Inflammation and transglutaminases in vascular remodeling and atherosclerosis
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NUCLEAR RECEPTOR NUR77 INHIBITS VASCULAR OUTWARD REMODELING AND REDUCES MACROPHAGE ACCUMULATION AND MATRIX METALLOPROTEINASE LEVELS

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**Aim**
Structural adaptation of the vessel wall in response to sustained alterations in hemodynamic forces is known as vascular remodeling. Detailed knowledge on the mechanism underlying this vascular response is limited and we aimed to study the function of Nur77 in smooth muscle cells (SMCs) in arterial remodeling.

**Methods and Results**
Carotid artery ligation in mice results in flow-induced, outward remodeling of the contralateral carotid artery and we observed enhanced Nur77 expression during this process. Transgenic mice that express Nur77 or its dominant-negative variant denoted as ‘ΔTA’ in arterial SMCs, were exposed to carotid artery ligation and after 4 weeks pressure-diameter relationships were measured. Structural outward remodeling is inhibited in Nur77-transgenic mice as compared to wildtype and ΔTA-transgenic mice. The key determinants of remodeling vascular tone and macrophage accumulation were studied. No difference in contractile and relaxant responses was detected in isolated aorta, carotid and mesenteric artery segments between transgenic and wild-type mice. SMC specific overexpression of Nur77 in transgenic mice reduced macrophage accumulation and repressed matrix metalloproteinase (MMP)-1 and -9 expression at early time points. MMP2 protein expression was reduced in Nur77 transgenic mice, whereas in ΔTA-transgenic mice MMP2 expression was increased.

**Conclusion**
Nur77 is induced during outward remodeling and inhibits this vascular adaptation in mice. Nur77-mediated inhibition of arterial remodeling involves a reduction in both macrophage accumulation and MMP expression levels.
INTRODUCTION

Variation in blood flow initially induces transient adaptation of the vessel wall through vasomotor responses, whereas prolonged change of hemodynamic forces results in structural adaptation of the vessel wall, which has been described as vascular remodeling. Flow-induced shear stress, altered blood pressure and/or cyclic circumferential stretch contribute to arterial remodeling in both physiologic vascular wall homeostasis and pathologic conditions like atherosclerosis, aneurysm formation and hypertension [1-3]. Limited information is available concerning the exact mechanism underlying vascular remodeling, but important determinants include vascular tone and the recruitment of inflammatory cells, which produce matrix metalloproteinases (MMPs) and growth factors [4].

In search for genes involved in smooth muscle cell (SMC) activation in vascular disease, we revealed induction of Nur77 expression in in vitro-activated human SMCs and demonstrated that Nur77 inhibits proliferation of SMCs involving increased expression of p27kip1 [5,6]. Furthermore, we have shown that NR4A nuclear receptors are expressed in SMCs and macrophages in human atherosclerotic lesions, but not in the media of normal arteries [6,7]. In transgenic mice overexpressing Nur77 under control of an arterial SMC-specific promoter we demonstrated that Nur77 protects against SMC-rich lesion formation [6].

Nur77 belongs to the NR4A nuclear receptor subfamily that comprises three members: Nur77 (also known as NR4A1, TR3, NGFI-B), Nurr1 (NR4A2, NOT), and NOR-1 (NR4A3, MINOR). The NR4A nuclear receptors are expressed as early response transcription factors upon stimulation by growth factors and pro-inflammatory stimuli [8]. Like other nuclear receptors, the NR4A nuclear receptors contain a central DNA-binding domain that binds the consensus response elements (RE) NBRE or NurRE in promoters of target genes [9,10]. The N-terminal transactivation domain is necessary to drive expression of target genes, whereas the C-terminal domain comprises a potential ligand-binding domain. In addition to direct regulation of gene expression, transcriptional regulation by transrepression mechanisms has been described [11,12]. Classical ligands for the NR4A subfamily have not yet been identified, classifying them as orphan receptors.

In the current study we assessed the expression and function of Nur77 in outward arterial remodeling. We report that Nur77 is induced during flow-induced, left carotid artery outward remodeling in response to contralateral carotid artery ligation in mice. In transgenic mice that overexpress Nur77 in arterial SMCs, contractile and relaxant responses of isolated vessel segments is normal, and outward remodeling is reduced. Both a reduction in macrophage accumulation and changes in MMP expression levels are involved in Nur77-mediated inhibition of outward remodeling.
METHODS AND MATERIALS

Immunohistochemistry and in-situ hybridization

Mouse carotid artery specimens were paraffin embedded, sectioned, and mounted on glass slides (Superfrost-Plus, Emergo, Amsterdam, The Netherlands). For characterization of mouse carotid arteries immunohistochemistry was performed to detect macrophages (Mac3, M3/84, BD, Pharmingen, Breda, The Netherlands), SMCs (1A4, DAKO), matrix metalloproteinase (MMP)-1 (Abbiotec, San Diego, CA, USA) and MMP2 (R&D, Minneapolis, MN, USA). Lawson and Sirius red staining was used to visualize elastic laminae and collagen, respectively. Nuclei were detected with hematoxylin. In-situ hybridization was performed with a gene-specific probe for SM22α as described previously [6]. Quantification of number of macrophages was performed by counting macrophages in 250 μm-separated tissue sections (5 μm) by two observers and was expressed as number of macrophages/section. For morphometry, elastic laminae were visualized by Lawson stain and medial, and adventitial surface areas were quantified by morphometric analysis (Leica Qwin, Germany). MMP positive surface areas were quantified by intensity analysis (Leica Qwin). Subsequently, MMP protein expression was expressed as MMP positive surface area corrected for total medial or adventitial surface area.

Transgenic mice

Animal care and experimental procedures were approved by the Animal Experimental Committee of the Academic Medical Center, University of Amsterdam and this investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). Transgenic mice expressing ΔTA or full-length Nur77 were generated in an FVB background (Broekman, Someren, the Netherlands) and characterized by Southern blotting as described [6]. SM22α promoter-driven expression of the transgene was used to achieve arterial SMC-specific expression [6,13]. Arteries of wild-type mice or mice homozygous for the transgene were used for experiments.
Flow-induced carotid artery remodeling experiments

At the age of 14 weeks the right carotid artery of female wild-type and transgenic (ΔTA or Nur77) mice were ligated (n=8-11) to induce left carotid artery outward remodeling as described [14-16]. Before surgery mice were anaesthetized with an intraperitoneal injection of 5 mg/kg midazolam (Roche, Basel, Switzerland), 0.5 mg/kg medetomidine (Orion, Helsinki, Finland) and 0.05 mg/kg phentanyl (Janssen, Geel, Belgium). Four weeks after ligation, mice were sacrificed and carotid arteries were harvested for analysis. Left carotid arteries were isolated, cannulated in papaverin 10^{-4} mol/l containing calcium-free MOPS buffer to achieve dilatation and passive pressure–diameter relationships were determined as described [17]. Structural remodeling is defined as the difference in the passive diameter at a given pressure between arteries from control and intervention groups. After cannulation arteries were fixed with formaldehyde for immunohistochemistry, macrophage quantification and in-situ hybridization. Next, a time course experiment was performed and left control and left outward remodeled carotid arteries were harvested at 0, 1, 7 and 14 days after right carotid artery ligation and placed in Trizol (Invitrogen, Breda, The Netherlands) for RNA isolation.

Isometric contraction and relaxation experiments

Contractile and dilatory responses were measured in mesenteric artery (n=6-7), carotid artery (n=3-5) and thoracic aorta segments (n=3-5) of transgenic and wild-type mice eight weeks of age, using a wire myograph setup. First branch mesenteric artery segments (internal diameter approximately 200-250 μm) and aorta and carotid artery segments (length approximately 2 mm) were prepared and processed as previously described with the following modifications [18]. For all preparations the diameter was determined by a normalization procedure [19]. In the aortic and carotid preparations the internal circumference was thereafter adjusted to a value which equals 90% of the diameter at an intraluminal pressure of 100 mmHg, whereas in the mesenteric preparations the passive wall tension was set to 5 mN according to Besnard et al. [20]. Preparations were exposed thrice to a depolarizing Tyrode’s solution (containing 40 mM (aorta and carotid) or 120 mM (mesenteric arteries) potassium chloride (KCl), equimolar substitution for NaCl) for 5 min with a 20-min interval. Hereafter, cumulative concentration response curves were constructed for L-phenylephrine and thromboxane A2 receptor agonist U46619. Endothelium-dependent and independent relaxation was studied by construction of cumulative
concentration-response curves for methacholine and isoprenaline respectively after pre-contraction with a sub-maximal concentration L-phenylephrine (1 μM). Using a computer program (Prism from GraphPad, San Diego, CA, USA), concentration-response curves for the different agonists were fitted to log concentration-response data of individual experiments.

**qRT-PCR gene expression analysis**

At the time points indicated, left carotid arteries of wild-type and transgenic mice (n=4-8) were lysed in Trizol and RNA was extracted. cDNA was synthesized (iScript, Biorad, Veenendaal, The Netherlands) and gene expression analyzed by qRT-PCR using gene-specific primers and SYBRGreen (MyiQ RT-PCR System, Biorad). All qRT-PCR data were normalized for equal amounts of cDNA by using expression data of housekeeping gene HKG36B4. qRT-PCR primer sequences used are mNur77: FW:5’-TTGAGTTCGGCAAGCCTACC-3’ RV:5’-GTGTACCCGTCCATGAAGGTG-3’ mMMP1: FW:5’-TGCCATTACTCAACAACATCCTC-3’ RV:5’-GAGACACAATATCGCCTTCC-3’ mMMP9: FW:5’-ACGGAGCACGGCAACGGGAAGG-3’ RV:5’-GCGTCCACTCGGTAGGGCAGAAG-3’

**Statistical Analyses**

The unpaired Student’s t test was used to calculate the statistical significance of expression ratios/numbers versus control. In animal experiments data are reported as mean ± SEM and were analyzed with ANOVA-test with a Dunnett’s (post hoc) test or unpaired Student’s t test (SPSS 12.0 for Windows, SPSS Inc, Chicago, Illinois, USA). p<0.05 was considered statistically significant (*p<0.05; **p<0.01; ***p<0.001).

**RESULTS**

**Nur77 inhibits flow-induced outward remodeling of carotid arteries in mice**

To study vascular remodeling, we used a mouse model in which the left carotid artery undergoes flow-induced outward remodeling in response to complete ligation of the right carotid artery [14-16]. Structural remodeling of left carotid arteries 4 weeks after right carotid artery ligation in comparison to control left carotid arteries
of wild-type mice was measured by pressure-diameter relationships of cannulated arteries (Figure 4-1A and Figure S4-1). Sections of remodeled carotid arteries were analyzed by Lawson staining and SMC-specific immunohistochemical staining for SMα-actin (Figure 4-1B). Furthermore, expression of the SMC-marker SM22α was analyzed by radioactive in-situ hybridization and shown to be homogeneous throughout the media in remodeling arteries (Figure 4-1B). Subsequently, we studied Nur77 mRNA expression at different time points of remodeling and demonstrated that Nur77 mRNA was induced over 3 fold with optimal expression 7 days after ligation (Figure 4-1C). To assess the function of Nur77 in arterial remodeling we applied our transgenic mice in which SMC-specific expression of Nur77 or its dominant-negative variant ΔTA is driven by the SM22α-promoter. To quantify
structural, outward remodeling of left carotid arteries, i.e. the change in passive diameter of arteries, pressure-diameter relationships (inner and outer diameter) were determined (Figure 4-2A-B). No difference in both inner and outer diameter increase was detected between ΔTA and wild-type mice. In Nur77-transgenic mice both the inner and outer diameter of left carotid arteries were significantly less outward remodeled as compared to ΔTA or wild-type mice at physiological blood pressure levels (Figure 4-2AB, Table 4-I). According to Poiseuille’s equation ($Q = \Delta P \pi r^4/8\eta l$; $Q$=flow; $P$=pressure; $r$=internal radius; $\eta$=viscosity; $l$=length of tube) the increase in diameter due to structural remodeling allows an approximate 49% increase in blood flow in wild-type and ΔTA-transgenic mice as compared to an approximate 27% increase in blood flow in Nur77 transgenic mice at 100 mmHg (Table 4-I).

![Figure 4-2](image_url)

Figure 4-2: Flow-induced left carotid artery remodeling is inhibited by SM22α-mediated transgenic Nur77 expression in mice. A-B) Pressure-diameter relationships (inner and outer diameter) were determined in Nur77-, ΔTA-transgenic or wild-type mice and the change in passive diameter was quantified. At physiological (blood) pressure levels both the inner (A) and outer (B) diameter of left carotid arteries of Nur77 transgenic mice were significantly less increased as compared to ΔTA or wild-type mice. No difference in diameter change in both inner and outer diameter was detected between ΔTA and wild-type mice. (n=8-11 mice/group, ±SEM; unpaired Student’s t test, p<0.05 Nur77 versus WT or ΔTA)
**Table 4-I:** Pressure-diameter analysis at 100 mmHg for remodeled left carotid arteries from wild-type, Nur77- and ΔTA-transgenic mice. *; p<0.05 compared to wild-type mice

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<th>Condition</th>
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<th>Outer diameter [μm ± SEM]</th>
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<td></td>
<td>n=8 664.6 ± 5.9</td>
<td>n=9 650.2 ± 5.1</td>
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<tr>
<td></td>
<td>n=8 725.7 ± 6.5</td>
<td>n=9 685.7 ± 3.2</td>
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<th>ΔDiameter Inner [μm (%)]</th>
<th>ΔDiameter Outer [μm (%)]</th>
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<tr>
<td>n=8 66.5 ± 9.6</td>
<td>n=9 38.0 ± 5.9</td>
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<tr>
<td>n=8 61.1 ± 9.6</td>
<td>n=9 35.5 ± 5.2</td>
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<table>
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<th>ΔFlow (%)</th>
<th>Wild-type n=8</th>
<th>Nur77 n=9</th>
<th>ΔTA n=8</th>
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<tbody>
<tr>
<td></td>
<td>49.8</td>
<td>27.4*</td>
<td>49.0</td>
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**Nur77 does not modulate vascular tone**

Since vascular tone is an important determinant of arterial remodeling, we assessed contractile and dilatory responses in artery segments of Nur77, ΔTA transgenic and wild-type mice in a myograph. The increase in wall tension of isolated thoracic aorta, carotid and mesenteric artery segments in response to high KCl concentrations was similar in preparations obtained from Nur77, ΔTA and control wild-type mice. Also the maximal response and the concentrations necessary to reach 50% of the maximal effect to L-phenylephrine and U46619 were not significantly different between mesenteric arteries taken from Nur77, ΔTA or control wild-type mice (Figure 4-3A and B). After pre-contraction with a sub-maximal concentration of L-phenylephrine the endothelium-dependent and -independent vasodilatation of mesenteric artery preparations to methacholine and isoprenaline, respectively, were measured, revealing that also maximal vasodilatation was not significantly different (Figure 4-3C and D). Similar non-significantly different contraction and vasodilatation response curves were obtained for isolated carotid artery and thoracic aorta segments derived from Nur77, ΔTA and wild-type mice (data not shown). These data demonstrate that overexpression or inhibition of Nur77 activity in arterial SMCs does not affect the vasoconstriction or vasodilatation responses that modulate vascular tone in isolated small resistance mesenteric, carotid and aorta preparations.
In addition to contractile and relaxant responses, macrophage accumulation and associated inflammatory responses that contribute to MMP expression are important determinants of structural arterial remodeling [21]. Macrophage accumulation was studied by counting numbers of macrophages detected by immunohistochemistry in remodeling carotid arteries of Nur77, ΔTA and wild-type mice. In line with
our previous observations \[4\], macrophages were predominantly present in the adventitia (Figure 4-4A). A significant 3.8 fold reduction in number of macrophages was observed in Nur77 transgenic mice as compared to wild-type mice. In contrast, a 1.9 fold increase in macrophages was detected in ΔTA transgenic mice as compared to wild-type mice (Figure 4-4B). In line with these data, time course MMP expression analyses demonstrated that Nur77 transgenic mice express lower MMP1 and MMP9 mRNA levels during outward arterial remodeling as compared to wild-type mice (Figure 4-5A and B). Optimal expression of both MMP1 and MMP9 was detected 1 day after initiation of outward remodeling. MMP1 and MMP2 protein expression was analyzed by immunohistochemistry at 28 days after carotid artery ligation. At this time point MMP1 and MMP2 expression was located in both medial SMCs and adventitia (Figure 4-6A). No significant difference in MMP1 expression
and collagen content (data not shown) was detected at this time point between Nur77, ΔTA transgenic and wild-type mice. In medial SMCs MMP2 protein expression was 3.2 fold reduced in Nur77 transgenic mice as compared to wild-type mice. In line with this observation, ΔTA transgenic mice showed a 1.8 fold increase in MMP2 protein in medial SMCs (Figure 4-6C). No significant difference in MMP2 protein expression was observed in the adventitia (Figure 4-6C).

**DISCUSSION**

Arterial remodeling is considered a critical process in vascular diseases such as hypertension, aneurysm formation and atherosclerosis. In atherosclerosis outward or expansive remodeling can compensate for luminal narrowing, a process which is referred to as Glagov’s phenomenon that is beneficial to preserve local blood flow [2,22]. However, outward remodeling has also been associated with plaque instability and unstable clinical cardiovascular syndromes [23,24]. In previous studies we described high expression levels of Nur77 in SMCs and macrophages in the shoulder region and adventitia of advanced, atherosclerotic plaques, which may point towards involvement of this nuclear receptor in vascular remodeling relevant to atherosclerosis [6,7,25]. In the current study we demonstrated that...
Nur77 transgenic mice exposed to flow-induced carotid artery remodeling show a substantial reduction in outward remodeling as compared to ΔTA or wild-type mice. No difference in outward remodeling between ΔTA transgenic and wild-type mice was observed, which is remarkable since ΔTA transgenic mice show an increase in macrophage accumulation in the adventitia of the non-ligated carotid artery. A likely explanation for this observation is that as soon as outward remodeling has restored blood flow and local shear stress is reduced to normal levels, there is no drive to further continue remodeling. The calculated approximate 49% increase in blood flow is similar to that observed in previous studies [2,26]. Both vascular tone and inflammation are key determinants in arterial remodeling [4,27,28]. Since α- and β-adrenergic receptor stimulation has been shown to regulate Nur77 expression

Figure 4-6: MMP1 and MMP2 protein expression in remodeled carotid arteries. (A) MMP1 and MMP2 protein was detected in both medial SMCs and to a lesser extent in the adventitia by immunohistochemistry after 28 days. (B) No difference in MMP1 protein expression levels was detected between Nur77, ΔTA transgenic and wild-type mice. (C) In medial SMCs MMP2 protein expression was 3.2 fold reduced in Nur77 transgenic mice as compared to wild-type mice. In ΔTA transgenic mice a 1.8 fold increase in MMP2 protein was observed in medial SMCs. No significant difference in MMP2 protein expression level was observed in the adventitia of the different mice groups (C). (n=5-7 mice/group, ±SD; Student’s t test; p<0.05)
in skeletal muscle, we anticipated that arterial contraction and relaxation involves Nur77 [29,30]. We clearly demonstrated, however, that contraction and relaxation of muscular, resistance mesenteric artery segments as well as elastic, conductance carotid artery and thoracic aorta segments are not affected by transgenic overexpression of Nur77 or full inhibition of endogenous Nur77 by transgenic ΔTA. Based on these data, we concluded that the inhibitory effect of Nur77 on outward arterial remodeling can not be explained by modulation of vascular tone.

Next to vascular tone, there is increasing evidence that inflammation is crucial in arterial remodeling, which is reflected by both increased macrophage accumulation and enhanced MMP expression levels [21]. In mesenteric arteries this inflammatory response is optimal between 0 and 4 days after initiation of outward remodeling and MMPs generated during this process are thought to contribute to a state of tissue plasticity through partial degradation of matrix, cell-matrix and cell-cell interactions which is necessary for structural remodeling [4]. In the current study, we demonstrate that outward remodeling of large, conductance carotid arteries is accompanied by a fast (day 1) increase of MMP1 and MMP9. Most significantly, SMC-specific overexpression of Nur77 inhibits carotid outward remodeling, which involves both a reduction in macrophage accumulation and MMP1 and MMP9 mRNA expression levels. In addition, MMP2 protein expression in medial SMCs was shown to be reduced in Nur77 transgenic mice. Nur77 has been shown to repress expression levels of an array of inflammatory genes in activated macrophages, including chemokines [7]. Therefore, we hypothesized that Nur77 overexpression in SMCs also reduces macrophage accumulation during arterial remodeling, which indeed is strongly supported by our data. The reduction in macrophage numbers may explain the reduced MMP1 and MMP9 levels detected, because macrophages are considered an important source of MMPs. Alternatively, as demonstrated by immunohistochemistry, also medial SMCs synthesize MMPs, most significantly MMP1 and MMP2 and a direct effect of Nur77 on SMC-derived MMP2 expression provides another explanation [31]. Indeed, Nur77 has been shown to transrepress NFκB [32,33] and Nurr1 a member of the NR4A subfamily, has been demonstrated to antagonize MMP expression during inflammation in chondrocytes and inflammatory cytokine expression in microglia [11,12]. Finally, we have demonstrated that Nur77 inhibits SMC proliferation, which will also contribute to the inhibitory function of Nur77 in carotid artery remodeling [6].

In conclusion, Nur77 inhibits flow-induced carotid artery remodeling in mice. Not vascular tone, but a reduction in both macrophage accumulation and changes in MMP expression may explain this observation. We propose that although Nur77 expression is enhanced in SMCs by increased blood flow during outward remodeling, this nuclear receptor has an inhibitory function in vascular adaptation, suggesting
that Nur77 is involved in a negative feedback mechanism operational in arterial remodeling. Nur77 is expressed in human atherosclerosis and therefore our data may implicate that Nur77 modulates vascular remodeling in atherosclerosis and other vascular pathologies.

FUNDING

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REFERENCES


Figure S4-1: Flow-induced left carotid artery outward remodeling is decreased in Nur77-transgenic mice. (A-B) Pressure-diameter relationships were determined for inner (A) and outer (B) diameters in Nur77-, ΔTA-transgenic or wild-type mice. At physiological (blood) pressure levels both the inner (A) and outer (B) diameter of left carotid arteries of Nur77 transgenic mice were less increased in diameter as compared to ΔTA or wild-type mice.