

## ORIGINAL ARTICLE

# Impaired flow-induced arterial remodeling in DOCA-salt hypertensive rats

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Arteries from young healthy animals respond to chronic changes in blood flow and blood pressure by structural remodeling. We tested whether the ability to respond to decreased (−90%) or increased (+100%) blood flow is impaired during the development of deoxycorticosterone acetate (DOCA)-salt hypertension in rats, a model for an upregulated endothelin-1 system. Mesenteric small arteries (MrA) were exposed to low blood flow (LF) or high blood flow (HF) for 4 or 7 weeks. The bioavailability of vasoactive peptides was modified by chronic treatment of the rats with the dual neutral endopeptidase (NEP)/endothelin-converting enzyme (ECE) inhibitor SOL1. After 3 or 6 weeks of hypertension, the MrA showed hypertrophic arterial remodeling (3 weeks: media cross-sectional area (mCSA):  $10 \pm 1 \times 10^3$  to  $17 \pm 2 \times 10^3 \mu\text{m}^2$ ; 6 weeks:  $13 \pm 2 \times 10^3$  to  $24 \pm 3 \times 10^3 \mu\text{m}^2$ ). After 3, but not 6, weeks of hypertension, the arterial diameter was increased ( $\emptyset$ :  $385 \pm 13$  to  $463 \pm 14 \mu\text{m}$ ). SOL1 reduced hypertrophy after 3 weeks of hypertension (mCSA:  $6 \times 10^3 \pm 1 \times 10^3 \mu\text{m}^2$ ). The diameter of the HF arteries of normotensive rats increased ( $\emptyset$ :  $463 \pm 22 \mu\text{m}$ ) but no expansion occurred in the HF arteries of hypertensive rats ( $\emptyset$ :  $471 \pm 16 \mu\text{m}$ ). MrA from SOL1-treated hypertensive rats did show a significant diameter increase ( $\emptyset$ :  $419 \pm 13$  to  $475 \pm 16 \mu\text{m}$ ). Arteries exposed to LF showed inward remodeling in normotensive and hypertensive rats (mean  $\emptyset$  between 235 and 290  $\mu\text{m}$ ), and infiltration of monocyte/macrophages. SOL1 treatment did not affect the arterial diameter of LF arteries but reduced the infiltration of monocyte/macrophages. We show for the first time that flow-induced remodeling is impaired during the development of DOCA-salt hypertension and that this can be prevented by chronic NEP/ECE inhibition.

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**Keywords:** dual NEP/ECE inhibition; endothelin-1; resistance artery; vascular hypertrophy; salt-sensitive hypertension

## INTRODUCTION

Blood flow is an important regulator of vascular function and structure. An acute alteration in blood flow initially leads to a change in vasomotor tone, which modifies the arterial diameter to normalize wall shear stress. When the increase in blood flow is sustained, arteries can structurally remodel to restore normal values of wall shear stress and circumferential wall stress.<sup>1</sup> Both flow-mediated dilatation and flow-mediated remodeling are endothelium-dependent processes.<sup>2,3</sup> Structural remodeling of the arterial wall in response to hemodynamic changes can occur under both physiological and pathological conditions, for example, in the uterine vascular bed during pregnancy and in hypertension, respectively.<sup>4–6</sup>

Because in established hypertension cardiac output is within the normal range, the increased blood pressure is mainly due to increased vascular resistance.<sup>7</sup> This is associated with an increased wall to lumen ratio in the systemic resistance arteries. In essential hypertension, this arterial remodeling is usually described as inward and eutrophic, that is, resulting from rearrangement of the medial material around a smaller diameter.<sup>8</sup> The arterial changes in secondary and experimental

hypertension, on the other hand, are mainly hypertrophic in nature and involve smooth muscle cell hypertrophy and/or hyperplasia.<sup>8,9</sup> Remodeling of the vascular wall in hypertension is also associated with changes in the composition of the extracellular matrix leading to increased stiffness.<sup>10</sup>

Although anti-hypertensive therapy is mainly focused on lowering the blood pressure, preventing or reversing the arterial structural changes might be more beneficial for the morbidity and mortality of hypertensive patients.<sup>11</sup> Angiotensin II is thought to have an important role in the pathological alterations in the vascular wall, as is supported by the partial reversal of these alterations by chronic treatment with inhibitors of angiotensin-converting enzyme or antagonists of AT1-receptors.<sup>12,13</sup> Endothelin-1 (ET1), another vasoactive peptide, might also be involved in the arterial structural changes in pathophysiological conditions. Apart from being a potent vasoconstrictor, ET1 is also a potent growth-promoting and proinflammatory agent.<sup>14–18</sup> Production of ET1 is reported to be increased in salt-dependent models of hypertension such as deoxycorticosterone acetate (DOCA)-salt-induced hypertension, a

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model showing pronounced endothelial dysfunction<sup>19</sup> and arterial hypertrophy.<sup>20,21</sup> ET1 can be formed through cleavage of big ET1 by endothelin-converting enzyme (ECE). Alternatively big ET1 can be hydrolyzed by chymase or MMP-2 to form ET1 (1-31) or ET1 (1-32), respectively. Both are further processed into ET1 by neutral endopeptidase (NEP).<sup>22–24</sup> Thus, a dual inhibitor of NEP and ECE reduces ET1 production by inhibiting all three pathways.<sup>25</sup> Recently Kalk *et al.*<sup>26</sup> reported that the dual NEP/ECE inhibitor SLV338 reduces smooth muscle hypertrophy, interstitial fibrosis and perivascular fibrosis in the two kidney one clip model, partly by suppression of TGF- $\beta$ 1 expression.

Here we tested the hypotheses that (1) flow-induced remodeling of resistance arteries is reduced during the development of DOCA-salt hypertension and that (2) treatment with a dual NEP/ECE inhibitor has beneficial effects on flow-induced arterial remodeling in this setting.

## METHODS

### Animals, model and surgery

Ten-week-old Wistar rats (Charles River, Maastricht, The Netherlands) were used in all experiments that were performed in accordance with the ethical committee for animal welfare of the Maastricht University.

All animals were anesthetized with isoflurane (1–4%) and the left kidney was removed. Blood flow-modifying surgery was performed in the animals by distal ligation of alternate first-order mesenteric arteries to create arteries chronically exposed to low blood flow (LF). The patent arteries between these then had a compensatory increased blood flow (HF). Arteries from a distant part of the intestine were considered control arteries exposed to normal flow (NF).<sup>9,27</sup> After a 1-week recovery period, a DOCA pellet (100 mg, Sigma Aldrich, Zwijndrecht, The Netherlands) ( $n=28$ ) or a control silicon pellet ( $n=29$ ) was implanted subcutaneously. All animals were given high-salt drinking water (1% NaCl and 0.2% KCl). In total, 16 normotensive rats and 14 hypertensive rats were investigated after 3 weeks and 13 normotensive and 14 hypertensive rats after 6 weeks.

Intra-arterial blood pressure was measured in conscious unrestrained rats via a heparinized (5 U ml<sup>-1</sup>) indwelling polyethylene 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, Honeywell, Amsterdam, The Netherlands) 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, Maastricht, The Netherlands).

Preliminary dose-finding experiments were carried out to identify appropriate doses 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](methyl)amino]-4-oxobutanoic acid; Abbott<sup>25</sup>). A dose of 50 mg kg<sup>-1</sup> per day drastically decreased urinary ET1 content (from 32.89  $\pm$  3.91 to 1.35  $\pm$  0.25 pg ml<sup>-1</sup>,  $P<0.001$ ) in DOCA-salt hypertensive rats ( $n=3$ ). Animals were randomly assigned to the treatment groups ( $n=7$ ). Osmotic minipumps (2ML4, Alzet, Cupertino, CA, USA) were implanted subcutaneously, simultaneously with the DOCA pellet, for chronic continuous drug treatment over the next 3 weeks. The vehicle group received osmotic minipumps containing a saline solution for the same period of time.

### Determination of arterial remodeling

Three or 6 weeks after the induction of hypertension, animals were euthanized with isoflurane (>4%) and arteries exposed *in vivo* to HF or LF conditions were isolated. Arteries located outside the region of ligation were used as normal flow controls (NF). Arteries were mounted on two glass micropipettes in an organ chamber filled with Ca<sup>2+</sup>-free Krebs Ringer solution (144 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 14.9 mM HEPES and 5.5 mM glucose; pH 7.4). Ten micromolar Na-nitroprusside (SNP, Sigma Aldrich) 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). After the experiment, vessels were fixed at 80 mm Hg in 4% phosphate-buffered formaldehyde solution for 1 h and stored in 70% ethanol.

Arterial distensibility was calculated by:  $DC = (1/\Delta P) \times (\Delta D/D_{n-1}) \times 100$  with DC = distensibility,  $P$  = intraluminal pressure and  $D$  = measured diameter.<sup>28</sup> A linear regression was performed on the pressure–distensibility curves between 20 and 80 mm Hg for every artery considered and the slope was calculated as an index of arterial stiffness.

### Histological analyses

Fixed vessels were embedded in paraffin and cross-sections (4  $\mu$ m) were stained with Lawson's solution (Boom, Meppel, The Netherlands). Media cross-sectional area (mCSA) 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 (# nuclei). The elastin density was determined in four areas of the media and four areas of the adventitia in every vessel using Leica Qwin software (Leica, Groot Bijgaarden, Belgium). Measurements were averaged for every layer individually in every vessel. Elastin content was calculated as percentage elastin-positive area per total area. To stain collagen, cross-sections were deparaffinized and incubated in phosphomolybdic acid (0.2%) for 5 min, followed by incubation with Sirius red. After washing with 0.1 M HCl for 2 min, sections were dehydrated and protected with coverslips. The content of collagen in the media and adventitia was quantified as described as for elastin.

To identify smooth muscle cells and monocyte/macrophages, immunoperoxidase staining was performed with primary monoclonal antibodies directed against smooth muscle  $\alpha$ -actin and rat monocyte/macrophages, respectively (ASMA (A-2547) and CD-68 (ED-1)).<sup>29</sup> (Sigma Aldrich, dilution 1:5000). After incubation with these monoclonal antibodies, the sections were incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako, Enschede, The Netherlands). Visualization was performed with DAB as a substrate.

### Statistical analysis

Results are expressed as means  $\pm$  s.e.m. Statistical significance of differences between groups was evaluated by analysis of variance (ANOVA for consecutive measurements for pressure–diameter curves) or one-way ANOVA followed by Bonferroni or paired *t*-test (graphpad 5.0). A value of  $P<0.05$  was considered statistically significant.

## RESULTS

### General characteristics

In normotensive rats, body weight and MAP did not change significantly between 14 and 17 weeks of age (Table 1). DOCA-salt treatment did not modify body weight but increased MAP after 3 weeks and to a greater extent after 6 weeks (Table 1). Administration of the dual NEP/ECE inhibitor SOL1 (50 mg kg<sup>-1</sup> per day) to DOCA-salt rats for 3 weeks did not modify body weight but attenuated the increase in MAP (Table 1).

In normotensive rats, the size of mesenteric small arteries (MrA) tended to increase between 14 and 17 weeks of age (Figures 1a,b and 2). The increases in lumen diameter ( $\varnothing$ : 385  $\pm$  13 to 414  $\pm$  20  $\mu$ m) in mCSA (10  $\pm$  1 to 13  $\pm$  2  $\times$  10<sup>3</sup>  $\mu$ m<sup>2</sup>) and in the number of nuclear profiles per medial section (# nuclei: 45  $\pm$  3 to 49  $\pm$  5) did, however, not reach statistical significance.

### Resistance artery remodeling in DOCA-salt hypertensive rats

MrA diameter was significantly increased after 3 weeks of DOCA-salt hypertension ( $\varnothing$ : 385  $\pm$  13 to 452  $\pm$  12  $\mu$ m,  $P<0.05$ ; Figures 1c and 2a). After 6 weeks of hypertension, MrA diameter tended to be increased ( $\varnothing$ : 414  $\pm$  20 to 451  $\pm$  12  $\mu$ m; Figures 1d and 2b) but this was no longer statistically significant compared with arteries from age-matched normotensive rats. After both 3 and 6 weeks of DOCA-salt hypertension, significant media hypertrophy was observed

**Table 1** General animal characteristics and overview of structural parameters

General characteristics	3 Weeks			6 Weeks	
	NT	HT	HT+ SOL1	NT	HT
Body weight (g)	353 ± 6	331 ± 6	359 ± 9	368 ± 12	338 ± 8
MAP (mm Hg)	118 ± 2	150 ± 5***	136 ± 2&	114 ± 3	173 ± 3***
<i>Diameter (μm)</i>					
Normal flow	385 ± 13	452 ± 12*	419 ± 13	414 ± 20	451 ± 12
High flow	463 ± 22###	471 ± 16	475 ± 16#	455 ± 21	486 ± 12
Low flow	270 ± 10###	290 ± 14###	253 ± 6###	265 ± 14###	265 ± 14###
<i>mCSA (10<sup>3</sup> μm<sup>2</sup>)</i>					
Normal flow	10 ± 1	16 ± 1**	14 ± 2	13 ± 2	24 ± 3*
High flow	15 ± 2#	16 ± 1	14 ± 2	14 ± 1	21 ± 2
Low flow	11 ± 3	12 ± 2#	7 ± 1&	11 ± 3	16 ± 2
<i># Nuclear profiles (media)</i>					
Normal flow	45 ± 3	58 ± 4	42 ± 3&	49 ± 5	66 ± 7
High flow	56 ± 3#	53 ± 5	45 ± 4	41 ± 3	53 ± 4
Low flow	65 ± 8#	57 ± 9	42 ± 4	40 ± 4	90 ± 13
<i>Stiffness factor (mm Hg<sup>-2</sup>)</i>					
Normal flow	-0.034 ± 0.002	-0.022 ± 0.002***	-0.021 ± 0.002	-0.031 ± 0.002	-0.016 ± 0.002***
High flow	-0.031 ± 0.002	-0.018 ± 0.002***	-0.019 ± 0.002	-0.027 ± 0.003	-0.017 ± 0.002**
Low flow	-0.003 ± 0.001###	-0.004 ± 0.001###	-0.002 ± 0.001###	-0.006 ± 0.001###	-0.001 ± 0.001###
<i>Elastin/collagen ratio</i>					
Normal flow	2.7 ± 0.8	1.3 ± 0.5	0.8 ± 0.5	5.7 ± 2.3	1.8 ± 0.3
High flow	2.1 ± 0.5	2.0 ± 0.7	3.5 ± 1.2	2.74 ± 0.6	1.8 ± 0.5
Low flow	9.1 ± 6.3	1.3 ± 0.4	2.8 ± 1.1	4.0 ± 0.9	6.0 ± 3.0#
<i>% ED-1-positive area</i>					
Normal flow	0.02 ± 0.01	0.17 ± 0.11	0.08 ± 0.02	0.03 ± 0.01	0.02 ± 0.01
High flow	0.03 ± 0.01	0.29 ± 0.11	0.11 ± 0.05	0.01 ± 0.01	0.03 ± 0.01
Low flow	1.24 ± 0.56#	5.89 ± 3.10##	0.24 ± 0.09&	0.38 ± 0.23	1.41 ± 0.63#

Abbreviations: HT, deoxycorticosterone acetate (DOCA)-salt hypertensive rats; MAP, mean arterial pressure; mCSA, media cross-sectional area; NT, normotensive rats; 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](methyl)amino]-4-oxobutanoic acid. Data are shown as mean ± s.e.m.

\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  NT vs. HT; # $P < 0.05$ ; ### $P < 0.001$  vs. NF; & $P < 0.05$  HT vs. HT + SOL1.

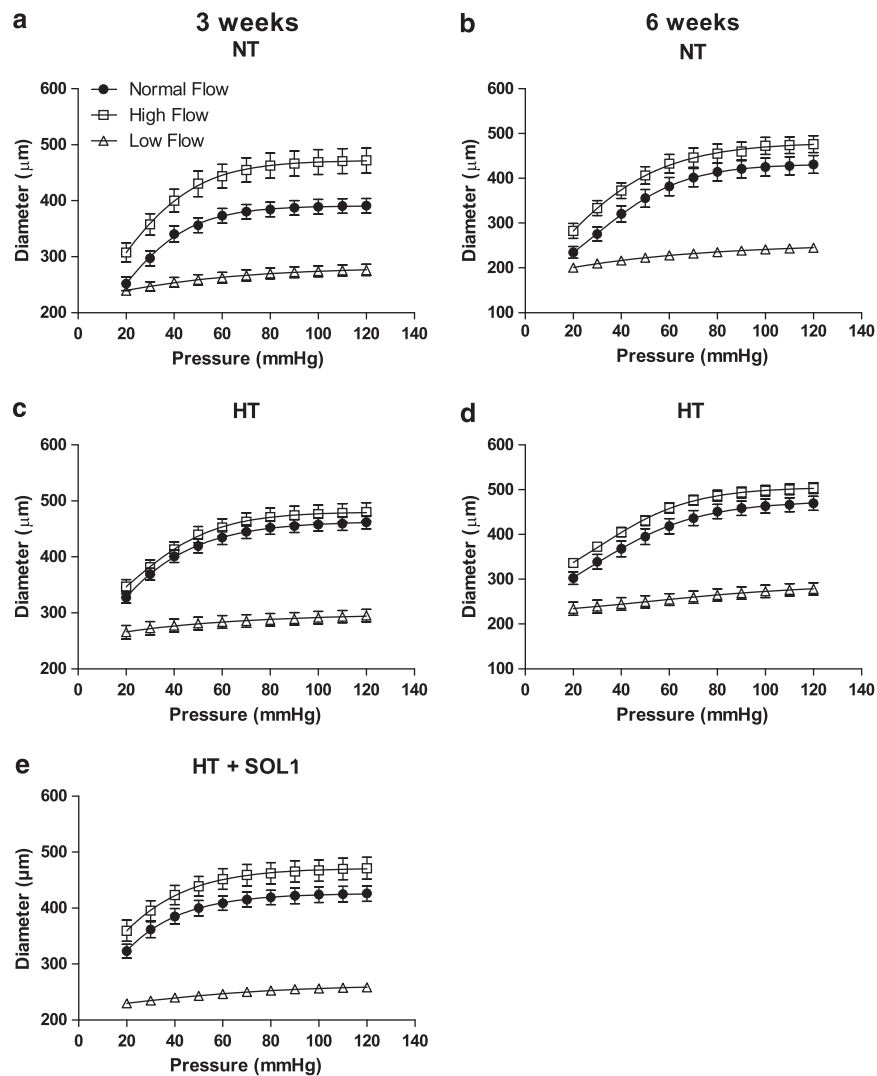
(mCSA: 3 weeks:  $10 \pm 1$  to  $16 \pm 1 \times 10^3 \mu\text{m}^2$ ,  $P < 0.01$ ; 6 weeks:  $13 \pm 2$  to  $24 \pm 3 \times 10^3 \mu\text{m}^2$ ,  $P < 0.01$ ; Figures 2c and d). This was accompanied by hyperplasia and by increased arterial wall stiffness. # nuclei averaged  $45 \pm 3$  and  $58 \pm 4$  at 3 weeks ( $P < 0.05$ ; Figure 2e) and  $49 \pm 5$  and  $66 \pm 7$  at 6 weeks (nonsignificant (NS); Figure 2f) and the stiffness factor averaged  $-0.034 \pm 0.002$  and  $-0.022 \pm 0.002 \text{ mm Hg}^{-2}$  at 3 weeks (Figure 2g) and  $-0.031 \pm 0.002$  and  $-0.016 \pm 0.002 \text{ mm Hg}^{-2}$  at 6 weeks (Figure 2h) in normotensive and hypertensive rats, respectively. The elastin to collagen ratio was, however, neither significantly modified (Table 1) nor there was a significant increase in monocyte/macrophages in the tunica media of MrA from DOCA-salt hypertensive rats (Table 1).

Three weeks treatment of DOCA-salt hypertensive rats with SOL1 tended to attenuate arterial outward remodeling ( $\emptyset$ :  $419 \pm 13 \mu\text{m}$ , NS, Figures 1e and 2a), but did not modify arterial hypertrophy (mCSA:  $14 \pm 2 \times 10^3 \mu\text{m}^2$ , Figure 2c), arterial stiffness ( $-0.021 \pm 0.002 \text{ mm Hg}^{-2}$ , Figure 2g) or elastin to collagen ratio ( $0.81 \pm 0.47$ ). Chronic treatment with the dual NEP/ECE inhibitor did, however, significantly reduce arterial hyperplasia (# nuclei:  $42 \pm 3$ ,  $P < 0.05$  vs. solvent-treated DOCA-salt rats) (Figure 2e).

### Flow-related arterial remodeling in normotensive rats

Increased local blood flow (HF) for 4 weeks in normotensive rats caused an upward shift of the pressure–diameter relationship (Figure 1a) and significantly increased lumen diameter ( $\emptyset$ : to  $463 \pm 22 \mu\text{m}$ ,  $P < 0.001$ ; Figure 2a), medial mass (mCSA: to  $15 \pm 2 \times 10^3 \mu\text{m}^2$ ,  $P < 0.05$ ; Figure 2c) and cellularity at 80 mm Hg (# nuclei: to  $56 \pm 3$ ; Figure 2e) compared with vessels exposed to normal blood flow (NF). This is in line with the flow-induced outward hypertrophic remodeling reported previously.<sup>9,27,30,31</sup> It was, however, not accompanied by alterations in arterial wall stiffness (Figure 2g), the elastin to collagen ratio or the macrophage count (Table 1). Surprisingly, after 7 weeks exposure to elevated blood flow, the outward remodeling (Figure 1b) and the increases in lumen diameter ( $\emptyset$ : to  $455 \pm 21 \mu\text{m}$ , NS, Figure 2b), in medial mass (mCSA: to  $14 \pm 1 \times 10^3 \mu\text{m}^2$ , NS, Figure 2d) and the hyperplasia (# nuclei: to  $41 \pm 3$ , NS; Figure 2f), were no longer statistically significant compared with control (NF) arteries of the same normotensive rats.

Reduced local blood flow (LF) for either 4 or 7 weeks in normotensive rats markedly reduced arterial diameter and markedly increased arterial wall stiffness (Figures 1a,b and 2a,b,g,h) without



**Figure 1** Relation between imposed pressure and diameter after 4 weeks of altered flow in mesenteric resistance arteries of (a) normotensive rats (NT), (c) DOCA-salt hypertensive rats (HT) and (e) HT+SOL1. Relation between imposed pressure and diameter after 7 weeks of altered flow in mesenteric resistance arteries in (b) NT and (d) HT. Data are shown as mean  $\pm$  s.e.m. Normal flow, high flow, low flow: arteries that had been exposed to normal, increased and reduced blood flow *in vivo*, respectively.

significant changes in medial mass (mCSA: to  $11 \pm 1$  and  $11 \pm 3 \times 10^3 \mu\text{m}^2$ , NS, Figures 2c and d) or medial cell number (# nuclei:  $40 \pm 4$  and  $90 \pm 13$ , after 4 and 7 weeks, respectively, NS, Figures 2e and f). At 3 weeks an elevated number of ED-1-positive monocyte/macrophages was observed in the LF vessels of normotensive rats, but this was no longer statistically significant at the later time point (Table 1).

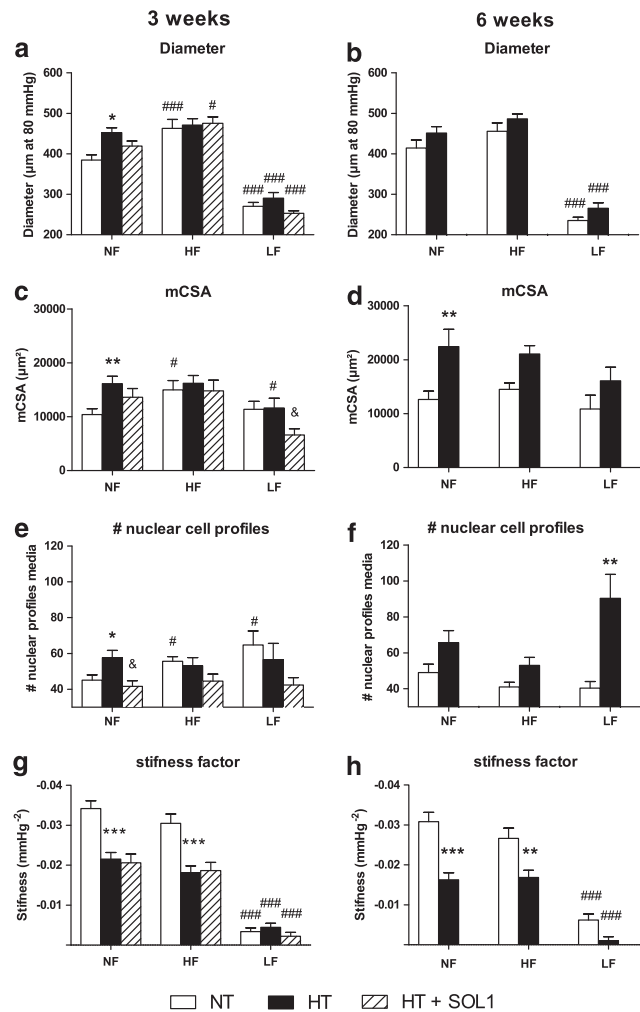
#### Flow-related arterial remodeling in DOCA-salt hypertensive rats

In MrA of DOCA-salt hypertensive rats, no additional outward hypertrophic remodeling occurred in HF arteries after 4 weeks ( $\text{Ø}$ :  $452 \pm 12$  to  $471 \pm 16 \mu\text{m}$ , mCSA:  $16 \pm 1$  to  $16 \pm 1 \times 10^3 \mu\text{m}^2$ , # nuclei:  $53 \pm 4$  to  $53 \pm 5$ ) (Figures 1c and 2a,c,e) and after 7 weeks ( $\text{Ø}$ :  $451 \pm 12$  to  $486 \pm 12 \mu\text{m}$ , mCSA:  $24 \pm 3$  to  $21 \pm 2 \times 10^3 \mu\text{m}^2$ , # nuclei:  $66 \pm 7$  to  $53 \pm 4$ ) (Figures 1d and 2b,d,f). Flow-induced outward remodeling was, however, improved by 3 weeks treatment with SOL1 ( $\text{Ø}$ : from  $419 \pm 13$  to  $475 \pm 16 \mu\text{m}$ ,  $P < 0.05$ ; Figures 1e and 2a) but no hypertrophy was found in HF arteries of DOCA-salt hypertensive

rats treated with SOL1 (mCSA from  $14 \pm 2$  to  $14 \pm 2 \times 10^3 \mu\text{m}^2$ ) (Figure 2c).

Although additional outward remodeling was blunted in DOCA-salt hypertensive rats, their LF arteries became markedly narrower (Figures 1c,d and 2a,b) and stiffer (Figures 2g and h). Lumen diameter was reduced from  $452 \pm 12$  to  $290 \pm 14 \mu\text{m}$  ( $P < 0.001$ ) and from  $451 \pm 16$  to  $265 \pm 14 \mu\text{m}$  ( $P < 0.001$ ) at 3 and 6 weeks of hypertension, respectively (Figure 2). Media mass was significantly reduced at 3 weeks (mCSA:  $16 \pm 1$  to  $12 \pm 2 \times 10^3 \mu\text{m}^2$ ,  $P < 0.05$ ) but not at 6 weeks of hypertension (mCSA:  $24 \pm 3$  to  $16 \pm 2 \times 10^3 \mu\text{m}^2$ , NS; Figure 2), a stage at which a marked increase in medial cell number was observed (Figure 2f). Despite the marked stiffening, the elastin to collagen ratio was significantly increased (Table 1).

Chronic treatment of DOCA-salt hypertensive rats with the dual NEP/ECE inhibitor SOL1 further decreased the diameter of LF arteries ( $\text{Ø}$ :  $253 \pm 6 \mu\text{m}$ ) and significantly decreased their mCSA ( $7 \pm 1 \times 10^3 \mu\text{m}^2$ ,  $P < 0.05$ ) and medial cell number (# nuclei:  $42 \pm 4$ ) but did not affect stiffness (Figure 2) or elastin/collagen (Table 1).



**Figure 2** The lumen diameter as measured at 80 mm Hg after (a) 4 and (b) 7 weeks of altered blood flow. The effect of altered blood flow on cross-sectional area of the medial layer after (c) 4 and (d) 7 weeks. The effect of altered blood flow on the number of medial cells per cross-section after (e) 4 and (f) 7 weeks. The effect of altered blood flow on the stiffness index after (g) 4 and (h) 7 weeks. Data are shown as mean  $\pm$  s.e.m. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 normotensive rats (NT) vs. DOCA-salt hypertensive rats (HT); # $P$ <0.05, ### $P$ <0.001 vs. NF, & $P$ <0.05 HT vs. HT+SOL1. mCSA: cross-sectional area of the medial layer; NF, HF, LF: arteries that had been exposed to normal, increased and reduced blood flow in vivo, respectively.

In an attempt to evaluate inflammation and characterize the nature of the hyperplastic changes, we performed immunohistochemistry for ED-1 and smooth muscle  $\alpha$ -actin (ASMA) (Figure 3). Significant numbers of monocyte/macrophages in the tunica media were observed only in LF arteries (Table 1). In arteries of normotensive rats this was a transient response, whereas in arteries of DOCA-salt hypertensive rats this was still statistically significant at 6 weeks (Table 1). Treatment with SOL1 significantly reduced this index of inflammation (Table 1). ED-1 staining was also observed in the adventitia of LF arteries (Figure 3b), but this could not be crisply quantified. ASMA staining indicates that the vast majority of cells in the hyperplastic tunica media of the LF arteries of DOCA-salt hypertensive rats were still differentiated smooth muscle cells (Figure 3d). Moreover, this approach revealed development of new blood vessels in this setting.

## DISCUSSION

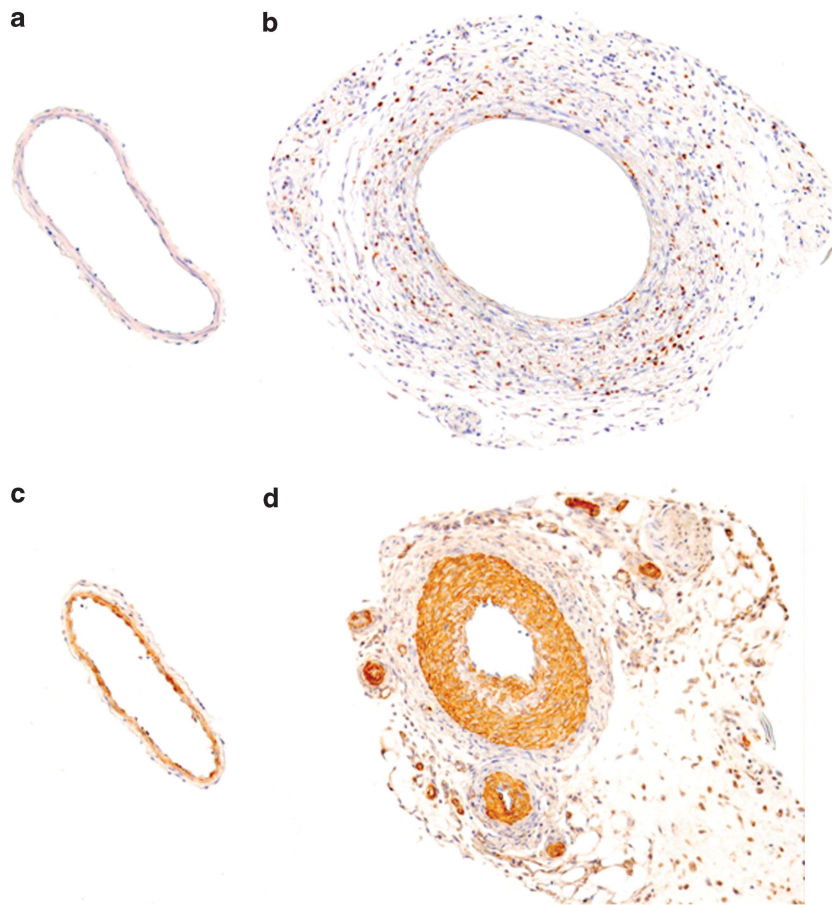
The main findings of this study are that in mesenteric resistance arteries of DOCA salt hypertensive rats flow-induced outward

remodeling is impaired. Moreover, the mesenteric resistance arteries of DOCA-salt hypertensive rats are hypertrophic and show a clear inflammatory component. These effects can partly be prevented by treatment with a dual NEP/ECE inhibitor.

DOCA-salt hypertension is a model of low renin/angiotensin but high ET1 levels that shows pronounced endothelial dysfunction<sup>19</sup> and arterial hypertrophy.<sup>20,21</sup> ET1 is not only a potent vasoconstrictor;<sup>14</sup> it is also a proinflammatory agent,<sup>17</sup> has mitogenic effects on vascular smooth muscle cells,<sup>15</sup> is pro-angiogenic<sup>18</sup> and promotes fibrosis.<sup>16</sup> An upregulation of the ET1 system has been linked to numerous pathologies, including salt-sensitive hypertension.<sup>20,21,32,33</sup> Because of these functions the endothelin system might in large part contribute to the hypertrophy and the endothelial dysfunction observed in DOCA-salt hypertension. In an attempt to test this we (i) treated DOCA-salt hypertensive rats for 3 weeks with the NEP/ECE inhibitor SOL1 and (ii) studied the endothelium-dependent structural effects of imposed changes in blood flow in DOCA salt hypertensive rats.

In this study MrA's of DOCA-salt hypertensive rats showed hypertrophic arterial remodeling after 3 and 6 weeks. At 3 weeks of





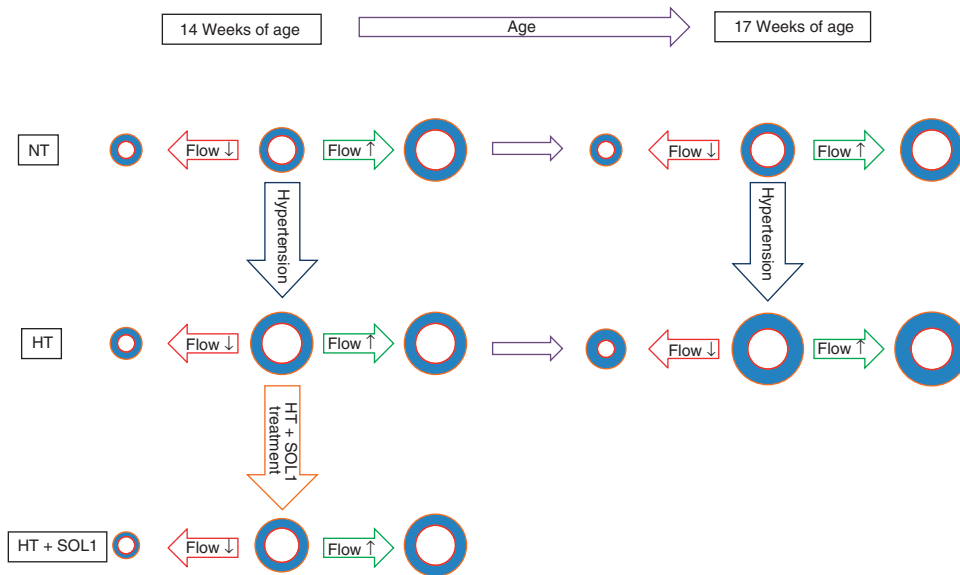
**Figure 3** Cross-sections of low-flow arteries of normotensive rats stained for (a) ED-1 and (c)  $\alpha$ -smooth muscle actin, and DOCA-salt hypertensive rats stained for (b) ED-1 and (d)  $\alpha$ -smooth muscle actin.

hypertension an increase in diameter was observed whereas at 6 weeks of hypertension diameter did not differ from that in arteries of normotensive rats that had increased as a result of development. Reports from other groups lean more to an unaltered or decreased arterial diameter.<sup>5,21</sup> This discrepancy might be because of differences in strain and age of the rats and in other aspects of the protocols used to induce DOCA-salt hypertension. A possible explanation for the increase in diameter after 3 weeks of hypertension is that in this experimental model blood pressure rises in a much shorter timeframe than in genetic hypertension<sup>8</sup> and that mesenteric blood flow may increase during the first weeks of DOCA-salt hypertension.<sup>34</sup> Although outward remodeling is generally not observed in models of hypertension, hypertrophic remodeling is often witnessed especially in models of non genetic hypertension and experimental hypertension. Also ET1 has been shown to contribute to the remodeling of large and small arteries in hypertension.<sup>35,36</sup> Hypertrophic remodeling rather than eutrophic remodeling of resistance arteries seems to occur in models associated with an upregulated endothelin system.<sup>5</sup> This is in line with the observation that chronic administration of the non-selective ET-receptor antagonist bosentan reduces the cross-sectional area of the arterial media in DOCA-salt hypertensive rats.<sup>5</sup> In contrast in mRen2 transgenic rats, a model of enhanced endogenous angiotensin II generation, inward eutrophic remodeling was observed, and bosentan had no effects on arterial structure.<sup>37</sup> A dual ACE/NEP inhibitor and an ACE inhibitor on the other hand caused a significant regression of

arteriolar structural changes.<sup>38–40</sup> Figure 4 shows a schematic overview of changes in arterial lumen diameter and medial mass in relation to hypertension (blue arrow) and age (purple arrow).

In the present study, 3 week treatment of DOCA-salt hypertensive rats with the NEP/ECE inhibitor SOL1 tended to reduce the arterial diameter and hypertrophy, and significantly reduced the arterial hyperplasia. Whether this is due to reduction of the direct arterial effects of ET1 or indirectly to the reduction of blood pressure, however, remains to be established. To discriminate between the effects of blood pressure lowering and of modified levels of vasoactive peptides, future studies with vasodilator compounds in a setting similar to the one used here may be considered. Figure 4 shows a schematic overview of changes in arterial lumen diameter and medial mass in relation to SOL1 treatment during hypertension (orange arrow).

Pourageaud and De Mey showed that in 6-week-old Wistar-Kyoto rats elevated blood flow resulted in outward hypertrophic remodeling and reduced blood flow in inward hypotrophic remodeling 4 weeks after flow altering surgery.<sup>30</sup> Similar observations were made by our group and others.<sup>27,30,41,42</sup> In the present study, we observed flow-induced outward hypertrophic remodeling but no hypotrophy in resistance arteries exposed to low blood flow in normotensive Wistar rats studied between 10 and 14 weeks of age. This indicates dependence of flow-related arterial remodeling on rat age<sup>43</sup> and strain.<sup>44</sup> Unexpectedly, no significant differences in diameter and media mass were observed between control arteries and arteries



**Figure 4** Schematic overview of changes in arterial lumen diameter and medial mass in relation to hypertension (blue arrow), imposed changes in flow (green arrow (high flow) and red arrow (low flow)), SOL1 treatment during hypertension (HT + SOL1: orange arrow) and age (purple arrow). NT: normotensive rats, HT: DOCA-salt hypertensive rats.

exposed to elevated blood flow for 7 weeks in normotensive rats. This seems to be due to the growth and development of the control arteries during this period time. We speculate in this respect that the marked flow-induced remodeling of young arteries represents an accelerated developmental process.<sup>45</sup>

In resistance arteries of DOCA-salt hypertensive rats, remodeling in response increased blood flow was blunted (see below) but structural responses to reduced blood flow were extensive. We observed a marked decrease in diameter that was, as in normotensive rats, not accompanied by a significant change in media mass. Unlike in normotensive rats, signs of marked and long-lasting inflammation were observed such as extensive ED-1 staining in tunica media and adventitia, and sprouting of new vessels in the adventitial layer. The observation that in DOCA-salt hypertensive rats treated with SOL1, mesenteric arteries exposed to decreased flow were hypotrophic and lack the inflammatory component, as well as the artery-like structures in the adventitia are in line with the mitogenic, proinflammatory and proangiogenic effects of ET1.<sup>15,17,18</sup>

Flow-induced outward arterial remodeling did not occur in DOCA-salt hypertensive rats. This implies that the physiological response to an increase in flow, outward remodeling, is impaired in DOCA-salt hypertensive rats. This model of hypertension is described to show pronounced endothelial dysfunction<sup>19</sup> as evidenced by increased infiltration of inflammatory cells and reduced endothelium-dependent vasodilatation and increased endothelium-dependent vasoconstriction. Several studies indicate a strong interplay between ET1 and endothelium derived nitric oxide.<sup>46</sup> Amiri *et al.*<sup>35</sup> showed that overexpressing the human *preproET1* gene in the endothelium in mice caused endothelial dysfunction and reduced NO bioavailability. Strong evidence indicates that endothelium-derived NO and other relaxing factors can be released from the endothelium in response to increased shear stress mediating flow-induced vasodilatation.<sup>2,3</sup> Endothelium-derived NO also has a role in the arterial remodeling of large conduit arteries in response to a chronic increase in blood flow.<sup>47,48</sup> In small resistance-sized arteries, the situation is less clear. Here NO is not the only endothelium-derived relaxing factor<sup>49</sup> and

chronic pharmacological inhibition of NO synthases does not inhibit flow-induced arterial remodeling.<sup>27</sup> In contrast to L-NAME-induced hypertension, flow-induced remodeling was inhibited in DOCA-salt hypertensive rats. This indicates that in DOCA-salt hypertension endothelium-dependent remodeling of resistance arteries is blunted independently from classical endothelial dysfunction. Outward remodeling might be independent of endothelium-dependent vasodilatation. Alternatively, the endothelial dysfunction in DOCA-salt hypertension might not be restricted to reduced bioavailability of NO and upregulation of ET1, but also involve another endothelium-derived relaxing factor such as EDHF contributing to structural changes. Another possibility is that due to the increase in diameter occurring during the development of the hypertension and due to the increased arterial stiffness, the dynamic range to respond to an imposed increase in flow might be considerably reduced in DOCA-salt hypertensive rats. Chronic SOL1 treatment during the development of DOCA-salt hypertension preserved the flow-induced outward remodeling response. Thus, NEP/ECE inhibition improves endothelium-dependent structural responses in mesenteric arteries of DOCA-salt hypertensive rats. Because a moderate inflammatory response seems necessary for the initiation of outward remodeling, the pharmacological effect is unlikely due to its anti-inflammatory consequences. The possible contributions of reduced blood pressure, reduced synthesis of ET1 and improved non-NO-mediated relaxation, however, remain to be established. Figure 4 shows a schematic overview of changes in arterial lumen diameter and medial mass in relation to an increase (green arrows) and a decrease (red arrows).

Our study has a number of limitations. The three-dimensional-dissector technique is more accurate to determine changes in cell number and size than the more rapid counting of nuclear profiles on hematoxylin–eosin-stained tissue sections.<sup>9</sup> Additional measurement of local transmural pressure and local blood flow at the various time points would have allowed to speculate on the extent to which normalization of wall stress and wall shear stress is impaired in mesenteric resistance arteries of DOCA-salt hypertensive rats.

In conclusion, in the resistance arteries of DOCA-salt hypertensive rats, we observed a selective reduction of flow-induced outward remodeling. This indicates that in this experimental model of hypertension, endothelial dysfunction is not limited to reduced endothelium-dependent vasodilatation. In addition some of our findings in the arteries of both normotensive and hypertensive rats suggest that experimentally induced and developmental flow-induced arterial remodeling have the same upper limit. This should be taken into account when, for instance, vasodilator therapies are being proposed for the reversal of inward arterial remodeling in essential hypertension.<sup>50</sup>

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