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ORIGINAL ARTICLE

Dual NEP/ECE inhibition improves endothelial function in mesenteric resistance arteries of 32-week-old SHR

Pieter Lemkens¹, Leon JA Spijkers², Merlijn J Meens^{1,3,4}, Jelly Nelissen¹, Ben Janssen¹, Stephan LM Peters², Paul MH Schiffers¹ and Jo GR De Mey^{1,5}

Endothelin 1 (ET-1), a potent vasoconstrictor, pro-mitogenic and pro-inflammatory peptide, may promote development of endothelial dysfunction and arterial remodeling. ET-1 can be formed through cleavage of big-ET-1 by endothelin-converting enzyme (ECE) or neutral endopeptidase (NEP). We investigated whether chronic treatment with the novel dual NEP/ECE inhibitor SOL1 improves functional and structural properties of resistance-sized arteries of 32-week-old male spontaneously hypertensive rats (SHR). SHR received a chronic 4-week treatment with SOL1, losartan or hydralazine. We then compared effects of inhibition of NO synthase (NOS) (100 μM L-NAME), blockade of ET_A- and ET_B-receptors (10 μM bosentan) and stimulation of the endothelium with 0.001–10 μM acetylcholine (ACh) in isolated third-order mesenteric resistance arteries. Losartan and hydralazine significantly lowered blood pressure. Losartan decreased the media-to-lumen ratio of resistance arteries. L-NAME (1) increased arterial contractile responses to K⁺ (5.9–40 mM) in the losartan, SOL1 and vehicle group and (2) increased the sensitivity to phenylephrine (PHE; 0.16–20 μM) in the SOL1 group but not in the losartan, hydralazine and vehicle group. Relaxing responses to ACh in the absence or presence of L-NAME during contractions induced by either 10 μM PHE or 40 mM K⁺ were not altered by any *in vivo* treatment. Acute treatment with bosentan did, however, significantly improve maximal relaxing responses involving endothelium-derived nitric oxide and -hyperpolarizing factors in the SOL1 group but not in the losartan, hydralazine or vehicle group. Thus, chronic inhibition of NEP/ECE improved basal endothelial function but did not alter blood pressure, resistance artery structure and stimulated endothelium-dependent relaxing responses in 32-week-old SHR.

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Keywords: bosentan; EDHF; endothelin-1; losartan; NO

INTRODUCTION

The endothelium can produce vasoactive compounds that influence the underlying layer of smooth muscle cells modulating vasomotor responses to control blood pressure and local blood flow. Endothelium-derived vasoactive factors include vasodilators, such as nitric oxide (NO), endothelium-derived hyperpolarizing factors (EDHF) and prostacyclin, and vasoconstrictors, such as endoperoxides and endothelin-1 (ET-1).^{1,2} Endothelial dysfunction is classically described as (i) an impairment of endothelium-dependent relaxing responses and (ii) an enhanced sensitivity to vasoconstrictors. In many forms of human and experimental hypertension, endothelial dysfunction and arterial remodeling are considered key players in the development and maintenance of high blood pressure.^{3–5} In spontaneously hypertensive rats (SHR) a well-described manifestation of endothelial dysfunction is an increased production of endothelium-derived contractile factors (EDCF).⁶

Drug-intervention studies have shown that some anti-hypertensive treatments, for instance with angiotensin AT₁ receptor antagonists, not only lower blood pressure but also cause an improvement of endothelial function and regression of structural changes in resistance arteries.^{7,8} Other anti-hypertensive treatments, for example, the selective β_1 -adrenoceptor antagonist atenolol, succeed in lowering blood pressure but did not reverse the structural changes or the endothelial dysfunction associated with hypertension. In other words, for reversing adverse effects associated with hypertension, lowering blood pressure alone is not sufficient.^{9,10}

Studies from our group showed that the level of the endothelium-derived peptide ET-1 is increased in several organs during the development of hypertension in SHR; importantly, the levels of ET-1 are increased in several organs.¹¹ Apart from being a potent vasoconstrictor, ET-1 is also a potent growth promoting and pro-inflammatory agent.^{12–16} Because of these actions, ET-1 is of

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interest in the development of endothelial dysfunction and hypertension-related arterial remodeling. ET-1 can be formed through cleavage of big-ET-1 by endothelin-converting enzyme (ECE). Alternatively, big-ET-1 can be hydrolyzed by chymase or matrix metalloproteinase 2 to form ET-1 (1–31) or ET-1 (1–32), respectively. Both are further processed into ET-1 by neutral endopeptidase (NEP).^{17–19} Thus, a dual NEP/ECE inhibitor, for example the novel compound SOL1, reduces ET-1 production by inhibiting all three pathways.²⁰ Recently, Kalk *et al.*²¹ reported that the dual NEP/ECE inhibitor daglutril (SLV338, an analog of SOL1) reduced cardiomyocyte hypertrophy, interstitial fibrosis and perivascular fibrosis in the 2-kidney 1-clip model of hypertension.

Here, we tested the hypothesis that ET-1 contributes to endothelial dysfunction, arterial remodeling and hypertension in established essential hypertension. We therefore compared the effects of chronic treatment with the vasodilator hydralazine, the AT₁ receptor antagonist losartan or the dual NEP/ECE inhibitor SOL1 on functional and structural properties of mesenteric resistance arteries from chronically hypertensive (32-week old) SHR.

METHODS

Animals, model and surgery

Twenty-eight-week-old male SHR were obtained from Charles River (Maastricht, NL, USA). All experiments were performed in accordance with the ethical committee for animal welfare of Maastricht University. The rats were anesthetized with isoflurane (1–4%) and osmotic minipumps (2ML4 pumps, Alzet, Cupertino, CA, USA) were subcutaneously implanted for chronic continuous drug treatment during the next 4 weeks. Preliminary dose-finding experiments were carried out to identify an appropriate dose of the dual NEP/ECE inhibitor SOL1 (2-[[1-((3S)-1-(carboxymethyl-2-oxo-2,3,4,5-tetrahydro-1H-1-benzazepin-3-yl)amino)carbonyl)cyclopentyl]methyl]-4-[[3-methylamino)propyl](methylamino)-4-oxobutanoic acid; Abbott, Hanover, Germany). A dose of 50 mg kg⁻¹ per day decreased urinary ET-1 content (from 32.89 ± 3.91 to 1.35 ± 0.25 pg ml⁻¹, *P* < 0.001). Losartan and hydralazine were administered at 20 mg kg⁻¹ per day and 9 mg kg⁻¹ per day, respectively. Additional hydralazine was added to the drinking water to keep the dose at 20 mg kg⁻¹ per day, because the maximal solubility of hydralazine was reached in saline. In untreated SHR, a dummy device (polyethylene (PE) tube of the same size as the 2ML4 pumps) was subcutaneously implanted.²² Animals were randomly assigned to treatment groups (*n* = 8 each).

At the end of the drug treatment, blood pressure was measured in conscious unrestrained rats via a heparinized (5 U ml⁻¹) indwelling PE catheter that was introduced into the left femoral artery 2 days before measurement. The arterial catheter was connected to a pressure transducer (Micro Switch 150 PC, Mouser Electronics, Munich Germany) and its output was sampled at 2.5 kHz. Mean arterial pressure (MAP) was calculated using the IDEEQ data-acquisition system (instrument services, Maastricht University). All animals were euthanized with isoflurane (> 4%).

Arterial pressure/diameter relationships

Animals were killed and first-order mesenteric arteries were isolated. Arteries were mounted on two glass micropipettes in an organ chamber filled with Ca-free Krebs Ringer solution (144 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 14.9 mM HEPES and 5.5 mM glucose; 37 °C, pH 7.4). An aliquot of 10 μM Na-nitroprusside was added to ensure maximal vasodilatation. A pressure–diameter relationship was established by recording the lumen diameter while gradually increasing the distending pressure (20–120 mm Hg, 10 mm Hg steps).²³ After the experiment, vessels were fixed at 80 mm Hg in 4% phosphate buffered formaldehyde solution for 1 h at 37 °C and stored in 70% ethanol.

Wire myography

Third-order mesenteric resistance arterial segments of ~2 mm in length were isolated and mounted in wire myographs for the recording of isometric force

development.²⁴ From a portion of the arterial segments, the endothelium was mechanically removed.²⁵ Each experiment started by progressively stretching the arterial segment to the diameter at which the largest contractile response to 10 μM noradrenaline (NA) could be obtained (optimal diameter).

To prevent the sensory nerves from releasing calcitonin gene-related peptide when stimulated during potassium induced depolarizations, all arterial segments were exposed to capsaicin (1 μM, during 20 min).^{26,27} Viability of the endothelium was tested by measuring acetylcholine (ACh; 10 μM)-induced relaxation during phenylephrine (PHE)-induced contraction. Contractile responses to increasing concentrations of K⁺ (5.9–40 mM) and PHE (0.16–20 μM) and relaxing responses to increasing concentrations of ACh (0.01–10 μM) were recorded. The effects of nitric oxide synthase inhibition (L-N^G-nitroarginine methyl ester (L-NAME), 100 μM), cyclo-oxygenase inhibition (indomethacin (INDO), 1 μM) and a non-selective ET-receptor antagonist (bosentan 10 μM) were evaluated.

After these experiments, vessels were fixed in 4% phosphate buffered formaldehyde solution for 1 h at 37 °C and stored in 70% ethanol.

Histological and morphometric analysis

Fixed first- and third-order arteries were embedded in paraffin and cross-sections (4 μm) were cut. All vessels were stained with Lawson's solution (Boom, Meppel, The Netherlands) to visualize the internal and external elastic laminae. The cross-sectional area of the media was determined from the circumferences of the internal and external elastic laminae. The average number of nuclear profiles in the media was determined by counting on haematoxylin–eosin stained cross-sections. Media thickness and media-to-lumen ratio were calculated as previously described.²³

Physiological solutions and drugs

The Krebs Ringer bicarbonate-buffered physiological salt solution (Krebs Ringer buffer (KRB)) that was continuously aerated with 95% O₂/5% CO₂ and maintained at 37 °C contained (in mM): 118.5 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.0 NaHCO₃ and 5.5 glucose. Bosentan was obtained from Actelion Pharmaceuticals (Allschwil, Switzerland) and was dissolved in DMSO. Capsaicin and INDO were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands) and dissolved in ethanol. ACh, NA and PHE were purchased from Sigma and dissolved in KRB. High K⁺-KRB solution consisted of KRB in which all of the NaCl was replaced by KCl. Solutions containing 10–40 mM K⁺ were prepared by mixing volumes of KRB and K⁺-KRB.

Statistical analysis

All contractile responses were expressed as a percentage of the maximal NA response prior to the administration of any pharmacological inhibitor. Individual concentration–response curves were fitted to a non-linear sigmoid regression curve (GraphPad Prism 5.0; La Jolla, CA, USA). Sensitivity (pEC₅₀) and maximal effect (*E*_{max}) are shown as mean ± s.e.m. Two-way analysis of variances were used to compare pEC₅₀ or *E*_{max}. A Bonferroni post-test was used to compare multiple groups. Morphology and pressure–diameter relations are expressed as mean ± s.e.m. Statistical significance of differences between groups was evaluated by analysis of variance (for consecutive measurements in pressure–diameter curves) or one way analysis of variance, followed by a Bonferroni or paired *t*-test (GraphPad Prism 5.0). A value of *P* < 0.05 was considered to denote a statistically significant difference.

RESULTS

General and arterial structural characteristics

Body weights were not significantly altered by any treatment. MAP was significantly reduced by losartan and hydralazine treatment. It tended to be reduced by SOL1, but this did not reach statistical significance (Table 1).

Lumen diameter at 80 mm Hg and cross-sectional area of the media of first-order mesenteric resistance arteries were comparable in vehicle-, SOL1-, losartan and hydralazine-treated animals. Media-to-lumen ratio of first-order mesenteric arteries was not altered in the

Table 1 General and arterial structural characteristics of first- and third-order mesenteric resistance arteries

	Vehicle	SOL1	Losartan	Hydralazine
Body weight (g)	353 ± 7	362 ± 8	366 ± 6	356 ± 9
MAP (mmHg)	191 ± 7	172 ± 3	125 ± 5 ^a	113 ± 14 ^a
Diameter 1st order (µm)	373 ± 21	369 ± 17	397 ± 19	398 ± 14
mCSA (10 ³ µm ²)	30 ± 3	26 ± 2	24 ± 3	28 ± 3
Media-to lumen ratio	0.184 ± 0.014	0.164 ± 0.006	0.144 ± 0.008 ^a	0.150 ± 0.012
NCC	55 ± 3	47 ± 2	49 ± 1	52 ± 3
Diameter 3rd order (µm)	273 ± 8	282 ± 10	244 ± 10 ^a	288 ± 7
Active tension (N m ⁻¹) ^b	4.91 ± 0.43	5.55 ± 0.45	4.65 ± 0.22	4.44 ± 0.37
mCSA (10 ³ µm ²)	13 ± 1	14 ± 1	11 ± 1	13 ± 1
Media-to lumen ratio	0.115 ± 0.005	0.140 ± 0.02	0.109 ± 0.004	0.109 ± 0.004

Abbreviations: MAP, mean arterial pressure; mCSA, cross-sectional area of the media; NA, noradrenaline; NCC, cell count of the media (nuclear profiles). Results are depicted as mean ± s.e.m.

^a*P* < 0.05 treatment vs. Vehicle.

^bMaximal contractile response induced by NA.

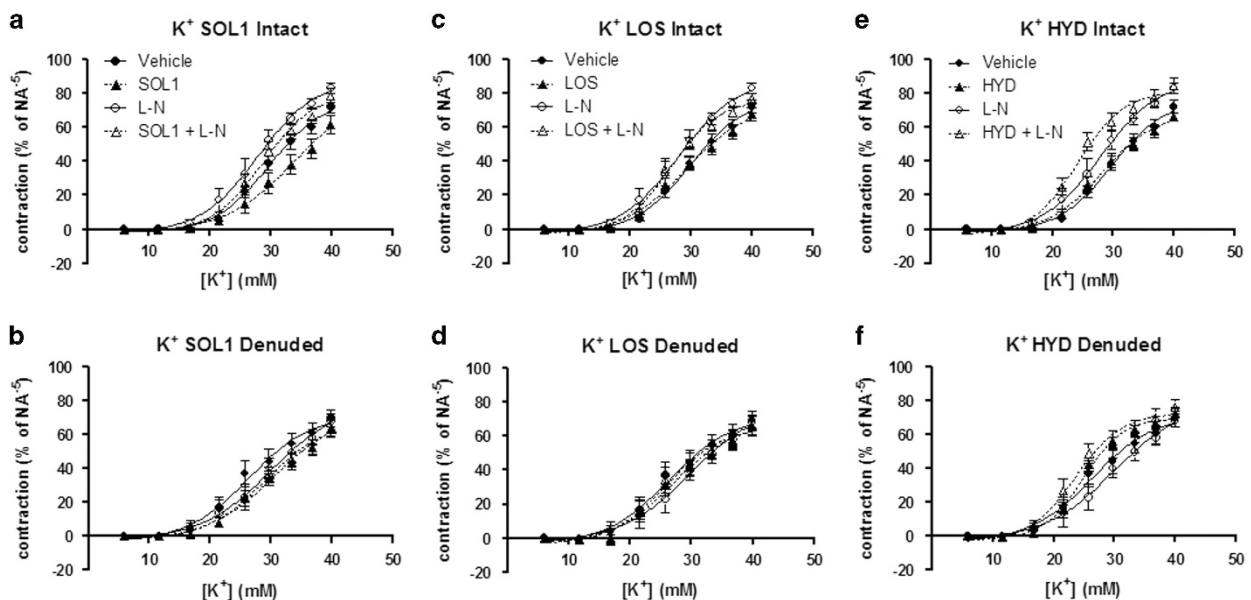


Figure 1 K⁺-induced contractile responses in intact (a, c, e) and denuded (b, d, f) arteries in presence of vehicle (all panels, solid lines and closed circles), SOL1 (a and b, dotted lines and closed triangles), losartan (c and d, dotted lines and closed triangles) or hydralazine (c and d, dotted lines and closed triangles). Open symbols: in the presence of L-NAME (L-N).

SOL1 and hydralazine groups and was significantly decreased in the losartan group (Table 1). Optimal diameter of third-order mesenteric resistance arteries was reduced by chronic treatment with losartan but was not affected by the other drugs. The cross-sectional area of the media and media-to-lumen ratio of third-order mesenteric resistance arteries were comparable in vehicle-, SOL1-, losartan- and hydralazine-treated animals. The maximal contractile response to NA was not altered in the losartan and hydralazine groups and tended to be increased in the SOL1-treated group (Table 1).

Effects of chronic treatments on K⁺-induced arterial contractions

In third-order mesenteric arteries, the pEC₅₀ and E_{max} of K⁺-induced contraction did not differ between treatment groups (Figure 1, Table 2). L-NAME did not modify the pEC₅₀ but increased E_{max} in all groups (saline: 72.3 ± 3.8% to 83.3 ± 3.0%; SOL1: 61.7 ± 5.5% to 78.9 ± 4.4%; losartan: 67.9 ± 4.1% to 77.3 ± 3.0%; hydralazine: 66.3 ± 2.2% to 84.7 ± 4.8%).

Endothelial denudation or bosentan (10 µM, non-selective ET_{A/B} antagonist) did not alter K⁺-induced contractile responses (Table 2).

In denuded arteries in all four treatment groups, L-NAME did not increase K⁺-induced contractions either (Figure 1, Table 2).

Effects of chronic treatments on phenylephrine-induced contractile responses

Sensitivity to PHE was significantly reduced by treatment with SOL1 or losartan (pEC₅₀, vehicle: 5.69 ± 0.03, SOL1: 5.53 ± 0.02; losartan: 5.50 ± 0.05) but was increased by treatment with hydralazine (pEC₅₀: hydralazine: 5.90 ± 0.03). L-NAME significantly increased the sensitivity to PHE in intact arteries of SOL1-treated animals (5.75 ± 0.02) but had no effect in arteries from vehicle-, losartan- or hydralazine-treated groups. Similarly, mechanical removal of the endothelium increased the sensitivity to PHE in SOL1-treated animals (pEC₅₀: 5.68 ± 0.07) but not in the vehicle-, losartan- or hydralazine-treated groups. L-NAME had no effects in denuded arteries (Figure 2, Table 3).

Bosentan decreased the sensitivity to PHE in arteries of SOL1-treated SHR in absence and presence of L-NAME (-L-NAME: 5.35 ± 0.03; +L-NAME: 5.65 ± 0.02). No effects of bosentan on

Table 2 pEC₅₀ and E_{max} of contractile responses to increasing concentrations of K⁺

	Vehicle	SOL-1	Losartan	Hydralazine
<i>Control</i>				
pEC ₅₀	1.52 ± 0.03	1.43 ± 0.10	1.52 ± 0.03	1.53 ± 0.03
E _{max} (%)	72.3 ± 3.8	61.7 ± 5.5	67.9 ± 4.1	66.3 ± 2.2
<i>+ L-NAME</i>				
pEC ₅₀	1.55 ± 0.02	1.53 ± 0.02	1.57 ± 0.01	1.60 ± 0.01
E _{max} (%)	83.3 ± 3.0 ^a	78.9 ± 4.4 ^a	77.3 ± 3.0	84.71 ± 4.8 ^a

Abbreviation: NA, noradrenaline.
Amplitude of contraction is expressed as % of the maximal response to NA. Results are shown as mean ± s.e.m.
^aP < 0.05 without vs. with L-NAME.

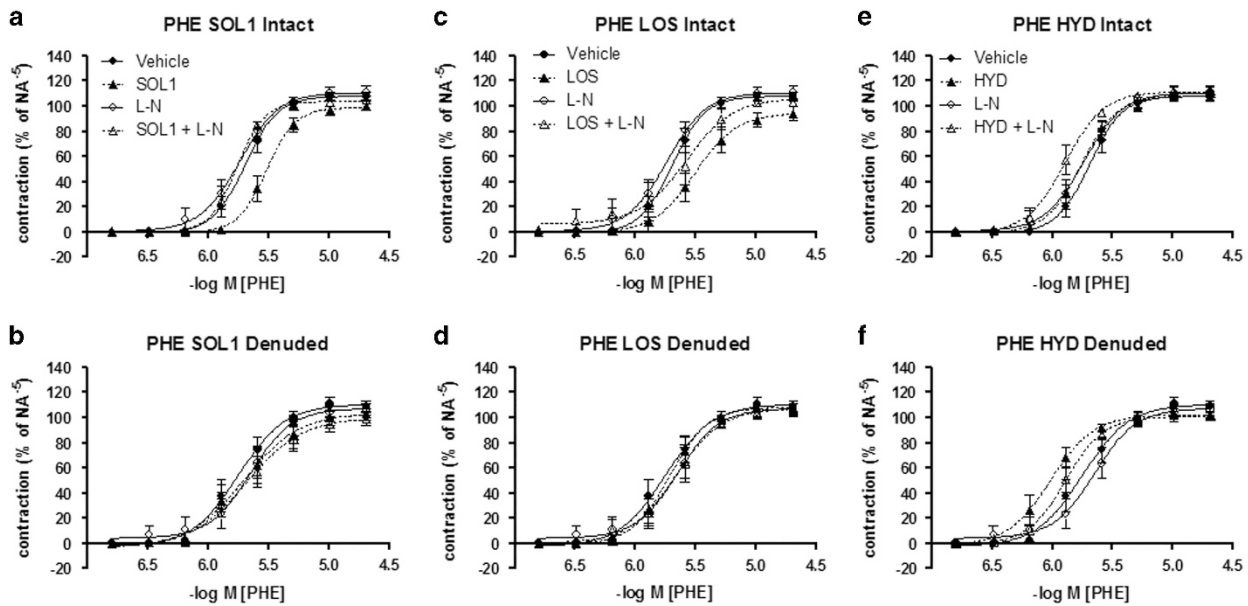


Figure 2 Contractile responses to PHE in intact (a, c, e) and denuded (b, d, f) arteries with vehicle (all panels, solid lines and closed circles), SOL1 (a and b, dotted lines and closed triangles), losartan (c and d, dotted lines and closed triangles) or hydralazine (c and d, dotted lines and closed triangles). Open symbols: in presence of L-NAME (L-N).

Table 3 pEC₅₀ and E_{max} as measured during PHE-induced contractile responses

	Vehicle	SOL-1	Losartan	Hydralazine
<i>Control</i>				
pEC ₅₀	5.69 ± 0.02	5.54 ± 0.03 ^a	5.50 ± 0.05 ^a	5.90 ± 0.03 ^a
E _{max} (%)	107.9 ± 3.5	100.3 ± 3.2	94.3 ± 5.3	110.9 ± 2.7
<i>+ L-NAME</i>				
pEC ₅₀	5.71 ± 0.04	5.75 ± 0.02 ^b	5.58 ± 0.08	5.75 ± 0.02
E _{max} (%)	111.0 ± 5.5	105.4 ± 3.2	103.0 ± 3.3	112.0 ± 3.3

Results are depicted as mean ± s.e.m.
^aP < 0.05 treatment vs. Vehicle.
^bP < 0.05 without vs. with L-NAME.

PHE-induced contractile responses were observed in the vehicle-, losartan- or hydralazine-treated groups. Maximal responses to PHE were not modified by the chronic drug treatments and were not altered by L-NAME, bosentan or endothelium removal (Figure 2, Table 3).

Effects of chronic NEP/ECE inhibition and anti-hypertensive treatments on arterial responses to acetylcholine

To assess endothelium-dependent responses, arteries were contracted with PHE and exposed to increasing concentrations of ACh (0.001–10 μM). In third-order mesenteric resistance arteries of 32-week-old SHR, pEC₅₀ and E_{max} to ACh-induced relaxations did not differ significantly between treatment groups (Figure 3a). Notably, E_{max} tended to be decreased by chronic hydralazine treatment and tended to be increased by chronic losartan and SOL1 treatment. Maximal responses to ACh were not modified by acute bosentan administration (slight increase; Figure 3a and b). However, E_{max} to ACh-induced relaxations were significantly increased in all groups by acute INDO administration (Figure 3a–d). This effect is due to reduction by the COX inhibitor of the contractile response that is observed with high concentrations of ACh.

Effects of chronic NEP/ECE inhibition and anti-hypertensive treatments on EDHF-related arterial responses

To assess the contribution of EDHF to ACh (0.001–10 μM) induced relaxing responses, arteries were contracted with PHE (20 μM) in presence of INDO (to prevent formation of vasodilator and

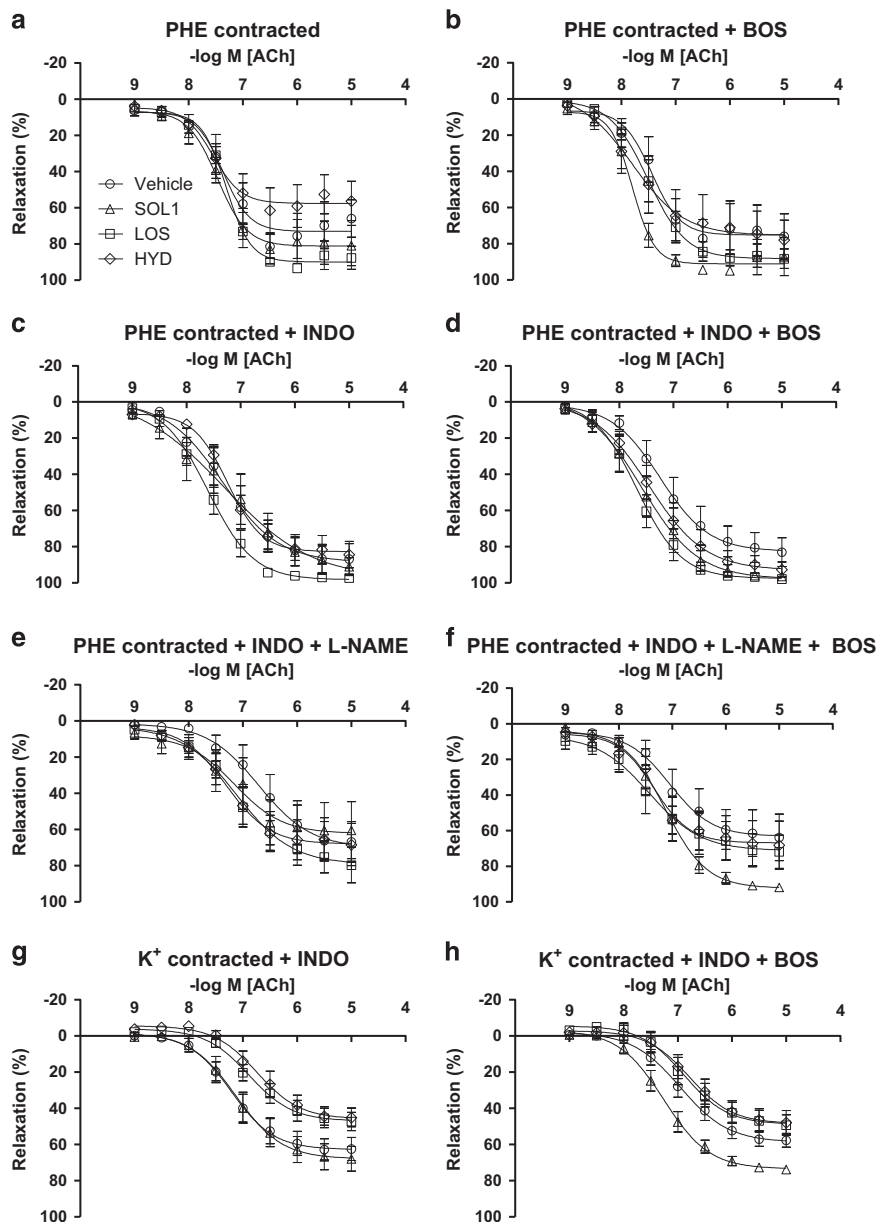


Figure 3 Relaxing responses of mesenteric resistance arteries of SHR to ACh in absence or presence of INDO. The top panels depict responses in absence and presence of INDO and absence (a, c) and presence (b, d) of bosentan (BOS) with vehicle (open circles), SOL1 (open triangles), losartan (open squares) or hydralazine (open diamonds). The middle panels depict responses to ACh during PHE-induced contractions in presence of INDO and L-NAME (EDHF-related responses) in absence (e) and presence (f) of BOS. The bottom panels depict responses to ACh during K⁺-induced contraction in presence of INDO, in absence (g) and presence (h) of BOS (EDNO-related responses). EDHF, endothelium-derived hyperpolarizing factor; EDNO, endothelium-derived nitric oxide; INDO, indomethacin; PHE, phenylephrine.

vasoconstrictor prostaglandins) and L-NAME (to prevent involvement of NO). Obviously, the remaining ACh-induced response was mediated by EDHF.

Sensitivity and maximal response did not differ significantly between groups. Bosentan significantly increased maximal EDHF-type responses in the SOL1-treated animals ($57.0 \pm 13.1\%$ to $91.9 \pm 2.0\%$) but not in the vehicle-, losartan- or hydralazine-treated animals. In the presence of bosentan, maximal EDHF-type responses were significantly larger in the SOL1-treated animals compared to vehicle ($63.9 \pm 13.1\%$ to $91.9 \pm 2.0\%$; Figure 3e and f; Table 4).

Effects of chronic NEP/ECE inhibition and anti-hypertensive treatments on arterial responses to endothelium-derived NO

To investigate the contribution of NO to ACh-induced (0.001 – $10 \mu\text{M}$) relaxing responses, arteries contracted with 40 Mm K^+ in the presence of INDO (to prevent formation of vasodilator and vasoconstrictor prostaglandins). A high extracellular K⁺ concentration effectively prevents EDHF-related responses.²⁸ The remaining ACh-induced response was abolished in the presence of L-NAME and was thus considered to be due to endothelium-derived NO (EDNO). Sensitivity and maximal responses did not differ significantly between groups. The maximal ACh-induced EDNO response in presence of bosentan

Table 4 pEC₅₀ and E_{max} of the relaxing response induced by increasing concentrations of ACh

	Vehicle	SOL1	Losartan	Hydralazine
EDNO				
Control				
pEC ₅₀	7.18±0.15	7.11±0.18	6.89±0.12	6.71±0.13
E _{max} (%)	62.6±6.4	68.0±6.8	47.4±5.1	45.1±5.2
Bosentan				
pEC ₅₀	6.95±0.11	7.22±0.08	6.88±0.15	6.78±0.20
E _{max} (%)	58.4±4.3	71.0±3.4 ^a	49.4±5.7	48.0±6.7
EDHF				
Control				
pEC ₅₀	6.72±0.28	7.14±0.39	7.04±0.19	7.31±0.25
E _{max} (%)	66.9±11.2	60.43±15.8	79.8±8.9	68.4±11.7
Bosentan				
Log EC ₅₀	7.04±0.27	7.13±0.10	7.43±0.31	7.31±0.25
E _{max} (%)	63.9±13.1	91.86±2.0 ^a	71.9±9.4	68.1±13.1

Abbreviations: EDHF, endothelium-derived hyperpolarizing factor (precontraction with 20 μM PHE in presence of L-NAME); EDNO, endothelium-derived nitric oxide (precontraction with 40 mM K⁺).

Results are depicted as mean±s.e.m.

^aP<0.05 treatment vs. Vehicle.

was increased in SOL1-treated animals vs. vehicle-treated animals (58.4±4.2% to 71.0±3.4%) and was unaltered in losartan- or hydralazine-treated groups (Figure 3g and h; Table 4).

DISCUSSION

This study demonstrates that chronic SOL1 treatment can restore several aspects of endothelial function, even though an effect on blood pressure was minimal. In contrast, more classic anti-hypertensive drugs, such as losartan or hydralazine, were both effective in lowering blood pressure but had little to no effect on endothelial dysfunction, even though losartan succeeded in partly reversing the resistance artery structural changes associated with hypertension.

ET-1 is involved in the pathophysiology of experimental and clinical pulmonary hypertension, chronic renal failure and heart failure. The situation in essential hypertension is, however, less clear.²⁹ In SHR, the role of the renin-angiotensin system is well established.³⁰ A potential role of the endothelin system in the arterial functional and structural changes is, however, less clear.³¹ Montagnani *et al.*³² reported that while there is no difference in ET-1-induced contractions between 5-week-old SHR and Wister Kyoto rats, they noted a marked increase in ET-1-induced contractile responses in 12-week-old SHR. Furthermore, our group reported increased levels of ET-1 in several organs of 8- and 12-week-old SHR.¹¹ This points to a transient upregulation of the endothelin system during the development of hypertension. ET-1 is not only a very potent vasoconstrictor¹² but is also a pro-inflammatory agent,¹⁵ has mitogenic effects on vascular smooth muscle cells,¹³ is pro-angiogenic,¹⁶ pro-oxidant³³ and promotes fibrosis.¹⁴ Moreover, endothelium restricted overexpression of ET-1 causes vascular remodeling and endothelial dysfunction in the absence of a hemodynamic effect.³³ The pro-oxidant and pro-inflammatory functions might, at least in some forms of hypertension, be more important than the pressor effect. Because of the aforementioned functions, the ET-1 system emerges as a potential target for normalizing functional and structural arterial properties that are associated with hypertension.

We tested the hypothesis that ET-1 contributes to endothelial dysfunction, arterial remodeling and hypertension in 32-week-old SHR. To evaluate this, we treated adult SHR with the dual NEP/ECE inhibitor SOL1, which effectively decreases big-ET-1 to ET-1 conversion,³⁴ and compared the effects with a 4-week treatment with an AT₁ receptor antagonist (losartan) and a 4-week treatment with a vasodilator (hydralazine). Decreasing ET-1 production might be helpful in conditions where either the intravascular levels of ET-1 and/or the vascular effects of the peptide are increased.^{32,35–37} Moreover, inhibiting NEP might have added beneficial value by chronically inhibiting the breakdown of other vasoactive peptides, such as natriuretic peptides and calcitonin gene-related peptide.^{38–41} We investigated both structural and functional effects of SOL1 in mesenteric resistance arteries.

ET-1 has been shown to contribute to remodeling of large and small arteries in hypertension. Hypertrophic remodeling rather than eutrophic remodeling of resistance arteries, seems to occur in models associated with an upregulated ET system suggesting that ET-1 system upregulation leads to vascular hypertrophy.⁴²

In the current study, we observed no significant change in diameter, media thickness, cross-sectional area of the media, nuclear cell count or media-to-lumen ratio in first-order mesenteric resistance arteries of 32-week-old SHR caused by reducing blood pressure by means of a 4-week treatment with a vasodilator (hydralazine). This means that a decrease in blood pressure alone cannot reverse structural changes in these arteries. These observations are consistent with earlier reports.^{43–45}

Chronic losartan treatment did not cause a significant increase in diameter or a decrease in cross-sectional area of the media or, the nuclear cell count in first-order mesenteric arteries of SHR. These arteries do, however, show a decrease in media-to-lumen ratio. Despite differences in experimental design and treatment timing, our findings are similar to earlier reports from other groups.^{8,43,44,46} Studies with chronic AT₁ receptor antagonists have shown that treatment for a longer period than 4 weeks is superior for establishing beneficial effects on vascular remodeling.⁴⁷

In contrast to chronic losartan treatment, SOL1 treatment was not successful in reducing blood pressure or arterial structural changes. The absence of an anti-hypertensive effect is consistent with observations made by Kalk *et al.*⁴⁸, who did not observe a blood pressure reduction with daglutril (SLV338; a SOL1 analog)²¹ in the 2-kidney 1-clip model. Similarly, Sharkovska *et al.* did not observe a blood pressure reduction with daglutril in rats chronically treated with L-NAME. In addition, as with losartan, a 4-week treatment with the NEP/ECE inhibitor might have been too short to fully correct structural changes in resistance arteries.

To assess arterial and endothelial function and the effects of a chronic SOL1 treatment, we investigated third-order mesenteric resistance arteries *in vitro*. Functional properties of resistance-sized arteries are often altered in hypertension in relation to dysfunctional endothelium. Endothelial dysfunction is frequently regarded as a reduced bioavailability or function of EDNO, blunted EDHF responses and hypersensitivity to vasoconstrictors.⁴⁹ In SHR, specifically EDCFs are described as a manifestation of endothelial dysfunction and these EDCF-mediated contractions in response to stimulation of the artery with ACh are fully inhibited by a cyclo-oxygenase inhibitor (INDO). In 32-week-old SHR investigated in this study, a moderate EDCF component was observed. The prostanoids PGH₂, PGI₂ and thromboxane A₂ (TXA₂) have been suggested to be responsible for increased vascular tone in hypertension.⁵⁰ Recently, Spijkers *et al.* reported endothelium-dependent contractions as a result of increased TXA₂ release in arteries of SHR. Moreover, they observed an increased expression of COX-1 in smooth

muscle cells of SHR compared to Wister Kyoto rats and an increased expression of iPLA₂ (substrate delivery to COX-1) and TXA₂-synthase in the endothelium of SHR compared to Wister Kyoto rats.^{22,51} In the present study, the EDCF response (indicated by a reduced maximal relaxing response to ACh) was abolished by cyclo-oxygenase inhibition and moderately inhibited by acute ET-receptor antagonism and by chronic losartan or SOL1 treatment.

No effects of hydralazine, losartan or SOL1 were observed on arterial smooth contractile responses to K⁺-induced depolarization. Sensitivity to smooth muscle activation was similar in all groups. Except for the hydralazine-treated animals, there was no effect of L-NAME on pEC₅₀ or E_{max}. Normally, NO synthase inhibition causes a leftward shift in the concentration–response curve and an increased maximal response.⁵² These effects are absent in the arteries investigated in this study, meaning that the NO production under basal conditions (for example, without agonist-induced stimulation) is blunted. None of the treatments could restore the L-NAME induced leftward shift and only hydralazine restored the increase in maximal contraction induced by L-NAME.

A similar L-NAME-induced leftward shift can be expected for PHE-induced concentration–response curves.^{25,52} Again, this shift was completely absent in vehicle-, losartan- or hydralazine-treated groups. After 4 weeks of chronic dual NEP/ECE inhibition, however, L-NAME caused a significant leftward shift. This can be considered an improvement of basal endothelial function because to some extent chronic SOL1 treatment succeeds in restoring basal NO production. The molecular mechanism of this effect of a 4-week treatment with the NEP/ECE inhibitor might involve changes in the dimerization and phosphorylation of endothelial NO synthase, but this remains to be established.⁵³

In this study, the agonist-induced EDNO-type responses were not significantly altered by any of the chronic treatments. Surprisingly, acute administration of the non-selective ET_{A/B} receptor antagonist bosentan improved the maximal EDNO-type responses in SOL1-treated animals. This might indicate that intra-arterial production of ET-1 is rapidly restored *in vitro* after wash-out of the ECE/NEP inhibitor that had been administered for 4 weeks. Another EDRF, EDHF causes endothelial hyperpolarization with subsequent vascular smooth muscle cell hyperpolarization. The contribution of EDHF is most marked in small arteries.^{24,54} In mesenteric resistance arteries, this response is blocked by depolarization with high extracellular [K⁺] or inhibitors of endothelial small- and intermediate-conductance calcium-activated K⁺ channels.^{24,28,55,56} EDHF-type responses were not significantly affected by SOL1, losartan or hydralazine treatment. Acute administration of the non-selective ET_{A/B} receptor antagonist bosentan improved the maximal EDHF-type relaxing responses in SOL1-treated animals. This means that both EDNO- and EDHF-type responses in the SOL1-treated group were improved by acute ET-receptor antagonism. Because SOL1 is easily removed from organs,²⁰ conversion of big-ET-1 to ET-1 may recommence before endothelial function is investigated. Subsequently, ET-1 might impair endothelium-derived relaxing responses through a similar myo-endothelial coupling mechanism, as proposed by Hilgers and De Mey.²⁵ This might explain the acute bosentan effect on the EDHF response in the SOL1-treated animals.

In conclusion, chronic treatment with an AT₁ receptor antagonist in 32-week-old SHR caused regression of the changes in arterial structure and a significant lowering of the blood pressure. Chronic dual NEP/ECE inhibition, on the other hand, caused an improvement of several aspects of endothelial function. While the effects of losartan and SOL1 are different, they could be considered beneficial in both

instances. A combination of an AT₁ receptor antagonist, or alternatively an inhibitor of angiotensin converting enzyme (ACE), with a dual NEP/ECE inhibitor could provide superior results and might be subject of future studies. Triple vasopeptidase (ACE/ECE/NEP) inhibitors are becoming available (for review, Duall *et al.*⁵⁷) and deserve detailed investigation in terms of anti-hypertensive effects and restoration of structural and endothelial properties in resistance arteries.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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