American Journal of Pathology, Vol. 168, No. 6, June 2006

DOI: 10.2353/ajpath.2006.050932

Vascular Biology, Atherosclerosis and Endothelium Biology

TR3 Nuclear Orphan Receptor Prevents Cyclic Stretch-Induced Proliferation of Venous Smooth Muscle Cells

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In coronary artery bypass surgery, the patency of arterial grafts is higher than that of venous grafts because of vein-graft disease, which involves excessive proliferation of venous smooth muscle cells (SMCs) and subsequent accelerated atherosclerosis. We studied the function of TR3 nuclear orphan receptor (TR3) in the early response of SMCs to mechanical strain, a major initiator of vein-graft disease. We demonstrate that TR3 expression is induced in human saphenous vein segments exposed *ex vivo* to whole-blood perfusion under arterial pressure. Cultured venous SMCs challenged by cyclic stretch displayed TR3 induction and enhanced DNA synthesis, whereas SMCs derived from the internal mammary artery remained quiescent. Small-interfering RNA-mediated knockdown of TR3 and adenovirus-mediated overexpression of TR3 in venous SMCs enhanced and abolished stretch-induced DNA synthesis, respectively. Accordingly, in organ cultures of wild-type murine vessel segments exposed to cyclic stretch, $p27^{Kip1}$ was down-regulated, whereas expression of this cell cycle inhibitor was unaffected by cyclic stretch in TR3-transgenic vessels, concordant with a lower proliferative response. Finally, stretch-mediated proliferation was inhibited by 6-mercaptopurine, an agonist of TR3. In conclusion, TR3 represents inhibitory mechanisms to restrict venous SMC proliferation and may contribute to prevention of vein-graft disease. *(Am J Pathol 2006, 168:2027–2035; DOI: 10.2353/ajpath.2006.050932)*

Smooth muscle cells (SMCs) play a key role in vascular pathologies such as atherosclerosis, (in-stent) restenosis after angioplasty, and vein-graft disease after coronary artery bypass surgery.[1] Even though the first two types of vascular disease occur in the arterial vessel wall and the latter in the venous vessel wall, SMC hyperplasia is a critical factor in the onset and progression of these large vessel diseases. Various stimuli are involved in initiation of SMC proliferation, of which inflammatory pathways are well established.[2] Here, we study the distinct effect of cyclic stretch on proliferation of venous and arterial SMCs to delineate the molecular mechanisms underlying the different responses to this physical force.

Bypass surgery is an established intervention to treat coronary artery disease, and both the internal mammary artery and the saphenous vein are applied as bypass material. The arterial bypass has a better patency than the venous bypass, but because of its relatively short length, its availability is limited. Venous material suffers from frequent development of vein-graft disease, resulting in vein-graft failure in 10 to 30% per year.[1,3] Vein-graft disease is the result of excessive SMC proliferation that is conceivably caused by cyclic stretch,[4,5] followed by activation of the mitrogen-activated protein kinase (MAPK)[6] and the nuclear factor κB[7] signal transduction pathways, for example, and the subsequent modulation of expression of a number of genes.[8,9] However, limited information is available on the functional involvement of genes

Supported by the Netherlands Heart Foundation (grants 99-167, M93.007, 2003B199, M93.001, and 2001D038) and by the Bekales Foundation, European Union 6FP Network of Excellence EVGN LSHM-CT-2003-503254.

Accepted for publication February 17, 2006.

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regulated by mechanical activation of SMCs except for *IEX-1* and *nuclear factor κB.*[10,11]

Previous studies on the transcription factor TR3, a member of the superfamily of nuclear receptors[12] and also known as nerve growth factor-induced protein B (NGFI-B), NR4A1, or Nur77, indicate a role of TR3 in vascular pathologies.[13,14] Originally, TR3 was found to induce T-cell apoptosis.[15] Yet, in vascular endothelial cells and SMCs, TR3 acts as an antiproliferative transcription factor, involving enhanced expression of an inhibitor of cell-cycle progression $p27^{Kip1}$ and resulting in cell-cycle arrest.[13,14] In the carotid artery ligation model, a murine model for SMC-rich lesion formation, we have shown that TR3 overexpression inhibits SMC hyperplasia.[13] Recently, it has been shown that the transcriptional activity of TR3 and its subfamily members Nurr1 (NR4A2) and NOR-1 (NR4A3) is enhanced by 6-mercaptopurine (6-MP), a nontraditional agonist that increases the activity of TR3-like factors without direct binding to these nuclear receptors.[16,17]

To define the relative contribution of cyclic stretch in initiation of vein-graft disease and the underlying mechanism of this stimulus in venous SMC hyperplasia compared with SMCs derived from the internal mammary artery, we studied the expression of the early-response gene TR3 in distinct SMC stretch models. First, cultured SMCs derived from both saphenous veins and internal mammary arteries were exposed to cyclic stretch, and in accordance with published data, we show that venous SMCs become proliferative, whereas arterial SMCs remain quiescent.[4] Second, both human and (transgenic) mouse vessels were studied in dedicated organ culture models in which arterial (pulsatile) pressure was applied. Third, we demonstrate functional involvement of TR3 in inhibition of stretch-induced proliferation by overexpressing the gene, inhibiting the expression of endogenous TR3 with small-interfering RNA (siRNA), and by enhancing TR3 activity with 6-MP.

Materials and Methods

Human Tissue Specimens

The *ex vivo* perfusion model in which human saphenous vein segments were exposed to whole blood under arterial pressure was described previously.[18] Briefly, vein segments ($n = 6$ for each time point; 1 and 6 hours) were placed in a loop of the extracorporeal circulation during bypass surgery and were exposed to autologous blood under flow (nonpulsatile), and the intraluminal pressure was allowed to approximate the arterial pressure of the patient. To study the effect of overdistension on bypass veins, vein segments were perfused in the presence or absence of an external stent (polytetrafluoroethylene graft).[18] After 1 and 6 hours of perfusion, the vein segments were harvested, fixed in formalin, and embedded in paraffin for histological examination. Patients included in this study gave their informed consent, and the local medical ethical committee approved the study. Anesthesia and cardiopulmonary bypass surgery were performed according to routine protocols.

In Situ *Hybridization*

In situ hybridizations were performed as described previously.[19] TR3 and plasminogen activator inhibitor 1 (PAI-1) probes were synthesized as follows: TR3, GenBank no. L13740, bp 1221 to 1905; PAI-1, GenBank no. X12701, bp 52 to 1308. The probes were labeled with [^{35}S]UTP (Amersham Biosciences, Buckinghamshire, United Kingdom). Paraffin sections (5 μm) of control and perfused saphenous vein segments were mounted on SuperFrost Plus slides (Menzel-Glaser, Braunschweig, Germany). After hybridization and stringent washing, the *in situ* sections were covered with nuclear research emulsion (ILFORD Imaging U.K. Limited, Cheshire, United Kingdom), exposed for 2 to 9 weeks, and then developed and counterstained with hematoxylin and eosin. Matching sense riboprobes were assayed for each gene and revealed neither background nor a nonspecific signal. As a control for the integrity of RNA, *in situ* hybridizations were performed with an antisense riboprobe for thrombin receptor PAR-1 (GenBank no. M62424, bp 3076 to 3472). PAR-1 was abundantly expressed in SMCs of control and perfused vein segments, indicating that the integrity of the RNA was comparable in all specimens (data not shown).

Immunohistochemistry

Paraffin sections (5 μm) were deparaffinized, rehydrated, incubated with 0.3% (v/v) hydrogen peroxide, and blocked with 10% (v/v) preimmune goat serum (DAKO, Glostrup, Denmark) in 10 mmol/L Tris-HCl (pH 8.0) and 150 mmol/L NaCl (Tris-buffered saline [TBS]). To detect endothelial cells, sections were incubated overnight at 4°C with biotinylated Ulex europaeus Agglutinin (Vector Laboratories Inc., Burlingame, CA) (1:50 dilution) in TBS, whereas $p27^{Kip1}$ expression was detected with a specific antibody from BD Biosciences (Alphen a/d Rijn, The Netherlands) followed by a biotin-conjugated secondary antibody. Biotin was detected with streptavidin-horseradish peroxidase conjugates (DAKO), and sections were subsequently developed with amino-ethylcarbazole and hydrogen peroxide. TR3 was detected after antigen retrieval with antibody M210 (Santa Cruz, Biotechnology, Santa Cruz, CA), followed by polyhorseradish peroxidase-goat-anti-mouse incubation (ImmunoLogic, Duiven, The Netherlands), and sections were developed with NovaRed (Vector). After counterstaining with hematoxylin, sections were embedded in glycergel (Sigma, St. Louis, MO).

SMC Culture and Immunofluorescence

Venous and arterial SMCs were cultured from explants of saphenous vein (SV) and internal mammary arteries (IMAs) in Dulbecco's modified Eagle's medium, containing 10% (v/v) fetal bovine serum with penicillin and streptomycin

(GIBCO-Invitrogen Life Technology, Breda, The Netherlands) and were used at passages 4 to 6. SMCs were characterized with monoclonal antibody 1A4, directed against SM α-actin (DAKO) and demonstrated to have homogenous fibrillar staining. SMCs were seeded in 6-well plates containing collagen I-coated flexible membranes (BioFlex culture plates; Dunn Labortechnik GmbH, Asbach, Germany) and were stretched in the Flexercell FX3000 apparatus (Dunn Labortechnik) for 1 hour up to 24 hours at 10% stretch at 0.5 Hz or without stretch (controls). To assess TR3 protein expression in control and stretched cells (5 hours), the cells were fixed in 4% (w/v) paraformaldehyde and were subsequently incubated with TR3 antibody (M210, 1:200), which was detected with goat-anti-rabbit-Alexa568 (Molecular Probes, Eugene, OR).

[^{3}H]Thymidine Incorporation, Adenovirus Infection, siRNA Electroporation, and 6-MP Treatment of SMCs

SMCs were seeded in 6-well stretch plates, and after reaching confluency, the cells were made quiescent by exposing them for 16 hours to medium containing only 0.5% (v/v) fetal bovine serum. The plates were transferred into the loading station and stretched for 24 hours. Control plates, without stretch, were cultured under identical conditions. Thereafter, cells were radiolabeled for 4 hours with 0.5 μCi/ml [methyl-^{3}H]thymidine (Amersham Biosciences). Incorporated radioactivity was precipitated for 30 minutes at 4°C with 10% (w/v) trichloroacetic acid, washed twice with 5% (w/v) trichlorioacetic acid, and dissolved in 0.5 N NaOH. [^{3}H]Thymidine incorporation was measured by liquid scintillation counting. On infection for 2 hours with mock- or TR3-containing adenovirus (3×10^8 plaque-forming units/10 cm^2), the cells were allowed to recover for 24 hours in complete medium before plating in the stretch plates.

The following siRNA sequences were used: TR3 siRNA, 5′-CAG UCC AGC CAU GCU CCU C dTdT-3′, as described previously[20]; and mutated control siRNA, 5′-CAG ACG AGC CUU GCU CGU C dTdT-3′ (Ambion Inc., Huntingdon, UK). Per Flexerplate well, 1 μg of siRNA was transfected into 5×10^5 SMCs using Nucleofector reagent for SMCs (Amaxa GmbH, Cologne, Germany) per the manufacturer's recommendations; the cells were subsequently placed in the stretch plates and treated as described above. 6-MP treatment (Sigma) was initiated 1 hour before stretch (stock at 50 mmol/L in dimethylsulfoxide).

Western Blotting Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with cell lysates (30 μg/lane). Proteins were transferred to nitrocellulose-Protran (Schleicher and Schuell, Hertogenbosch, The Netherlands). Expression of p27^{Kip1} (BD), p21^{Cip1} (BD), SM α-actin (DAKO), PAI-1 (MAI-12; Biopool, Umea, Sweden), TR3 (anti-mouse Nur77; BD Pharmingen), calponin (clone hCP; Sigma), and α-tubulin (Cedar Lane, Hornby, Ontario, Canada) was studied, using the indicated antibodies directed against these proteins. Primary antibodies were incubated overnight at 4°C in 5% Protifar plus (Nutricia, Cuijk, The Netherlands) in TBS. As secondary antibodies, horseradish peroxidase-conjugated goat anti-rabbit (for p27^{Kip1}) or goat anti-mouse (for all other antibodies) (Bio-Rad Laboratories Inc., Hercules, CA) in a 1:5000 dilution in TBS were used. Proteins were visualized by enhanced chemiluminescence detection (Lumi-Light^{PLUS}; Roche Diagnostics GmbH, Mannheim, Germany). Quantitative analysis was performed by the Lumi-Imager (Boehringer Mannheim, Mannheim, Germany). α-Tubulin staining served as a control for equal loading.

Real-Time Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated using Trizol reagent (GIBCO). cDNA was synthesized by reverse transcription from 1 μg of total RNA with SuperScript II (GIBCO) and 0.5 μg $(dT)_{12-18}$ primer. Real-time polymerase chain reaction was performed with the use of the FastStart DNA Master SYBR Green I kit (Roche) in the LightCycler System (Roche). Primers for TR3 were as follows: forward, 5′-GTTCTCTGGAGGTCATCCGCAAG-3′, and reverse, 5′-GCAGGGACCTTGAGAAGGCCA-3′. As a control for equal amount of first-strand cDNA in different samples, we determined hypoxanthine phosphoribosyl transferase mRNA levels with the following primers: forward, 5′-TAATTATGGACAGGACTGAACG-3′, and reverse, 5′-CACAATCAAGACATTCTTTCCAG-3′.

Organ Cultures of Mouse Carotid Arteries

Organ culture was performed as described previously.[21] Briefly, left and right carotid arteries were obtained from TR3-transgenic mice[13] or wild-type littermates and were tied on both ends to glass cannulas. The vessels were equilibrated to 37°C in Leibovitz medium (GIBCO), and the left carotid artery was exposed for 48 hours to pulsatile stretch (60–140 mm Hg) at 1 Hz, whereas the right carotid artery was maintained unloaded in the same medium. The medium was recirculated at low flow (1 ml/min). After 48 hours, the vessels were removed from the organ bath, fixed in 4% (v/v) paraformaldehyde, and embedded in paraffin for subsequent histological analyses.

Statistical Analysis

Values are mean ± SD, and Student's *t*-test was applied for paired or unpaired analyses to reveal statistical significance. Data comprising multiple groups were analyzed by analysis of variance.

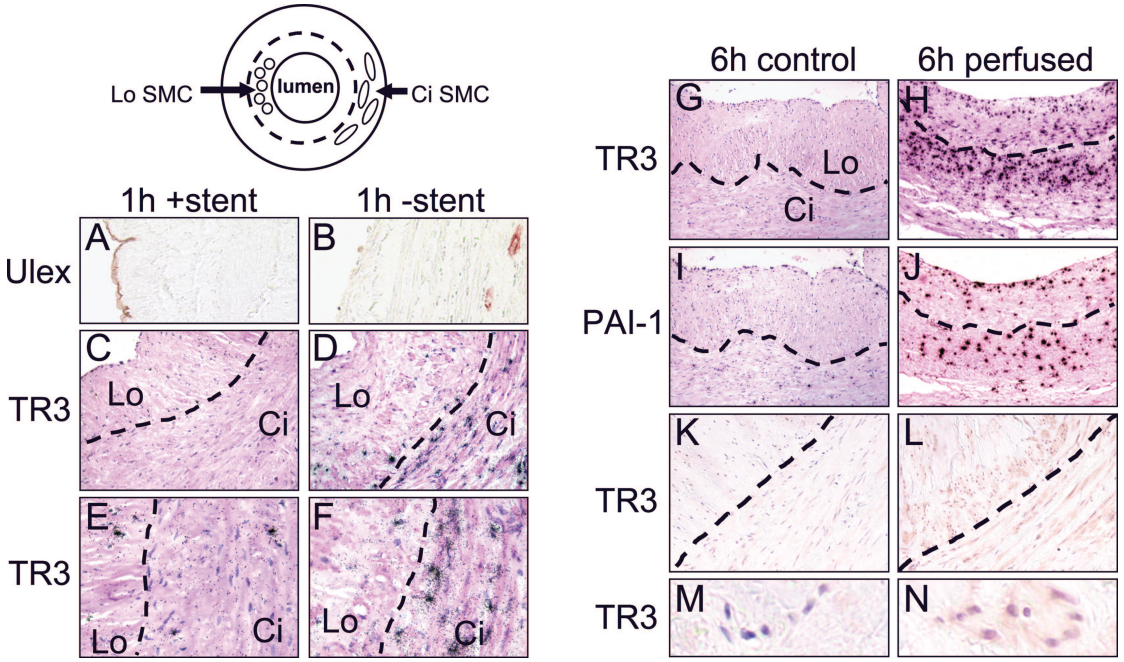

Figure 1. Endothelial cell-specific immunohistochemistry and TR3 and PAI-1 expression in perfused human vein segments. A schematic drawing of the venous vessel wall structure shows the longitudinally oriented inner layer (Lo) and the outer layer in which the SMCs are oriented circularly (Ci). Human vein segments were placed in a loop of the extracorporal circulation during bypass surgery with or without the support of an external stent. **A:** After 1 hour of perfusion, endothelium integrity was preserved in the stented segments (**red monolayer**). **B:** Loss of endothelium was observed in the nonsupported segments, whereas capillary endothelial cells were detected near the adventitia. TR3 mRNA expression was studied by radioactive *in situ* hybridization (**black dots**). Scarce TR3 expression was observed in stented vein grafts (**C**, ×200; **E**, ×400). In nonstented veins after 1 hour of perfusion, TR3 expression is only observed in the circular (Ci) SMC layer (**D**, ×200; **F**, ×400), whereas after 6 hours of perfusion, TR3 is expressed throughout the vessel wall (**H**, ×100). As a control, PAI-1 mRNA expression was assayed and shown to be induced in response to 6 hours of perfusion under arterial pressure (**J**, ×100). In nonperfused vein grafts, no expression of TR3 or PAI-1 mRNA was observed (**G** and **I**, ×100). **K–N:** Immunohistochemistry to demonstrate TR3 protein expression only in perfused vein segments (**K** and **L**, ×200; **M** and **N**, ×630). TR3 protein is predominantly expressed in the nucleus (compare **M**, no TR3 expression, and **N**, with stretch-induced TR3 expression). **Dotted lines** indicate the border between Lo and Ci SMC layers. Nuclei were counterstained in purple. Similar results were found in vessel segments derived from six individuals undergoing coronary artery bypass grafting.

Results

TR3 Expression in Perfused Human Saphenous Vein Segments

To study the molecular processes causing vein-graft disease, we applied an *ex vivo* perfusion model in which segments of saphenous veins are placed in the extracorporeal circulation during coronary artery bypass surgery. The arterial pressure generated substantial distension of the nonstented saphenous veins, which resulted in an almost complete loss of the endothelial cell layer and damage to the circular (outer) SMC layer after 1 hour of perfusion under arterial pressure.[18] Veins protected against excessive cyclic stretch, because of placement of an external stent, contained intact endothelium after perfusion (as illustrated by endothelium-specific immunohistochemistry; Figure 1A). In the nonstented vein segments, endothelial cell-specific staining revealed the presence of endothelial cells in capillaries in the adventitia, whereas the luminal endothelium had disappeared (Figure 1B).

The SMC organization of saphenous veins differs from that of the arterial wall because veins contain two layers of SMCs that are oriented in perpendicular directions, whereas arteries contain only circularly oriented SMCs. A layer of longitudinally oriented SMCs is situated close to the lumen of the vein, and a circular SMC layer (like in arteries) is present adjacent to the adventitia (Figure 1).

To search novel genes involved in vein-graft disease, we assayed mRNA expression of the early-response gene TR3 in *ex vivo* perfused vein segments by radioactive *in situ* hybridization. After 1 hour of perfusion, TR3 expression was detected in a few endothelial cells and SMCs of the stented vein segments (Figure 1, C and E). However, extensive TR3 expression was detected predominantly in the outer circular SMC layer of the nonstented vein segments (Figure 1, D and F). TR3 expression was virtually absent in the nonperfused control vein segment (Figure 1G), yet after 6 hours of perfusion, TR3 was abundantly expressed throughout the entire vessel, in both the longitudinal and circular SMC layers of the nonstented perfused vein (Figure 1H).

As a control, PAI-1 mRNA expression was analyzed because it has been shown that PAI-1 is induced by cyclic stretch in cultured SMCs.[8,9] In control, nonstretched veins, PAI-1 was present in a few endothelial cells and SMCs (Figure 1I), and PAI-1 expression was strongly increased in the venous vessel wall after 6 hours of perfusion (Figure 1J). Furthermore, TR3 protein expression was assayed in specimen derived before and after perfusion (Figure 1, K–N). TR3 protein expression is clearly induced in the venous vessel wall in response to stretch, and TR3 protein is localized predominantly in the nucleus (Figure 1, M and N; magnifications in K and L).

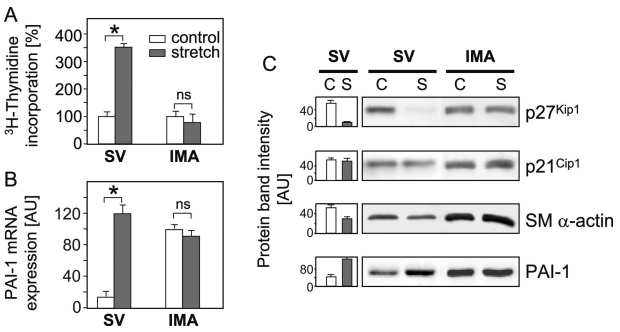

Figure 2. Cyclic stretch-induced [^{3}H]thymidine incorporation in venous SMCs and changes in gene and protein expression. **A:** DNA synthesis increased in response to a 24-hour period of cyclic stretch in venous SMCs, whereas arterial SMCs were unresponsive to stretch. **B:** PAI-1 mRNA was increased in venous SMCs after 4 hours of stretch but not in IMA SMCs. Similar data were obtained in SV and IMA SMCs derived from 12 and 6 independent donors, respectively, in **A** and **B**. **C:** p27^{Kip1} protein was down-regulated after 24 hours of stretch in venous SMCs, whereas p21^{Cip1} expression levels remained similar as demonstrated by Western blotting. In addition, SM α-actin was down-regulated in response to stretch in venous SMCs, whereas PAI-1 levels were up-regulated. In arterial cell lysates, the expression of these proteins was unchanged. α-Tubulin expression served as control for equal loading (not shown), and intensity of protein bands corrected for α-tubulin was given for the protein expression levels in venous SMCs in arbitrary units (AU). SV, saphenous vein SMCs; IMA, internal mammary artery SMCs; C, control; S, stretch; $^*P < 0.01$; ns, not statistically significant.

In conclusion, TR3 and PAI-1 mRNA and TR3 protein are expressed in SMCs in human saphenous vein grafts subjected to perfusion under arterial pressure, and TR3 mRNA expression is initially localized in the circularly oriented SMCs. The circular SMC layer is the outer part of the venous vessel wall, indicating that TR3 expression is presumably not induced by a circulating factor in blood or in response to endothelial cell damage but rather that the key stimulus is cyclic stretch.

Cyclic Stretch-Induced Proliferation in Venous SMCs

To explain the better performance of bypass material derived from mammary artery than that from saphenous veins and to investigate the underlying mechanism, the intrinsic difference between SMCs derived from these distinct vessels was studied in response to cyclic stretch. We applied an experimental stretch device (Flexercell FX-3000 apparatus) to subject SMCs of arterial and venous origin to cyclic stretch. Standardization of this model involved analysis of DNA synthesis and PAI-1 expression.[4,8,9] SMCs, derived from mammary arteries or saphenous veins, were subjected for 24 hours to 10% cyclic stretch (0.5 Hz), and [^{3}H]thymidine incorporation was monitored. In line with published data,[4] we observed that stretch increased DNA synthesis in venous SMCs 3.5-fold, whereas arterial SMCs derived from the same individual remained quiescent (Figure 2A). PAI-1 mRNA was measured as a marker gene for stretch sensitivity and was shown to be induced 8.1-fold in venous SMCs by cyclic stretch, whereas in arterial SMCs, PAI-1 expression is virtually unaffected by cyclic stretch. Basal PAI-1 mRNA expression is relatively high in mammary artery SMCs compared with venous SMCs (Figure 2B). To reveal potential changes in cell-cycle progression, the expression level of two inhibitors of the cell cycle was analyzed in cell lysates of stretched SMCs of venous and arterial origin. Cyclin-dependent kinase inhibitor p27^{Kip1} was decreased in venous SMCs on stretch (Figure 2C). In accordance with the DNA synthesis data, stretch did not alter the expression of p27^{Kip1} in arterial SMCs. The expression of another cell-cycle inhibitor, p21^{Cip1}, was not affected by stretch in either venous or arterial SMCs. SM α-actin protein expression was assayed as a measure for quiescence of SMCs and was moderately decreased in venous SMCs after stretch. In addition to mRNA levels, we also established that PAI-1 protein levels were increased in stretched venous cells. In line with the mRNA expression data, relatively high levels of PAI-1 protein are synthesized in arterial SMCs independent of cyclic stretch. In conclusion, cyclic stretching induces a proliferative phenotype in saphenous vein SMCs, whereas mammary artery SMCs remain quiescent.

TR3 Overexpression Decreases and siRNA-TR3 Enhances Stretch-Induced Proliferation of Venous SMCs

To extend the observations made in SV segments exposed to perfusion (Figure 1), we determined whether TR3 mRNA is also expressed by *in vitro* cultured SMCs in response to cyclic stretch. Saphenous vein and mammary artery SMCs were stretched for periods of 1, 2, 4, or 6 hours, whereas nonstretched cells served as controls. TR3 mRNA was up-regulated in arterial SMCs (Figure 3A). However, in venous SMCs, TR3 mRNA expression was induced 14.2 ± 1.7-fold after 1 to 2 hours cyclic stretch to a significantly higher level than in arterial SMCs. TR3 protein expression was analyzed by immunofluorescence (Figure 3C, a–d), demonstrating only background signal in mammary artery SMCs without and with stretch (Figure 3C, a and b). In saphenous vein SMCs transfected with a control siRNA, TR3 protein expression is robustly induced after 5 hours of stretch (Figure 3C, c and d). Again, mammary artery-derived SMCs appear to be distinct from venous SMCs and seem less responsive to cyclic stretch.

In previous studies, we described and applied a dominant-negative variant of TR3 (ΔTA) that inhibits the activity of all TR3-like factors.[13,14] In the current study, we chose to specifically knockdown TR3 expression in venous SMCs by TR3-specific siRNA. Clearly, TR3-specific siRNA reduced endogenous TR3 mRNA expression after 2 hours of cyclic stretch to approximately 30% of the expression in the presence of a control siRNA (Figure 3B). TR3 protein expression was also reduced by siRNA knockdown as shown in Figure 3C by immunofluorescence (compare d and f). Significantly, this reduction in TR3 expression resulted in an increased proliferative response of the cells in response to stretch as shown by [^{3}H]thymidine incorporation experiments (Figure 3D).

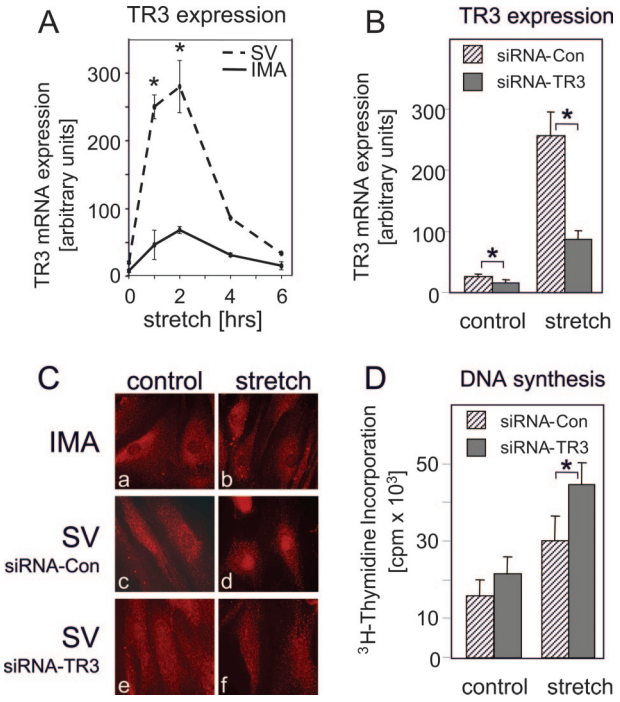

Figure 3. Stretch-induced TR3 expression in venous SMCs and enhanced DNA synthesis after TR3 siRNA knockdown. **A:** TR3 mRNA expression was increased optimally in venous SMCs 1 to 2 hours after stretch. TR3 mRNA expression was corrected for equal mRNA content by correcting for the extent of hypoxanthine phosphoribosyl transferase mRNA expression. **B:** siRNA transfection of venous SMCs resulted in down-regulation of TR3 mRNA expression after 2 hours of cyclic stretch with TR3 gene-specific siRNA sequences, compared with a control siRNA. **C:** TR3 protein expression was detected by immunofluorescence. In IMA SMCs, only background signal was detected (**a** and **b**). TR3 protein expression is increased in response to stretch (5 hours) in SV SMCs (compare **c** and **d**) and is down-regulated by TR3-siRNA (compare **d** and **f**). **D:** Knockdown of endogenous TR3 expression by siRNA-TR3 results in enhanced DNA synthesis in response to stretch as measured by [^{3}H]thymidine incorporation, compared with SMCs transfected with siRNA-Con. SV, saphenous vein SMCs; IMA, internal mammary artery SMCs. *$P < 0.01$. The results in **A** were obtained in SV-SMCs derived from six independent donors; in **B** and **C**, the experiments were repeated in three distinct SV-SMCs cultures and in **D**, in five distinct SV-SMCs cultures.

Subsequently, TR3 was overexpressed in venous SMCs by infection with TR3-containing adenovirus, and after 24 hours of stretch, the infected cells were assayed for DNA synthesis. Mock virus-infected cells showed a 2.7-fold increase in DNA synthesis on stretch, similar to the response in noninfected venous SMCs (compare Figure 4A with Figure 2A). However, in TR3-overexpressing SMCs, the proliferative response under conditions of cyclic stretch was repressed (Figure 4A). TR3 protein expression was confirmed by Western blotting analysis after 24 hours of stretch; at this time point, no endogenous TR3 was visible (Figure 4B). Furthermore, after stretch, TR3 virus-infected SMCs showed a more differentiated (contractile) SMC phenotype, as evidenced by increased expression of SM α-actin, calponin, and 27^{Kip1} protein compared with mock virus-infected cells (Figure 4B). In conclusion, inhibition of endogenous TR3 enhances DNA synthesis, whereas overexpressed TR3 results in complete inhibition of this adverse stretch response.

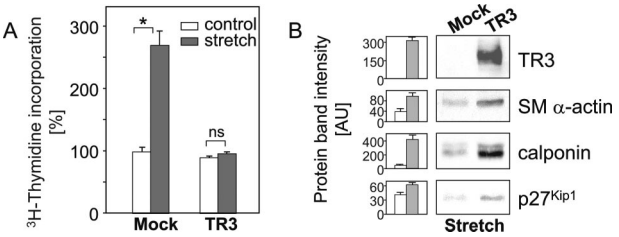

Figure 4. Adenovirus-mediated TR3 overexpression inhibits stretch-induced DNA synthesis. **A:** [^{3}H]Thymidine incorporation is increased after 24 hours of cyclic stretch in mock virus-infected SMCs compared with infected, unstretched control cells. Adenovirus-mediated overexpression of TR3 completely blocked stretch-induced DNA synthesis (data from experiments in SV-SMCs derived from four independent donors). **B:** The expression of TR3 protein after infection of stretched, venous SMCs with TR3-encoding adenovirus as demonstrated by Western blotting (a typical example is shown after 24 hours of stretch). In stretched, TR3-infected cells, SM α-actin, calponin, and 27^{Kip1} protein levels are higher than in mock-infected cells. α-Tubulin served as control for equal loading (not shown), and intensity of protein bands, corrected for the amount of α-tubulin protein, is given in arbitrary units (AU). *$P < 0.01$; ns, not statistically significant.

TR3 Prevents P27^{Kip1} Down-Regulation in the Murine Carotid Organ Model

To further substantiate functional involvement of TR3 in protection against SMC proliferation in response to cyclic stretch, we exposed segments of (transgenic) mouse vessels in an organ culture model that reproduces *in vivo* mechanical conditions of cyclic stretch. We applied vessels of transgenic mice in which TR3 overexpression is directed to arterial SMCs by a well-described part of the SM22α promoter.[13] Because TR3 is not overexpressed in the veins of these mice, we studied TR3-related stretch responses in carotid arteries of which the medium is relatively thin with only three to four layers of SMCs. For each mouse, we cannulated one carotid artery and exposed it for 48 hours to cyclic stretch (60 to 140 mmol/L Hg, 1Hz, minimal flow) and maintained the other carotid artery under similar culture conditions, without stretch. Subsequently, $p27^{Kip1}$ immunohistochemical analyses were performed as a measure for proliferation, and we compared the relative number of $p27^{Kip1}$-positive cells in nonstretched and stretched carotids in paired analyses as a measure for a proliferative response (Figure 5). In nonstretched carotids, the percentage of $p27^{Kip1}$-positive nuclei was similar for wild-type ($n = 4$) and TR3-transgenic mice ($n = 4$) (53.8 ± 4.6 and 46.3 ± 9%,

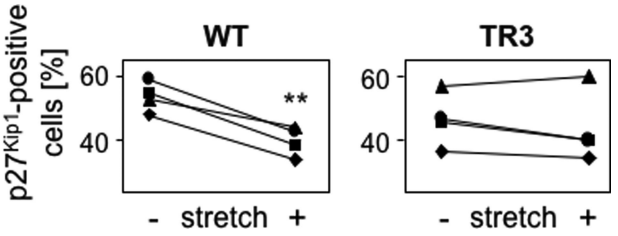

Figure 5. Down-regulation of $p27^{Kip1}$ expression in mouse vessel segments in response to cyclic stretch is prevented by transgenic TR3 overexpression. Carotid arteries derived from wild-type or TR3-transgenic mice ($n = 4$ each) were maintained in organ cultures during 48 hours under cyclic stretch (60 to 140 mm Hg, 1 Hz) or left unstretched. Histological sections were subsequently assayed for $p27^{Kip1}$ expression by immunohistochemistry, and the relative number of positive cells was counted. **$P = 0.004$ in paired *t*-test analysis.

respectively). However, in wild-type carotid arteries $p27^{Kip1}$ expression was significantly down-regulated by cyclic stretch (to 40.0 ± 4.5%, $P = 0.004$), whereas the percentage of $p27^{Kip1}$-positive SMCs did not change significantly in stretched TR3-transgenic carotids (to 43.3 ± 11.6%, $P = 0.28$) (Figure 5). In conclusion, enhanced TR3 expression also prevents a proliferative response to cyclic stretch in intact vessel segments.

6-MP Decreases Stretch-Induced Proliferation in Venous SMCs

To provide additional evidence that endogenous TR3 directly affects stretch-induced SMC proliferation and to propose a therapeutic strategy to prevent vein-graft disease, we assayed the effect of the TR3 agonist 6-MP on cyclic stretch-induced DNA synthesis. 6-MP is the active metabolite of the immunosuppressive drug azathioprine (Imuran) that induces apoptosis of T cells and acts as agonist of TR3-like factors.[16,22] This immunosuppressive drug is well tolerated during chronic use, although at high doses, 6-MP is cytotoxic because of the ability of its metabolites to incorporate into DNA. To exclude the possibility that 6-MP would be applied to SMCs at concentrations that would induce apoptosis or cytotoxicity, venous SMCs were cultured with 25 μmol/L 6-MP or, as a control, with the apoptotic agent carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (at 10 μmol/L), which is known to induce apoptosis. CCCP-treated cells demonstrated less cell spreading and more shrunken nuclei (a mild apoptotic phenotype), whereas cells treated with 6-MP and control cells showed normal morphology as revealed by SM α-actin staining and the presence of large, round nuclei, indicative for the absence of apoptosis (data not shown). To determine whether 6-MP influences stretch-induced proliferation, venous SMCs were treated with 6-MP at various concentrations. Untreated venous SMCs, subjected to 24 hours of stretch, showed a 2.5-fold induction of [^{3}H]thymidine incorporation, whereas the effect on DNA synthesis was reduced in a dose-dependent manner by 6-MP treatment (Figure 6A). At 25 μmol/L 6-MP, stretch-induced DNA synthesis was completely inhibited. Analogous to TR3 overexpression, 6-MP also increases SM α-actin and calponin as well as $p27^{kip1}$ protein expression under stretch conditions (Figure 6B). To reveal the relative contribution of TR3 in 6-MP-mediated inhibition of stretch-induced proliferation, we assayed the effect of 6-MP on DNA synthesis in SMCs transfected with TR3-siRNA (Figure 6C). Knockdown of TR3 by siRNA completely abolishes the effect of 6-MP on stretch-induced DNA synthesis. These data unambiguously demonstrate that TR3 mediates the inhibitory effect of 6-MP on the proliferative response of SMCs in stretch.

In conclusion, we show that 6-MP prevents excessive SMC proliferation of venous SMCs through activation of TR3. Moreover, this experiment demonstrates that endogenous TR3 levels are sufficient to prevent stretch-induced DNA synthesis when an agonist enhances its activity.

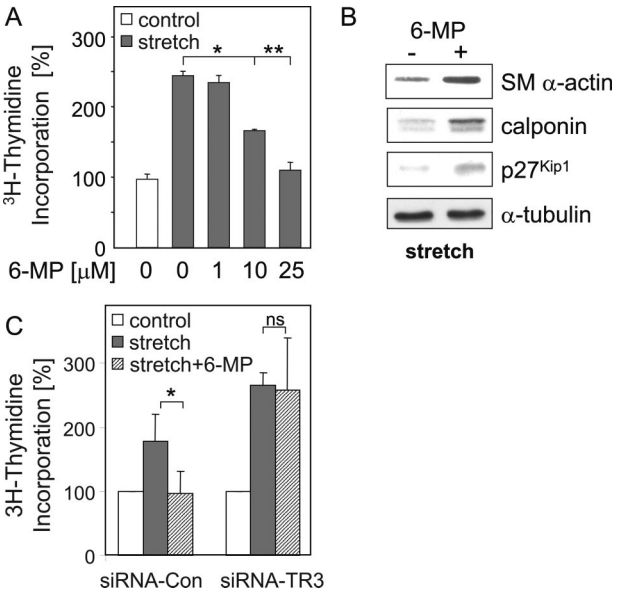

Figure 6. 6-MP inhibits DNA synthesis in venous SMCs exposed to cyclic stretch. **A:** [^{3}H]Thymidine incorporation was increased in SMCs in response to 24 hours of cyclic stretch, whereas 6-MP reduced stretch-mediated proliferation in a dose-dependent manner. [^{3}H]Thymidine incorporation was expressed as percentage of the control value. **B:** The expression of SMα-actin, calponin, and $p27^{Kip1}$ protein in stretched, venous SMCs treated without (−) or with (+) 25 μmol/L 6-MP is detected by Western blotting. α-Tubulin served as control for equal loading. **C:** When TR3 expression is knocked down by siRNA-TR3, 6-MP no longer inhibits DNA synthesis, which was measured by [^{3}H]thymidine incorporation. Analysis of variance analysis of the data revealed significance of the data. $^*P < 0.05$, $^{**}P < 0.01$; NS, not significant.

Discussion

Coronary artery bypass surgery relieves patients from angina pectoris and may prevent myocardial infarction. However, when saphenous veins are applied as bypass material, excessive SMC hyperplasia may cause vein-graft disease, representing a significant drawback for a substantial number of patients.[1,3] In contrast, mammary artery bypass grafts display a better patency than saphenous vein grafts. Resolving the molecular differences between these bypass specimens may lead us to a rational design of targets to combat or prevent SMC-based pathologies.[3]

In the human *ex vivo* perfusion model applied in this study, the early-response gene TR3 was not expressed in control veins, whereas TR3 mRNA was up-regulated in the circular SMC layer after 1 hour of perfusion under arterial pressure and throughout the vessel wall after 6 hours. We concluded that TR3 mRNA expression is presumably not induced by a circulating factor or in response to endothelial cell damage but rather that the key stimulus is cyclic stretch. Anatomical differences between the internal mammary artery and saphenous vein, ie, the lack of elastic laminae in veins, may in part explain the observed graft damage by overdistension of vein segments under arterial pressure.[18] Vein segments that were protected against overdistension by an external stent displayed normal morphology as illustrated by preserved luminal endothelium and by the absence of TR3 mRNA induction.

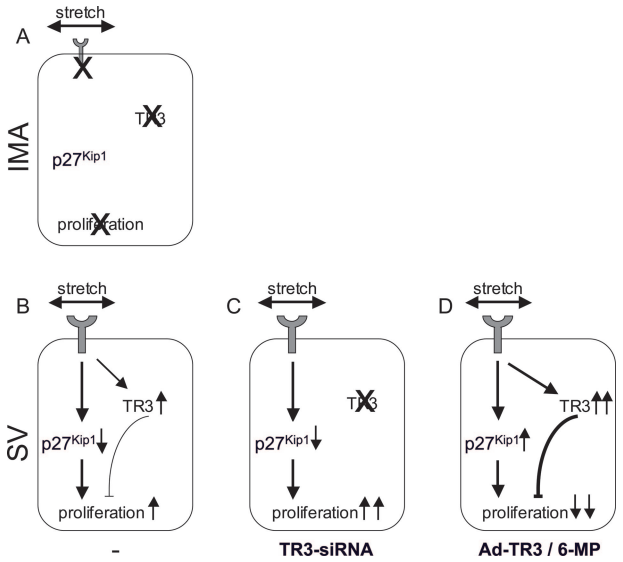

Figure 7. Schematic model of mechanical strain responses in IMA and SV SMCs. **A:** IMA-SMCs remain quiescent when exposed to stretch, which corresponds with relatively high $p27^{Kip1}$ protein expression levels and unaffected proliferation. **B:** In SV-SMCs, integrin signaling is activated in response to mechanical strain resulting in down-regulation of $p27^{Kip1}$ protein expression and induction of proliferation. Simultaneously, feedback mechanisms are initiated in SV-SMCs involving enhanced TR3 expression to prevent an excessive proliferative response. **C:** The importance of TR3 in such endogenous inhibitory pathways has been revealed by knocking down TR3 expression (siRNA), which results in enhanced proliferation of SV-SMCs after mechanical strain. **D:** Overexpression of TR3 or enhancement of the activity of endogenous TR3 with the agonist 6-MP results in a reduction of stretch-induced proliferation.

In addition, intrinsic differences between mammary artery and saphenous vein SMCs play a crucial role in the response of SMCs to external stimuli. It has been established that saphenous vein SMCs are more responsive to mitogenic stimuli such as platelet derived growth factor, thrombin, or cyclic stretch than internal mammary artery SMCs.[4,23,24] In the present study, we investigated the role of transcription factor TR3 with regard to intrinsic differences in stretch-related responses of these two types of SMCs in an *in vitro* stretch model, and the data obtained in this study are summarized in the scheme in Figure 7. We observed robust up-regulation of TR3 expression in venous SMCs, in contrast to internal mammary artery SMCs (Figure 7, A and B). The abundant stretch-mediated expression of TR3 in vein grafts in the *ex vivo* model confirmed the relevance of the data found *in vitro*. Previously, we reported that TR3 decreases serum-induced DNA synthesis in SMCs, which prompted us to study functional involvement of TR3 in stretch-induced proliferation.[13] siRNA knockdown experiments show that down-regulation of TR3 expression enhances DNA synthesis in stretched SMCs (Figure 7C). Significantly, adenovirus-mediated TR3 overexpression in venous SMCs results in full inhibition of stretch-induced DNA synthesis (Figure 7D). Moreover, also in vessels derived from TR3-transgenic mice, a lower proliferative response was observed after cyclic stretch compared with wild-type vessels. Based on these data, we propose that TR3 protects venous SMCs from excessive proliferation. In addition, the SMC-specific differentiation markers SM α-actin and calponin were up-regulated in stretched SMCs that were infected with adenoviruses mediating TR3 overexpression. These data imply that TR3 not only prevents proliferation of venous SMCs but also enhances the differentiated, contractile phenotype of these cells. Hence, these data support the concept that TR3 may act as a target for intervention in vein-graft disease. This is further strengthened by our observations with 6-MP, a recently identified agonist of TR3 that modulates TR3 activity.[16] In T cells, it has been shown that 6-MP enhances apoptosis through inactivation of rac-1, a GTP-binding protein with an anti-apoptotic function in T cells.[22] Activation of the ras/rac-1 pathway and subsequent p38 MAPK phosphorylation has been implicated in cyclic strain-induced proliferation of SMCs.[6] We show that 6-MP decreased stretch-induced SMC proliferation in a dose-dependent way and increased $p27^{Kip1}$ levels and that TR3 expression is crucial in these cellular responses to 6-MP (Figures 6C and 7D).

In conclusion, vein-graft disease is the result of excessive SMC proliferation in response to biomechanical stimulation of venous bypass grafts. Venous SMCs respond to cyclic stretch by initiation of proliferation, while at the same time, cell-cycle inhibitory feedback systems are also activated, such as the recently described IEX-1 pathway[10] and the TR3 transcription factor pathway identified in this study. The activity of endogenous TR3 is enhanced by 6-MP, and we hypothesize that 6-MP modulates biomechanical intimal thickening after bypass surgery as a means to prevent excessive SMC proliferation and subsequent vein-graft disease.

Acknowledgments

Simone A.R. van de Graaf and Chris M. van der Loos are acknowledged for technical assistance.

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