and washed extensively. Membranes were incubated with Chemiluminescence Blotting Substrate (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's protocol, and exposed to film that was immediately

**Table 2. Morphological Properties of Mesenteric Small Arteries in 8 and 72 Weeks Old Rats**

| Age | | WKY | SHR | SHR-Los | SHR-Spiro |
|---|---|---|---|---|---|
| 8 weeks | CSA ($\mu m^2$) | n.d. | 5,511±291 | 5,896±534 | 6,509±607 |
| | Mt (μm) | n.d. | 8.2±0.4 | 8.2±0.5 | 8.8±0.6 |
| | Diameter (μm) | n.d. | 205±2.3 | 219±7 | 224±11 |
| | *W*/*L* | n.d. | 0.08±0.01 | 0.07±0.01 | 0.08±0.01 |
| 72 weeks | CSA ($\mu m^2$) | 16,909±496 | 24,812±2,490* | 23,476±1,703* | 24,496±1,139* |
| | Mt (μm) | 16.5±0.5 | 23.6±2.0* | 24.7±2.6* | 24.4±1.5* |
| | Diameter (μm) | 305±11 | 296±13 | 318±17 | 290±6 |
| | *W*/*L* | 0.11±0.01 | 0.16±0.01** | 0.15±0.02* | 0.17±0.01** |

WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats; SHR-Los, prehypertensively losartan treated SHR; SHR-Spiro, prehypertensively spironolactone treated SHR; CSA, media cross-sectional area; Mt, media thickness; *W*/*L*, wall-to-lumen ratio. *$p<0.05$, **$p<0.01$, all SHR groups *vs*. WKY.

**Table 3. Reactivity of Mesenteric Small Arteries of 72 Weeks Old Rats**

| | WKY | SHR | SHR-Los | SHR-Spiro |
|---|---|---|---|---|
| AWT | | | | |
| $K_{max}$ (N/m) | 1.7±0.1 | 1.9±0.2 | 3.1±0.3***,$\$\$$ | 2.6±0.2* |
| $NA_{max}$ (N/m) | 4.6±0.1 | 6.5±0.3** | 7.4±0.3*** | 6.9±0.6*** |
| $EFS_{max}$ (N/m) | 0.9±0.7 | 3.8±0.5* | 5.0±1.2** | 5.0±1.0** |
| $EFS_{max}/NA_{max}$ | 0.17±0.12 | 0.65±0.07 *** | 0.65±0.16*** | 0.68±0.11*** |
| AWS | | | | |
| $K_{max}$ ($N/m^2$) | 50±4 | 48±7 | 126±12 ***,$\$\$\$$ | 109±10 ***,$\$\$\$$ |
| $NA_{max}$ ($N/m^2$) | 265±9 | 258±15 | 319±12 *,$\$$,# | 216±16 |

WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats; SHR-Los, prehypertensively losartan treated SHR; SHR-Spiro, prehypertensively spironolactone treated SHR; AWT, wall tension; AWS, wall stress; $K_{max}$, maximal response to 40 mmol/L $K^+$ effective potassium dosage; $NA_{max}$, maximal response to noradrenaline; EFS, electric field stimulation. *$p<0.05$, **$p<0.01$, ***$p<0.001$, all SHR groups *vs*. WKY. $^{\$}p<0.05$, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$, prehypertensively treated SHR *vs*. SHR. $^{\#}p<0.001$, SHR-Los *vs*. SHR-Spiro.

developed. The film was detected with a Zeiss Axioscope (Zeiss) and a standard CCD camera (Stemmer) and saved to a computer. Band intensity was measured by computer analysis, using the LeicaQwin program.

### Statistics

Contractile responses are expressed as active wall tension (increase in force divided by twice the length of the vessel segment, N/m) and as active wall stress (active wall tension divided by media thickness, $N/m^2$) to correct for differences in wall thickness. Data on WKY, SHR and transiently prehypertensive treated SHR were compared by ANOVA with post-hoc Dunnett's *t*-test. Data are expressed as mean±SEM. Values of $p<0.05$ were considered to indicate statistical significance.

## Results

### Blood Pressure

Blood pressure results are summarized in Table1. SBP was significantly higher in untreated SHR than in WKY at 8 weeks of age. SHR treated from 4 to 8 weeks of age had significantly lower SBP than untreated SHR at 8 weeks of age. Comparable blood pressure lowering was obtained with losartan and spironolactone.

In 72-week-old animals (64 weeks after drug withdrawal) SHR had significantly elevated SBP as compared to WKY. Prehypertensively treated SHR showed SBP values comparable to those in untreated SHR (Table 1).

### Morphological Properties of Small Mesenteric Arteries

In 8-week-old SHR, lumen diameter, media CSA, media thickness and wall-to-lumen ratio (*W*/*L*) were comparable between the untreated and prehypertensively treated groups (Table 2). In 72-week-old animals, SHR showed increased values as compared to WKY. Prehypertensively treated SHR did not show different values as compared to untreated SHR. Prehypertensive losartan treatment led to a slightly smaller *W*/*L* than spironolactone treatment when compared to WKY (Table 2).

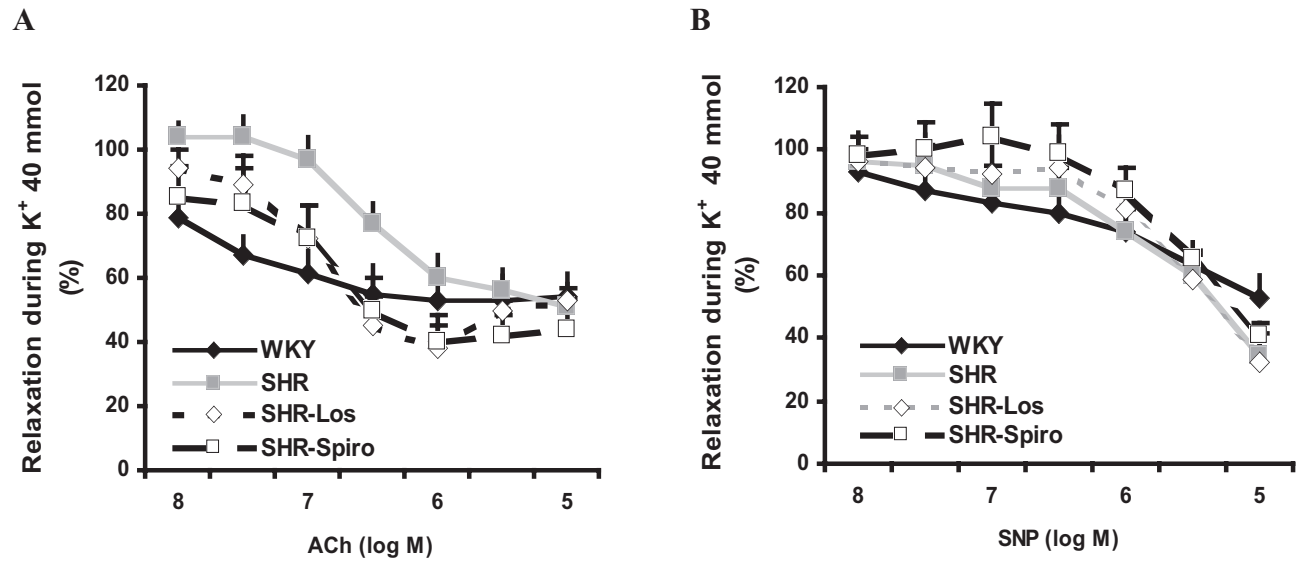


**Fig. 1.** *Endothelium-dependent (A) and -independent (B) relaxation in small mesenteric arteries of 72-week-old untreated WKY and SHR and 72-week-old SHR that were prehypertensively treated with losartan or spironolactone. A: The concentration of ACh needed to half maximally relax potassium precontracted small mesenteric arteries was significantly lower in WKY (−log $EC_{50}$: 7.7±0.1; $p<0.001$) and both prehypertensively treated SHR groups (−log $EC_{50}$: 7.0±0.2, 6.9±0.1; $p<0.05$) as compared to untreated SHR (−log $EC_{50}$: 6.5±0.1). B: Administration of the NO donor SNP resulted in comparable responses in all groups.*

## Contractile Responses and Active Wall Stress

To assess receptor-dependent, receptor-independent and neurogenic contractile responses, isolated small mesenteric arteries of young and aged animals were treated with potassium ($K^+$), NA, and EFS, respectively (Table 3).

In 8-week-old SHR the responses to $K^+$, NA and EFS were comparable between untreated and prehypertensively treated. In 72-week-old animals depolarization lead to comparable contraction in untreated SHR and WKY. Prehypertensively treated SHR showed significantly increased contractile responses to depolarization as compared to untreated SHR and WKY. Responses were accentuated in prehypertensively losartan-treated SHR. The values of the active wall stress (AWS) during depolarization (after correction for alterations in media thickness) were comparable between untreated SHR and WKY. Prehypertensively treated SHR showed significantly increased AWS to depolarization as compared to untreated SHR and WKY. Responses were similar between the two prehypertensively treated SHR groups.

In 72-week-old animals NA and EFS led to larger contractile responses in untreated SHR than in WKY. Responses of prehypertensively treated SHR were non-significantly elevated as compared to SHR and significantly increased as compared to WKY. Responses were similar in both prehypertensively treated SHR groups. The AWS during NA (correcting the AWT for alterations in media thickness) were comparable between untreated SHR and WKY. Prehypertensively losartan-treated SHR showed significantly increased AWS as compared to untreated SHR, WKY and in particular SHR-Spiro. The ratio of $EFS_{max}/NA_{max}$ as an estimate of sympathetic neuroeffector function in untreated SHR was significantly increased as compared to WKY. Ratios were similar in the two prehypertensively treated SHR groups.

## Relaxation Responses

In 8-week-old SHR the responses of ACh and SNP during $K^+$-induced contraction were comparable between the untreated and prehypertensively treated groups (data not shown). In 72-week-old animals endothelium-dependent relaxation was significantly lower in SHR as compared to WKY (Fig. 1; $p<0.001$). Prehypertensively treated SHR showed significantly ameliorated endothelium-dependent relaxation as compared to untreated SHR ($p<0.05$). Reponses were similar in prehypertensively treated SHR groups. The endothelium-independent relaxant agent SNP roduced similar relaxations irrespective of strain and prehypertensive treatment.

## Quantification of Aortic eNOS

Using aortic tissue eNOS density was quantified in untreated and treated SHR by Western blotting. Untreated SHR showed 50% less eNOS as compared to WKY ($p<0.01$). SHR-Los and SHR-Spiro also demonstrated less eNOS than WKY and did not differ significantly from untreated SHR ($p<0.05$) (Fig. 2).

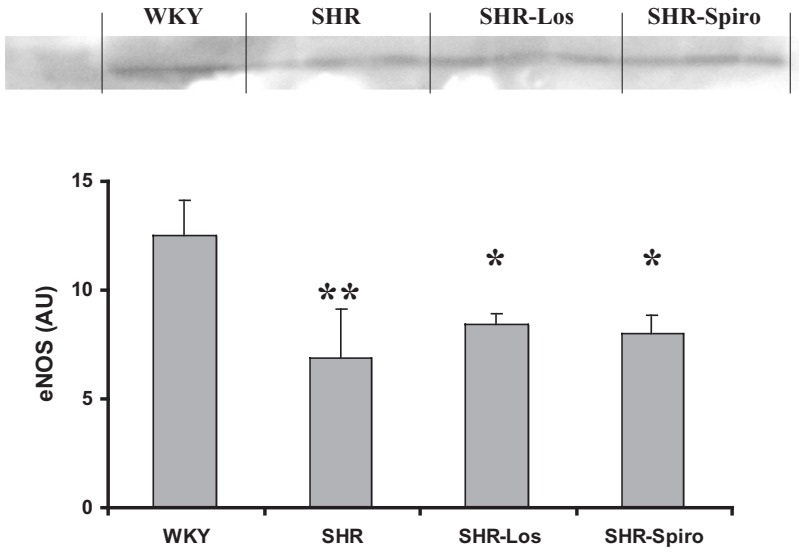


**Fig. 2.** *Western blotting of eNOS in aortic tissue of 72-week-old WKY, untreated and prehypertensively treated SHR. Untreated SHR, and SHR that were prehypertensively treated with losartan or spironolactone demonstrate similar densities of eNOS. However significant differences in comparison with WKY were apparent between untreated (**$p < 0.01$) and prehypertensively treated SHR (*$p < 0.05$). The blot shows WKY rats and three rats per SHR group.*

### Localization of eNOS in Mesenteric Arteries

Whole vessel segments were investigated for eNOS density and intracellular distribution using TPLSM (Fig. 3). Semi-quantitative analysis of the TPLSM are summarized in Table 4. In 72-week-old SHR significantly less endothelial eNOS was apparent as compared to WKY and endothelial cells were stained above the detection limit. In SHR the fluorescent signal was less intense, while in WKY the majority of the signal was localized in one intracellular area located on one side of the nucleus. Prehypertensively treated SHR showed similar amounts of eNOS as compared to untreated SHR. The amounts were similar in prehypertensively treated SHR groups. In contrast to the blurred localization in untreated SHR, prehypertensively treated SHR displayed plasmalemmal and perinuclear distributions of eNOS that were comparable to that in WKY.

## Discussion

This study demonstrates that short-term RAAS blockade in young SHR resulted in altered reactivity of small mesenteric arteries at 64 weeks after withdrawal of the treatment. The alterations consisted of increased vasoconstriction and reduced endothelial dysfunction and were not associated with a persistent reduction of arterial hypertrophy and blood pressure. Effects were largely similar after transient prehypertensive therapy with losartan or spironolactone. These findings suggest that early inhibition of the RAAS might lead to a long lasting resetting of this humoral axis that slows the deterioration of small artery function with aging.

Prehypertensive RAAS blockade was induced by losartan or spironolactone from 4 to 8 weeks of age. At the end of these treatments, blood pressure was reduced but contractile responses and endothelial dysfunction were not modified. These findings suggest that brief antihypertensive RAAS blockade in prehypertensive SHR had no immediate effects on vascular structure and reactivity despite significant blood pressure lowering. This was unexpected as prehypertensive SHR are considered to demonstrate increased RAAS mediated contraction, angiotensin II receptor density and hypertrophic remodeling (*15*, *16*). This might be explained by the short duration of the treatment and by the relatively small blood pressure lowering (*10*).

We (*17*) and others (*3*, *4*) demonstrated previously that blood pressure lowering after prehypertensive treatment persists into adulthood (*i.e.*, 36 weeks of age). In contrast to earlier studies that were terminated at this age we continued to monitor blood pressure and observed a restoration of hypertension at later stages of life (*17*). This is supported by our present results that at 72 weeks of age, blood pressure no longer differ between prehypertensively treated and untreated SHR. This demonstrates that the blood pressure lowering effects of this treatment strategy were not life-long effects. The similar blood pressure values between the three 72-week-old SHR groups led us to conclude that the arterial changes observed in the old SHR treated at young age were not the direct consequence of a lower blood pressure (*6*, *10*).

In line with the absence of a blood pressure lowering effect, small artery structure did not differ. In contrast to these findings prehypertensive treatment resulted in increased maximal contractile responses to all stimuli investigated. When the

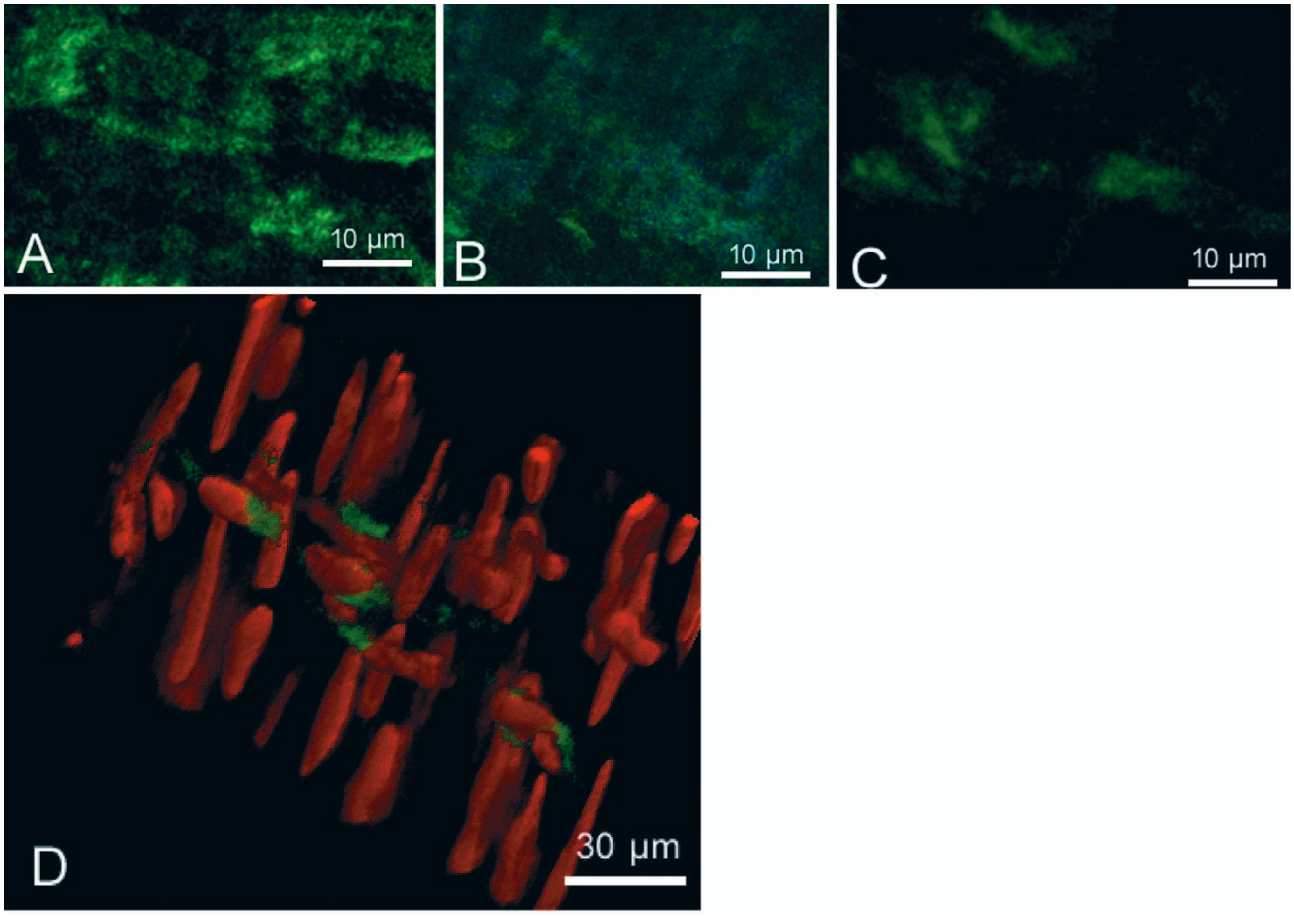


**Fig. 3.** *Two photon light scanning microscopy (TPLSM) of eNOS in the endothelial cell layer of 72-week-old WKY (A), SHR (B) and prehypertensively losartan treated SHR (C). WKY show intense staining with a notable concentration in a perinuclear compartment, while SHR show little staining. The blue background is related to the maximal scanning intensity that had to be used for SHR and resulted in autofluorescence of the underlying lamina eslastica interna. Prehypertensively treated SHR show perinuclear eNOS staining with less intensity than WKY. D: Additional nuclear staining was performed. Below the endothelial cell layer smooth muscle cells of the tunica media are displayed to demonstrate the 3-dimensional context (longer nuclei perpendicular to the endothelial nuclei and to the long axis of the vessel). Green: eNOS; red: nuclei.*

thickness of the tunica media was taken into account, differences between old SHR with and without prehypertensive treatment persisted. This indicates that prehypertensive RAAS blockade resulted in increased contractility of the arterial smooth muscle in old SHR which was more marked after prehypertensive treatment with losartan than spironolactone. Whether this reflects a predominantly AT1 receptor dependent alteration or a dosage phenomenon remains unclear. Recently Demirci *et al*. described a reduction of contractile responses during aging of SHR which was partially antagonized by continuous losartan treatment (*18*). Our data now suggest that not only continuous but also transient prehypertensive RAAS blockade might decelerate the loss of arterial contractility with aging in a blood pressure independent manner. Nakaya *et al*. proposed a role for resetting of the RAAS in the prevention of the premature aging of the kidney (*5*).

Unlike the results previously reported for smooth muscle, the neuroeffector mechanisms in the present study did not seem to be modified by the prehypertensive treatments. While the ratio of the maximal response to nerve stimulation to the maximal response to noradrenaline was markedly higher in SHR compared to WKY, this functional index of sympathetic nerve density did not differ between untreated and prehypertensively treated SHR. Periarterial innervation, which in the rat is established during the first 2 weeks of life, thus seems not to be persistently altered by RAAS blockade between 4 and 8 weeks of age (*19*).

With respect to the endothelium we recorded responses to acetylcholine during $K^+$-induced contraction in order to concentrate on nitric oxide (NO) in the absence of influences of endothelium-derived hyperpolarizing factor. At 72 weeks of age these responses were ameliorated in prehypertensively

**Table 4. Semiquantitative Analysis of eNOS Staining**

| | WKY | SHR | SHR-Los | SHR-Spiro |
|---|---|---|---|---|
| Total | ++ | + | + | + |
| Cytoplasma | ++ | + | – | – |
| Perinuclear | ++ | – | + | + |

eNOS, endothelial nitric oxide synthase; WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats; SHR-Los, prehypertensively losartan treated SHR; SHR-Spiro, prehypertensively spironolactone treated SHR. –, poor; +, good; ++, very good.

treated SHR as compared to untreated SHR. Thus, not only contractile responses but also endothelium-dependent responses were improved by this treatment strategy in a blood pressure independent manner. Reversal of decreased bioavailability or synthesis of endothelium-derived NO, or of the dysfunction of vascular smooth muscle in aging hypertensive animals, may have contributed to these improved responses. The latter possibility can be dismissed by the finding that SNP, an NO donor, produced an equal degree of relaxations in SHR small mesenteric arteries, irrespective of treatment. Losartan and spironolactone both inhibit NAD(P)H oxidase–mediated $O_2^-$ synthesis and enhance antioxidant superoxide dismutase activity in the small mesenteric arteries (*20*, *21*). As a consequence, this increases NO bioavailability (*22*, *23*). We therefore suggest that the beneficial effects of prehypertensive treatments were at least partially mediated by enhanced endothelial supply of NO.

We demonstrated that transient losartan and spironolactone had similar long-term consequences on the endothelial function. Similar results were obtained with chronic AT1 receptor blockade and aldosterone antagonism, where treatment increased eNOS and reduced oxidative stress (*9*, *24*, *25*). Thus, one explanation could be that transient losartan and spironolactone treatment affect the same pathway as chronic treatment. It may be additionally speculated that both treatments could stimulate the angiotensin II type 2 (AT2) receptor, thereby promoting NO release (*26*). Independent of the underlying mechanism, an improved endothelial function reduces cardiovascular risk (*27–29*).

To substantiate that the enhanced responses in prehypertensively treated SHR were related to the synthesis of NO, we investigated eNOS in aortic tissue. Using Western blotting we demonstrated that the concentration of eNOS was lower in untreated SHR than in WKY, and that this was not significantly altered by prehypertensive treatment. Thus other mechanisms could be involved in the enhanced responses. As NO synthesis depends on the intracellular distribution of eNOS (*30*) we investigated the endothelial cell layer of mesenteric arteries in untreated and treated SHR using TPLSM and its semiquantitative analysis. As expected, the density of eNOS was larger in WKY than in SHR with dense staining in a perinuclear region at one side of the nucleus, which was most likely the Golgiapparatus (*30*). This perinuclear staining was absent in the endothelium of untreated SHR. Prehypertensively treated SHR demonstrated similar densities of eNOS as untreated SHR, thereby confirming our Western blot results. However, prehypertensively treated SHR demonstrated a perinuclear staining at the same perinuclear region as WKY, albeit at a lower concentration. The consequences of this eNOS localization remain uncertain.

However, because the distribution, but not the amount, of eNOS is associated with endothelial function in untreated and prehypertensively treated SHR, the specific eNOS distribution might have functional consequences (*31*). Recent studies suggest that such a distribution can be modulated by wall shear stress (*30*). Furthermore, the perinuclear eNOS distribution can attenuate oxidative stress and peroxynitrite production as the therefore necessary NADPH oxidases are commonly distributed at the cell membrane (*32*). In hypertension, oxidation of tetrahydrobiopterin by NADPH oxidase culminates in increased production of reactive oxygen species by NO synthases (*22*). The combination of eNOS expression and intracellular distribution could result in the enhanced endothelium-dependent responses seen in prehypertensively treated SHR.

In summary, we demonstrated that prehypertensive RAAS inhibition resulted in small mesenteric arteries of old SHR in increased maximal contractile responses and ameliorated endothelial function as compared to untreated SHR. This process was blood pressure independent. As explanation we propose a long-lasting pharmacological resetting of the RAAS that culminates in deceleration of the premature aging of arterial function in SHR.

## References

1. Harrap SB, Van der Merwe WM, Griffin SA, Macpherson F, Lever AF: Brief angiotensin converting enzyme inhibitor treatment in young spontaneously hypertensive rats reduces blood pressure long-term. *Hypertension* 1990; **16**: 603–614.
2. Berecek KH, Nagahama S, Oparil S: Effect of central administration of MK-422 (the diacid form of enalapril) on the development of hypertension in the spontaneously hypertensive rat. *J Hypertens Suppl* 1984; **2**: S63–S66.
3. Regan CP, Bishop SP, Berecek KH: Early, short-term treatment with captopril permanently attenuates cardiovascular changes in spontaneously hypertensive rats. *Clin Exp Hypertens* 1997; **19**: 1161–1177.
4. Bergstrom G, Johansson I, Wickman A, Gan L, Thorup C: Brief losartan treatment in young spontaneously hypertensive rats abates long-term blood pressure elevation by effects on renal vascular structure. *J Hypertens* 2002; **20**: 1413–1421.
5. Nakaya H, Sasamura H, Hayashi M, Saruta T: Temporary treatment of prepubescent rats with angiotensin inhibitors suppresses the development of hypertensive nephrosclerosis. *J Am Soc Nephrol* 2001; **12**: 659–666.
6. Folkow B: Physiological aspects of primary hypertension. *Physiol Rev* 1982; **62**: 347–504.
7. Weber KT, Brilla CG: Pathological hypertrophy and car-

diac interstitium. Fibrosis and renin-angiotensin-aldosterone system. *Circulation* 1991; **83**: 1849–1865.
8. Neves MF, Virdis A, Schiffrin EL: Resistance artery mechanics and composition in angiotensin II–infused rats: effects of aldosterone antagonism. *J Hypertens* 2003; **21**: 189–198.
9. Virdis A, Neves MF, Amiri F, Viel E, Touyz RM, Schiffrin EL: Spironolactone improves angiotensin-induced vascular changes and oxidative stress. *Hypertension* 2002; **40**: 504–510.
10. Morton JJ, Beattie EC, MacPherson F: Angiotensin II receptor antagonist losartan has persistent effects on blood pressure in the young spontaneously hypertensive rat: lack of relation to vascular structure. *J Vasc Res* 1992; **29**: 264–269.
11. Rizzoni D, Porteri E, Piccoli A, *et al*: Effects of losartan and enalapril on small artery structure in hypertensive rats. *Hypertension* 1998; **32**: 305–310.
12. Brilla CG, Janicki JS, Weber KT: Cardioreparative effects of lisinopril in rats with genetic hypertension and left ventricular hypertrophy. *Circulation* 1991; **83**: 1771–1779.
13. Skov K, Fenger-Gron J, Mulvany MJ: Effects of an angiotensin-converting enzyme inhibitor, a calcium antagonist, and an endothelin receptor antagonist on renal afferent arteriolar structure. *Hypertension* 1996; **28**: 464–471.
14. van Zandvoort M, Engels W, Douma K, *et al*: Two-photon microscopy for imaging of the (atherosclerotic) vascular wall: a proof of concept study. *J Vasc Res* 2004; **41**: 54–63.
15. Endemann D, Touyz RM, Li JS, Deng LY, Schiffrin EL: Altered angiotensin II–induced small artery contraction during the development of hypertension in spontaneously hypertensive rats. *Am J Hypertens* 1999; **12**: 716–723.
16. Touyz RM, Endemann D, He G, Li JS, Schiffrin EL: Role of AT2 receptors in angiotensin II–stimulated contraction of small mesenteric arteries in young SHR. *Hypertension* 1999; **33** (1 Pt 2): 366–372.
17. Baumann M, Smits JFM, Struijker Boudier HAJ: Long-term echocardiographic follow up of brief and early losartan treated SHR reveals sustained cardiac protection. *Hypertension* 2005; **46**: 892 (Abstract).
18. Demirci B, McKeown PP, Bayraktutan U: Blockade of angiotensin II provides additional benefits in hypertension- and ageing-related cardiac and vascular dysfunctions beyond its blood pressure–lowering effects. *J Hypertens* 2005; **23**: 2219–2227.
19. Gillies LK, Lu M, Wang H, Lee RM: AT1 receptor antagonist treatment caused persistent arterial functional changes in young spontaneously hypertensive rats. *Hypertension* 1997; **30**: 1471–1478.
20. Touyz RM, Schiffrin EL: Increased generation of superoxide by angiotensin II in smooth muscle cells from resistance arteries of hypertensive patients: role of phospholipase D–dependent NAD(P)H oxidase–sensitive pathways. *J Hypertens* 2001; **19**: 1245–1254.
21. Rajagopalan S, Kurz S, Munzel T, *et al*: Angiotensin II–mediated hypertension in the rat increases vascular superoxide production *via* membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest* 1996; **97**: 1916–1923.
22. Landmesser U, Dikalov S, Price SR, *et al*: Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest* 2003; **111**: 1201–1209.
23. Wilcox CS: Oxidative stress and nitric oxide deficiency in the kidney: a critical link to hypertension? *Am J Physiol Regul Integr Comp Physiol* 2005; **289**: R913–R935.
24. Sanz-Rosa D, Cediel E, de las Heras N, *et al*: Participation of aldosterone in the vascular inflammatory response of spontaneously hypertensive rats: role of the NFkappaB/IkappaB system. *J Hypertens* 2005; **23**: 1167–1172.
25. Sanz-Rosa D, Oubina MP, Cediel E, *et al*: Eplerenone reduces oxidative stress and enhances eNOS in SHR: vascular functional and structural consequences. *Antioxid Redox Signal* 2005; **7**: 1294–1301.
26. Cosentino F, Savoia C, De Paolis P, *et al*: Angiotensin II type 2 receptors contribute to vascular responses in spontaneously hypertensive rats treated with angiotensin II type 1 receptor antagonists. *Am J Hypertens* 2005; **18** (4 Pt 1): 493–499.
27. Kanematsu Y, Tsuchiya K, Ohnishi H, *et al*: Effects of angiotensin II type 1 receptor blockade on the systemic blood nitric oxide dynamics in $N^{\omega}$-nitro-L-arginine methyl ester–treated rats. *Hypertens Res* 2006; **29**: 369–374.
28. Mori Y, Ohyanagi M, Koida S, Ueda A, Ishiko K, Iwasaki T: Effects of endothelium-derived hyperpolarizing factor and nitric oxide on endothelial function in femoral resistance arteries of spontaneously hypertensive rats. *Hypertens Res* 2006; **29**: 187–195.
29. Mensah GA, Ryan US, Hooper WC, *et al*: Vascular endothelium summary statement II: Cardiovascular disease prevention and control. *Vascul Pharmacol* 2007; **46**: 318–320.
30. Cheng C, van Haperen R, de Waard M, *et al*: Shear stress affects the intracellular distribution of eNOS: direct demonstration by a novel *in vivo* technique. *Blood* 2005; **106**: 3691–3698.
31. Zhang Q, Church JE, Jagnandan D, Catravas JD, Sessa WC, Fulton D: Functional relevance of Golgi- and plasma membrane–localized endothelial NO synthase in reconstituted endothelial cells. *Arterioscler Thromb Vasc Biol* 2006; **26**: 1015–1021.
32. Forstermann U, Munzel T: Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation* 2006; **113**: 1708–1714.