TR3 nuclear orphan receptor in cardiovascular disease
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Chapter 7

Normal arterial responsiveness and remodeling after modulation of TR3 orphan receptor expression


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Abstract

Background
Smooth muscle cell (SMC) function is important for physiological arterial responsiveness and in vascular remodeling during collateral artery formation. In addition, SMCs are pivotal in intimal thickening in atherosclerosis and restenosis. We have shown that TR3-like nuclear orphan receptors are expressed in human atherosclerotic lesions and that TR3 nuclear orphan receptor (TR3) inhibits SMC proliferation in the murine carotid artery ligation model of restenosis. In this study we assayed the effect of TR3 on vascular contraction and relaxation as well as a potential function in arteriogenesis.

Methods and Results
Responsiveness to vasoconstrictor or vasodilator agents were determined in the aortas and small resistance mesenteric arteries of both transgenic mice overexpressing TR3 or a dominant-negative variant of TR3 (ATA) and wild-type mice. No differences in arterial contraction and dilation between the different mice were established in either vessel type. To study the effect of TR3 on arteriogenesis, mice that express full-length TR3 (n=13), or ATA (n=15) and their wild-type littermates (n=14) underwent femoral artery occlusion. No significant differences in hindlimb perfusion were observed one week after arterial ligation, as assessed with fluorescent microspheres. Immunohistochemistry revealed no obvious differences in the proliferative activity of medial SMCs in TR3 or ATA transgenic mice after ligation.

Conclusions
Neointima formation caused by intimal SMC hyperplasia is reduced upon overexpression of TR3, without negative effects on arterial responsiveness and vascular remodeling during collateral artery formation. These findings are important when considering TR3 as a therapeutic target to limit vascular lesion formation.
Normal arterial responsiveness and remodeling in TR3 mice

Introduction

Percutaneous coronary intervention may cause early thrombus deposition, cellular inflammation, and ultimately intimal smooth muscle cell (SMC) proliferation, leading to restenosis. The introduction of stents has reduced the incidence of restenosis, however, in-stent restenosis evolved as a novel SMC-specific pathology. We have identified three transcription factors that are differentially expressed in cultured SMCs upon activation with an atherogenic stimulus; TR3 orphan receptor (TR3), Mitogen-induced nuclear orphan receptor (MINOR) and Nuclear receptor of T-cells (NOT), which form a sub-family of the nuclear orphan receptors denoted NGFI-B or NR4A. TR3, MINOR and NOT are expressed in human atherosclerotic lesions in neointimal SMCs, endothelial cells and to lesser extent in macrophages. TR3 inhibits SMC proliferation, whereas a dominant-negative variant of TR3 (ΔTA) that lacks the transactivation domain and inhibits all three family members, enhances SMC growth. Transgenic mice were generated expressing TR3 or the dominant-negative variant ΔTA in arterial SMCs. Vascular lesion formation was studied in a murine carotid artery ligation model and TR3 was shown to inhibit intimal SMC hyperplasia, whereas ΔTA aggravated lesion formation. It was hypothesized that TR3-like transcription factors are potential therapeutic targets to prevent (in-stent) restenosis. However, this potential new treatment modality to inhibit SMC proliferation may involve an important adverse effect, since SMC proliferation plays a key role in processes of vasoconstriction or vasodilatation and in arterial remodeling during collateral artery formation. The purpose of this study was to evaluate the effect of modulation of TR3 expression on in-vitro arterial responsiveness to vasoactive substances and on in-vivo collateral artery growth in the mouse ischemic hindlimb model.

Methods

Animals

Mice

Animal care and experimental procedures were approved by the Animal Experimental Committee at our institution. Transgenic mice expressing ΔTA or full-length TR3 cDNA under the control of a specific part of the SM22α promoter, which directs transgene expression to SMCs of the arterial vascular wall, were generated in an FVB-background, as described before. The animals were housed in standard cages and fed water and chow ad libitum.

Chemicals

The following drugs were used: (acetyl-b)methacholine chloride, 9α-epoxymethano-prostaglandin F2α (U46619), isoprenaline bitartrate and L-phenylephrine HCl which were
obtained from Sigma Chemical (St. Louis, Mo., USA). All drugs were dissolved in distilled water and kept frozen at -20°C prior to use, except for isoprenaline, for which solutions were freshly prepared prior to the experiments.

Assessment of Stimulated Isometric Contractile and Relaxation Responses

The direct vasoconstrictor and relaxant effects of phenylephrine (n=3), the thromboxane A2 receptor agonist U46619 (n=5), methacholine (n=5) and isoprenaline (n=3) on aorta segments of transgenic and wild-type mice of eight weeks of age were investigated using an isometric wire myograph. Also direct vasoconstrictor and relaxant effects of phenylephrine (n=6-7), U46619 (n=6-7), methacholine (n=6) and isoprenaline (n=6-7) on small resistance mesenteric arteries of transgenic and wild-type mice were investigated. After decapitation, the thoracic aorta and the mesenteric vascular bed were isolated, dissected free from its connective tissue and transferred to physiological Tyrode’s solution of the following composition (mM) NaCl 118.5, KCl 4.7, MgCl2 1.2, CaCl2 2.5, KH2PO4 1.2, NaHCO3 25, glucose 5.5 and EDTA 0.024. The organ bath contained Tyrode’s that was oxygenated by carbogen (95% O2 + 5% CO2), at room temperature. Small segments (length approximately: 2mm) of aorta or first branch mesenteric artery (internal diameter approximately 200-250 μm) were prepared. Subsequently, a 40 μm (for aortic preparations) or a 25 μm (for mesenteric segments) stainless steel wire was inserted to the lumen and the preparations were mounted in an isometric wire myograph according to Mulvany and Halpern.10, 11 The vessel segments were fixed to a micrometer screw and after insertion of a second wire to an isometric force transducer. Hereafter the preparations were equilibrated for 15 minutes in Tyrode’s solution at 37°C and oxygenated with carbogen. Subsequently, the diameter was determined by a normalization procedure. In this normalization procedure the passive wall tension was adjusted to a level comparable to an intraluminal pressure of 100 mmHg. In the aortic 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.12. After an additional 15 min equilibration period, the preparations were exposed thrice to a depolarizing Tyrode’s solution (containing 40 mM (aorta) or 120 mM (mesenteric arteries) KCl, equimolar substitution for NaCl) for 5 min with a 20-min interval. Hereafter cumulative concentration response curves were constructed for the α agonist L-phenylephrine and the thromboxane A2 receptor agonist U46619. Endothelium-dependent and endothelium-independent relaxation was studied by construction of cumulative concentration-response curves for methacholine and isoprenaline, respectively after precontraction with a sub-maximal
Normal arterial responsiveness and remodeling in TR3 mice

concentration phenylephrine (1 μM). Using a computer program (GraphPad, Institute for Scientific Information, San Diego, Calif., USA), concentration-response curves for the different agonists were fitted to log concentration-response data of individual experiments.

Measurements of Collateral Dependent Hind Limb Perfusion

Hind limb blood flow was quantified after 7 days of ligation (ATA: n = 15; full-length TR3: n = 13 and WT: n = 14). For this purpose, a pressure-controlled perfusion of the isolated hind limbs was performed, using fluorescent microspheres. Briefly, a catheter was inserted in the abdominal aorta for perfusion of the hind limbs with 4 differently fluorescent labeled microspheres (Molecular Probes, Eugene, Oregon, USA). These microspheres were put in a buffer solution of NaCl 0.9%, adenosine (5 mg/liter) and Tween 20% (1 ml/liter). Each differently colored microsphere was infused at a specific pressure level (70, 80, 90 and 100 mm Hg) that was generated via an automated, computer driven exogenous perfusion system. Hereafter, the tissue from the peripheral hind limb (m. gastrocnemius and m. peroneus) was harvested for digestion of the tissue, and subsequent counting of the microspheres using FACS analysis. Perfusion was expressed as a percentage of the ligated compared to the non-ligated hind limb.

Immunohistochemistry and in Situ Hybridization of Collateral Arteries

Proximal hind limb muscle tissue (m. quadriceps and m. adductor) were harvested 7 days after femoral artery ligation (ATA: n = 5; full-length TR3: n = 5 and WT: n = 5) and snap-frozen in methylbutane that was cooled with liquid nitrogen at -150 to -160 °C. Finally, the tissue was stored at -80 °C until further processing. Some samples were fixed in formalin and embedded in paraffin. For all histological examinations 5 μm sections were used. Micrographs were taken with a Leica microscope (Leica, Wetzlar, FRG) equipped with a Sony DXC-950 3CCD digital video camera. TR3 and ATA are expressed under control of the SM22α-promoter and to evaluate transgene expression in this model we assessed expression of endogenous SM22α by radioactive in situ hybridization assays that were performed as described. The following probe was synthesized for in situ hybridization: SM22α, Genbank NM_011526, bp 330-582. A matching sense riboprobe was assayed and was shown to give neither background nor an aspecific signal. The sections were exposed for 5 days. Antibody 1A4 (DAKO) that recognizes SM α-actin was used to detect vascular SMCs. An antibody directed against PCNA (DAKO) was used to detect proliferating SMCs in the vascular wall. For pretreatment, the sections were rehydrated, incubated with 0.3% hydrogen peroxide to block endogenous peroxidase activity, and blocked with 10% (vol/
vol) preimmune goat serum (DAKO) in Tris-buffered saline (TBS; 10 mmol/L Tris (pH 8.0), 150 mmol/L NaCl). Subsequently, the sections were incubated with specific antibodies, followed by incubation with biotinylated secondary antibodies, which were detected with streptavidin-horseradish peroxidase conjugates (DAKO). Peroxidase activity was visualized with aminoethylcarbazole and hydrogen peroxide. After counterstaining with hematoxylin, the sections were embedded in glycergel (Sigma).

Statistical Analysis
Data are expressed as mean ± SEM. Continuous variables were compared using a Student T-test using SPSS version 11.0 (SPSS Inc, Arlington, USA). A multiple comparison was made between the control and two treatment groups, using an ANOVA-test with a Dunnett’s (post) test. A p-value <0.05 was considered statistically significant.

Results
In vitro vasodilation and vasoconstriction
Vasoconstrictor responses
The increase in wall tension (N/mm) of isolated thoracic aorta and mesenteric segments in response to a high potassium chloride concentration (40 mM) was similar in preparations obtained from TR3, ATA and control groups (1.79 ± 0.09, 1.75 ± 0.03 and 1.879 ± 0.11 N/mm, respectively for mesenteric segments).

The maximal response and pD₂ values of phenylephrine-mediated α₁-adrenoreceptor activation were not significantly different in small resistance mesenteric arteries taken from TR3, ATA or wild-type mice as shown in Fig. 1A. Also the concentration response curves for the thromboxane A2 receptor agonist U46619 were not different for the three experimental groups (Fig. 1B). Furthermore, the maximal response to phenylephrine or U46619 was not significantly different in aorta segments taken from TR3, ATA or wild-type mice (data not shown).

Vasodilator responses
Endothelium-dependent vasodilator responses of small resistance mesenteric artery (Fig. 1C) or thoracic aorta (data not shown) preparations to metacholine proved not to be significantly altered in the TR3, ATA and wild-type animals. After preconstriction with phenylephrine (1 µM) the relative relaxant responses were the same in these three groups. The methacholine-induced responses of the thoracic aorta preparations and the small resistance mesenteric arteries (maximal relaxation 81.5 ± 4.7%) appeared not to be influenced by TR3. Concentration-response curves for isoprenaline showed no difference in maximal
Normal arterial responsiveness and remodeling in TR3 mice

vasodilator response (75.7 ± 3.5%) or sensitivity of small resistance mesenteric arteries of the different mice (Fig. 1D). Thoracic aortas of the various mice also showed no difference in their response to isoprenaline (data not shown).

Our results indicate that TR3 or ΔTA do not induce major changes in the responsiveness of isolated aorta segments or small resistance mesenteric arteries to different vasoactive substances.

**Figure 1.** The relaxant and vasoconstrictor effects of phenylephrine (n=6-7; A), thromboxane (U46619; n=6-7; B), methacholine (n=6; C) and isoprenaline (n=6-7; D) on segments of mesenteric arteries of transgenic and wild-type mice.
Chapter 7

Collateral artery dependent hind limb perfusion

To assess the effect of TR3 overexpression or inhibition of all three TR3-like factors by ΔTa overexpression, we challenged the transgenic mice with a femoral artery ligation. None of the mice showed a macroscopically visible loss of function or overt necrosis of the foot. Microsphere measurements revealed no statistical differences of hind limb perfusion between mice expressing ΔTa, full-length TR3 and the WT mice after 7 days of femoral artery ligation ΔTa: 54.6% ± 16%; full-length TR3: 58.8% ± 17% and WT: 58.5% ± 14%; Fig. 2; P = ns (not significant).

**Figure 2.** Microsphere perfusion measurements 7 days after femoral artery ligation of mice that express ΔTa, the dominant-negative variant of TR3 (n=15), mice expressing full-length TR3 (n=13) and wild-type littermates (n=14), respectively. Quantification of blood flow of the occluded leg is expressed as a percentage from the non-occluded leg. (ns = not significant)

In situ hybridisation and immunohistochemistry of collateral arteries

In the transgenic mice applied in this study the transgenes were under control of the SM22α promoter to direct expression specifically to arterial SMCs. To exclude the possibility that the absence of significant differences in collateral formation were observed due to downregulated expression of the transgenes in this ligation model, we evaluated the expression of SM22α expression in collateral vessels one week after ligation. Radioactive in situ hybridization showed clear expression specifically in all SMCs of the arterial vessel wall of the collateral arteries (Fig. 3a). Consequently, we assume that the transgenes are continuously expressed during collateral artery remodeling.

To further substantiate similarity of medial SMC proliferation in collaterals in response to ligation in the different (transgenic) mice, we assayed for proliferating cell nuclear antigen (PCNA) in sections of ligated hind limbs. Immunohistochemistry showed no differences in PCNA expression in TR3, ΔTa or wild-type mice after femoral artery ligation (Fig. 3b and 3c).
In situ hybridization

Immunohistochemistry

**Figure 3.** In situ hybridization to show SM22α expression 7 days after femoral artery ligation (a). Immunohistochemistry to demonstrate PCNA expression in collateral arteries obtained from ΔTA (b) or TR3 (c) mice after femoral artery ligation.

**Discussion**

In the present study we have shown that vascular responsiveness, as assessed in vitro using different vasoactive substances, was not different in aortic rings and small resistance mesenteric arteries derived from transgenic mice in which the nuclear receptor TR3 was either overexpressed or fully inhibited by overexpression of its dominant-negative variant ΔTA, compared to wild-type mice. Furthermore, modulation of TR3 activity did not cause a (pathological) effect on vascular remodeling in the process of collateral artery formation.

**TR3 in Contractile and Vasodilator Responses**

SMCs play a key role in physiological processes such as vasoconstriction or vasodilatation, as well as in vascular pathologies like atherosclerosis, (in-stent) restenosis, vein-graft disease and transplantation arteriosclerosis. In previous studies we have shown that TR3 (and possibly also MINOR and NOT) inhibit SMC proliferation in vitro as well as in an in vivo restenosis model and proposed that TR3-like transcription factors exhibit an inhibitory role in atherogenesis. More specifically, it was shown that TR3 affects protein expression levels of the cyclin-dependent kinase inhibitor p27^Kip1^ and thus promotes arrest of the cell cycle at G1.\(^7,16\) However, since SMCs are pivotal in the process of physiological vasoconstriction and vasodilation as well as in arteriogenesis, the inhibition of SMC proliferation may theoretically influence the functionality of these cells. This may create significant adverse effects, when considering the stimulation of TR3 expression as a novel therapeutic modality for the treatment of neointima formation, in-stent restenosis and/or atherosclerosis. For this reason, the influence of changes of TR3 expression on the vasoconstrictor and vasodilator arterial activity was evaluated in an in-vitro setup. The vasoconstrictor responses to high potassium concentrations (40 or 120 mM), phenylephrine as well as the thromboxane
analogue U46619 and the vasodilator responses to methacholine and isoprenaline appeared not to be influenced by TR3 overexpression or inhibition of all three endogenous TR3-like factors. This is of importance because recently it was discovered that another nuclear receptor, i.e. the retinoic acid receptor-related orphan receptor α (RORα), which is involved in atherogenesis, is required for a normal contractile phenotype of SMCs in small resistance arteries. Disruption of the RORα gene in the so-called staggerer mice causes a reduced contractile response to serotonin and phenylephrine of the small resistance mesenteric arteries. Also relaxation was impaired in these vessels, whereas these phenomena were not observed in aorta segments of the staggerer mice. In contrast, we show that TR3 nuclear orphan receptor is not required for normal contractile responses of SMCs in aorta and small resistance mesenteric arteries of mice.

Collateral artery formation after modulation of TR3 expression
Arteriogenesis is the process of remodeling of pre-existing arterioles to mature collateral arteries. This natural compensatory mechanism has been shown to limit the damage in patients with an acute myocardial infarction. In case of arterial obstruction, these collateral arteries are recruited, bypassing the vessel narrowing or occlusion. The redistribution of blood flow via arterioles that interconnect the different vascular territories causes a local increase of shear stress, initiating an activation of the endothelium. Subsequently, the endothelium starts the production of adhesion molecules and chemotactic cytokines like monocyte chemoattractant protein 1 (MCP-1), regulating the local attraction, adhesion and subsequent transendothelial migration of monocytes. After transformation into macrophages, these cells create an inflammatory environment (involving metalloproteinases and pro-inflammatory cytokines like tumor necrosis factor α) enabling the expansion of these pre-existing arterioles. Subsequently, perivascular macrophages generate mitogenic factors like basic fibroblast growth factor and transforming growth factor β that stimulate proliferation of SMCs around the remodeling arterioles. Ultimately, this results in an increase in lumen size of the arterioles and an increased number of perivascular SMC layers. As already discussed, the intracellular transcription factor TR3 inhibits SMC proliferation. Therefore, it was postulated that overexpression of TR3 or inhibition of all three TR3-like transcription factors in arterial SMCs may potentially influence the natural course of remodeling of arterioles in the process of arteriogenesis. However, in the present study no effects were observed in our established murine ligation model of arterial obstructive disease. These data clearly demonstrate that neointimal SMC hyperplasia as observed in restenosis is an intrinsically different process as the medial SMC expansion during
Normal arterial responsiveness and remodeling in TR3 mice

arteriogenesis. In this respect it is of interest to mention that even though substantial functional involvement of FGF-2 in neointimal SMC hyperplasia as well as in arteriogenesis has been proposed, it has recently been shown that targeted disruption of FGF-2 gene does not affect collateral remodeling.24

TR3 activity in the vascular wall

Since no effects were observed on the contractile and vasodilator responses of the normal arterial wall neither as on the natural course of the arteriogenesis process in response to modulation of TR3 activity, it seems likely that these processes do not utilize the transcription factor TR3. The rationale behind these findings remains speculative. One explanation may be the fact that the driving force of the process of intimal thickening is caused by a combination of thrombus deposition, cellular inflammation, and ultimately intimal SMC proliferation and migration. Moreover, progression of atherosclerotic lesion formation is sustained by the local transition of macrophages into lipid laden foam cells that are trapped in the vessel wall.1 This is substantially different from the driving force in vasoconstriction and vasodilation in our in-vitro setup (pharmacologically induced) and in the model of arteriogenesis (enhanced shear forces and lack of the presence of foam cells).8 The transcriptional activity of TR3-like factors is modulated by co-activators and co-repressors, as well as by the extent of hetero-dimerization with rexinoid receptors (RXR).25 The expression of each of these regulating proteins may be modulated distinct in the different processes involving SMC hyperplasia. Consequently, differences in gene expression profiles downstream of TR3-like factors may vary between these processes and experimental models. Second, no expression of TR3 is observed in the normal arterial wall. We have previously shown that TR3 (and MINOR and NOT) mRNA was exclusively expressed in neointimal SMCs and not in normal medial SMCs.7 Although the modified TR3 construct was also embedded in the medial layer of the arterial wall of the created mice, the site specific expression of TR3 in case of pathological conditions like intimal thickening may explain the ineffectiveness of modulation of TR3 expression in the in-vitro and in-vivo experimental procedures. In both conditions, SMC activation takes place in the (normal) medial layer of the vascular wall rather than in the subendothelial compartment where intimal hyperplasia takes place.

Conclusions

In conclusion, the efficacy of the inhibition of SMC-rich lesion formation using over-expression of the TR3 receptor is associated with normal arterial responsiveness of both
aorta and small resistance mesenteric arteries and vascular remodeling. TR3-like factors are members of the Nuclear Receptor superfamily, which comprises proteins of which the transcriptional activity in general is regulated by small, non-protein ligands. At present, traditional ligand(s) of the orphan TR3-like receptors are still unidentified. Our current findings are important when considering TR3-like factors as therapeutic targets in the treatment of atherosclerosis and/or restenosis.

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Normal arterial responsiveness and remodeling in TR3 mice

References


