Activation of Nuclear Receptor Nur77 by 6-Mercaptopurine Protects Against Neointima Formation

Nuno M.M. Pires, MSc*; Thijs W.H. Pols, MSc*; Margreet R. de Vries, BSc; Claudia M. van Tiel, PhD; Peter I. Bonta, MD; Mariska Vos, BSc; E. Karin Arkenbout, MD, PhD; Hans Pannekoek, PhD; J. Wouter Jukema, MD, PhD; Paul H.A. Quax, PhD; Carlie J.M. de Vries, PhD

Background—Restenosis is a common complication after percutaneous coronary interventions and is characterized by excessive proliferation of vascular smooth muscle cells (SMCs). We have shown that the nuclear receptor Nur77 protects against SMC-rich lesion formation, and it has been demonstrated that 6-mercaptopurine (6-MP) enhances Nur77 activity. We hypothesized that 6-MP inhibits neointima formation through activation of Nur77.

Methods and Results—It is demonstrated that 6-MP increases Nur77 activity in cultured SMCs, which results in reduced [3H]thymidine incorporation, whereas Nur77 small interfering RNA knockdown partially restores DNA synthesis. Furthermore, we studied the effect of 6-MP in a murine model of cuff-induced neointima formation. Nur77 mRNA is upregulated in cuffed arteries, with optimal expression after 6 hours and elevated expression up to 7 days after vascular injury. Local perivascular delivery of 6-MP with a drug-eluting cuff significantly inhibits neointima formation in wild-type mice. Locally applied 6-MP does not affect inflammatory responses or apoptosis but inhibits expression of proliferating cell nuclear antigen and enhances protein levels of the cell-cycle inhibitor p27Kip1 in the vessel wall. An even stronger inhibition of neointima formation in response to local 6-MP delivery was observed in transgenic mice that overexpressed Nur77. In contrast, 6-MP does not alter lesion formation in transgenic mice that overexpress a dominant-negative variant of Nur77 in arterial SMCs, which provides evidence for the involvement of Nur77-like factors.

Conclusions—Enhancement of the activity of Nur77 by 6-MP protects against excessive SMC proliferation and SMC-rich neointima formation. We propose that activation of the nuclear receptor Nur77 is a rational approach to treating in-stent restenosis. (Circulation. 2007;115:493-500.)

Key Words: muscle, smooth • receptors, cytoplasmic and nuclear • restenosis • stents • transcription factors

Postangioplasty restenosis is a common complication after percutaneous coronary interventions and is mainly caused by excessive proliferation of vascular smooth muscle cells (SMCs). As a result, the coronary artery narrows again, which severely reduces beneficial effects of the intervention. Even though the introduction of drug-eluting stents has considerably decreased restenosis incidence, postangioplasty restenosis remains a persistent problem.

Clinical Perspective p 500

The phenotypic modulation of SMCs from quiescent differentiated cells into activated proliferative cells plays a key role in restenosis. In our search for genes involved in such phenotypic changes, we have revealed induction of Nur77 gene expression. Furthermore, we showed that Nur77 is expressed in SMCs in the diseased human vessel wall but not in healthy vessels. Paradoxically, overexpression of Nur77 in SMCs leads to a quiescent SMC phenotype, and mice that overexpress Nur77 in arterial SMCs are protected against SMC-rich lesion formation. Therefore, we propose Nur77 as a potential target for treatment of restenosis.

Nur77 (NR4A1, TR3, NGFI-B) is highly homologous to Nurr1 (NR4A2) and NOR-1 (NR4A3), and together they form the nuclear receptor subfamily 4 group A (NR4A) of nuclear hormone receptors. The NR4A transcription factors are implicated in diverse cellular events, such as apoptosis, differentiation, and proliferation. Nuclear receptors consist of an N-terminal activating function-1 domain, a DNA-binding domain, and a ligand-binding domain at the C-terminus. At this time no traditional ligands are known to activate Nur77-like factors through interaction with their ligand-binding domains, which designates Nur77, Nurr1, and...
NOR-1 as orphan receptors. Recently, however, it has been shown that 6-mercaptopurine (6-MP) increases Nur77 trans-activation via its activating function-1 domain without direct binding to Nur77. 6-MP is currently used for the treatment of leukemia, whereas azathioprine, the pro-drug of 6-MP, is prescribed at relatively low doses as a chronic immunosuppressive drug in inflammatory bowel disease as well as after organ transplantation.

A well-defined mouse model of neointima formation consists of placement of a nonconstrictive perivascular cuff around the femoral artery. Previously we showed that the nonconstrictive perivascular cuff may be constructed from a polymeric formulation suitable for controlled drug delivery. This drug-eluting cuff simultaneously induces reproducible intimal hyperplasia and allows confined delivery of drugs to the cuffed vessels. In the present study, drug-eluting cuffs were applied to evaluate the local effect of 6-MP on neointima formation. We demonstrate that enhancement of the activity of Nur77 by 6-MP inhibits SMC proliferation and activity of Nur77 by 6-MP inhibits SMC proliferation and facilitates cell death, favoring the resolution of the neointima.

Methods

SMC Culture

Human SMCs were explanted from umbilical cord arteries. Cells were cultured in Dulbecco’s Modified Eagle’s Medium (Invitrogen, Breda, the Netherlands) with 10% (v/v) fetal bovine serum (FBS) with penicillin and streptomycin (Invitrogen). Cells were used at passages 5 to 7. SMCs were characterized with a monoclonal antibody and directed against smooth muscle α-actin (1A4; DAKO, Glostrup, Denmark), and SMCs demonstrated uniform fibri-

Transfection Experiments and Luciferase Assay

Cells were electroporated according to the Amaxa method (Amaxa, Breda, the Netherlands) with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) fetal calf serum. 

DNA Synthesis Assay

SMCs were seeded in 24-well plates at 1 to 4×10^4 cells/well and reached 60% to 70% confluence after 24 hours. SMCs were made quiescent by incubation for 24 hours in FBS-free medium. 6-MP was dissolved in dimethylsulfoxide and applied 1 hour before FBS stimulation. SMCs were stimulated for 24 hours with 10% (v/v) FBS, and subsequently cells were labeled for 18 hours with 0.25 μCi/well [methyl-3H]thymidine (Amersham Biosciences, Buckinghamshire, UK). Incorporated [3H]thymidine was precipitated for 30 minutes at 4°C with 10% (wt/vol) trichloroacetic acid, washed twice with 5% (wt/vol) trichloroacetic acid, and dissolved in 0.5N NaOH (0.5 mL/well), and radioactivity was measured by liquid-scintillation count.

Small Interfering RNA Experiments

The following small interfering RNA (siRNA) sequences were used: Nur77 siRNA, 5’-CAGUCACGCAAUOCUCCUtGdTdT-3’, as described previously; 5’-CAGUCACGAAUOCUCCU- GCUCGUUtGdTdT-3’ (Ambion, Austin, Tex). Five micrograms of siRNA was transfected into 0.5 to 1×10^6 SMCs with Nucleofector reagent for SMCs (Amaxa) according to the manufacturer’s recommendations. Total mRNA was isolated 5 days after transfection with the Absolutely RNA Miniprep Kit (Stratagene, La Jolla, Calif). Subsequent cDNA synthesis was performed with the iScript cDNA synthesis kit (BioRad, Hercules, Calif). Real-time polymerase chain reaction (PCR) was performed with SYBR green mix (Biorad) in the MyIQ System (BioRad). Primers for Nur77 were as follows: forward, 5’-GTCTCTGGAAGTCATCGGCAAG-3’; reverse, 5’-GACGGGAC- CTGAGAAGGCCA-3’. As a control for equal amounts of first-strand cDNA in different samples, we checked for β-actin and phosphoprotein (PP) mRNA levels, which were determined with the following primers: forward, 5’-TCGCAATGGGACATCTAC-3’; reverse, 5’-ATCGCT- TCCACAGACAGG-3’.

Drug-Eluting Cuffs

Poly(e-caprolactone)–based drug-eluting cuffs were manufactured as previously described. Briefly, 6-MP was dissolved at different concentrations in blended, molten drug–polymer mix, and cuffs were designed to fit around the mouse femoral artery. Drug-eluting cuffs are shaped as longitudinally cut cylinders with an internal diameter of 0.5 mm, an external diameter of 1.0 mm, a length of 2.0 mm, and a weight of ~5.0 mg. Drug-eluting cuffs were loaded with 0.5% and 1% (w/w) 6-MP, and the in vitro release profiles were determined for a 4-week period as described before (n=5 per group). 6-MP showed a sustained and dose-dependent release. Total release values at 4 weeks were 11.3±2.3 μg (46.3%) and 30.0±3.5 μg (58.7%) for the 0.5% and 1% 6-MP–eluting cuffs, respectively.

Femoral Artery Cuff Murine Model

All animal studies were approved by the Academic Medical Center (Amsterdam, the Netherlands) institutional regulatory authority and carried out in compliance with guidelines issued by the Dutch government. Wild-type FVB mice, transgenic mice that expressed NOR-1 as orphan receptors, were housed under control of the SM22α promoter, which directs the expression of transgenes specific to SMCs in an FVB background were used for experiments. Male mice that were 10 to 12 weeks old were fed a standard chow diet. At the time of surgery, mice were anesthetized 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 fentanyl (Janssen, Geel, Belgium). The femoral artery was dissected from its surroundings and loosely sheathed with a nonconstrictive cuff. Either an empty control cuff or a 6-MP–eluting cuff (0.5% or 1% w/w) was used (n=6 per group).

Nur77 mRNA Expression in Cuffed Mouse Femoral Artery

Male wild-type mice underwent femoral artery cuff placement as described above. Animals were euthanized at different time points after surgery (0, 6, 24, 48, and 72 hours, and 7 days), with 4 mice per group. Femoral arteries were isolated, harvested, and snap-frozen. Total RNA was isolated with the RNeasy Fibrous Tissue Mini-Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer’s protocol. cDNA was made from all RNA samples with Ready-To-Go Forward, 5’(P0) mRNA levels, which were determined with the following primers:

DNA Synthesis Assay

SMCs were seeded in 24-well plates at 1 to 4×10^4 cells/well and reached 60% to 70% confluence after 24 hours. SMCs were made quiescent by incubation for 24 hours in FBS-free medium. 6-MP was dissolved in dimethylsulfoxide and applied 1 hour before FBS stimulation. SMCs were stimulated for 24 hours with 10% (v/v) FBS, and subsequently cells were labeled for 18 hours with 0.25 μCi/well [methyl-3H]thymidine (Amersham Biosciences, Buckinghamshire, UK). Incorporated [3H]thymidine was precipitated for 30 minutes at 4°C with 10% (wt/vol) trichloroacetic acid, washed twice with 5% (wt/vol) trichloroacetic acid, and dissolved in 0.5N NaOH (0.5 mL/well), and radioactivity was measured by liquid-scintillation count.

Small Interfering RNA Experiments

The following small interfering RNA (siRNA) sequences were used: Nur77 siRNA, 5’-CAGUCACGCAAUOCUCCUtGdTdT-3’, as described previously; 5’-CAGUCACGAAUOCUCCU-GCUCGUUtGdTdT-3’ (Ambion, Austin, Tex). Five micrograms of siRNA was transfected into 0.5 to 1×10^6 SMCs with Nucleofector reagent for SMCs (Amaxa) according to the manufacturer’s recommendations. Total mRNA was isolated 5 days after transfection with the Absolutely RNA Miniprep Kit (Stratagene, La Jolla, Calif). Subsequent cDNA synthesis was performed with the iScript cDNA synthesis kit (BioRad, Hercules, Calif). Real-time polymerase chain reaction (PCR) was performed with SYBR green mix (Biorad) in the MyIQ System (BioRad). Primers for Nur77 were as follows: forward, 5’-GTCTCTGGAAGTCATCGGCAAG-3’; reverse, 5’-GACGGGAC-CTGAGAAGGCCA-3’. As a control for equal amounts of first-strand cDNA in different samples, we checked for β-actin and phosphoprotein (PP) mRNA levels, which were determined with the following primers: forward, 5’-TCGCAATGGGACATCTAC-3’; reverse, 5’-ATCGCTTCCACAGACAGG-3’.

Drug-Eluting Cuffs

Poly(e-caprolactone)–based drug-eluting cuffs were manufactured as previously described. Briefly, 6-MP was dissolved at different concentrations in blended, molten drug–polymer mix, and cuffs were designed to fit around the mouse femoral artery. Drug-eluting cuffs are shaped as longitudinally cut cylinders with an internal diameter of 0.5 mm, an external diameter of 1.0 mm, a length of 2.0 mm, and a weight of ~5.0 mg. Drug-eluting cuffs were loaded with 0.5% and 1% (w/w) 6-MP, and the in vitro release profiles were determined for a 4-week period as described before (n=5 per group). 6-MP showed a sustained and dose-dependent release. Total release values at 4 weeks were 11.3±2.3 μg (46.3%) and 30.0±3.5 μg (58.7%) for the 0.5% and 1% 6-MP–eluting cuffs, respectively.

Femoral Artery Cuff Murine Model

All animal studies were approved by the Academic Medical Center (Amsterdam, the Netherlands) institutional regulatory authority and carried out in compliance with guidelines issued by the Dutch government. Wild-type FVB mice, transgenic mice that expressed NOR-1 as orphan receptors, were housed under control of the SM22α promoter, which directs the expression of transgenes specific to SMCs in an FVB background were used for experiments. Male mice that were 10 to 12 weeks old were fed a standard chow diet. At the time of surgery, mice were anesthetized 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 fentanyl (Janssen, Geel, Belgium). The femoral artery was dissected from its surroundings and loosely sheathed with a nonconstrictive cuff. Either an empty control cuff or a 6-MP–eluting cuff (0.5% or 1% w/w) was used (n=6 per group).

Nur77 mRNA Expression in Cuffed Mouse Femoral Artery

Male wild-type mice underwent femoral artery cuff placement as described above. Animals were euthanized at different time points after surgery (0, 6, 24, 48, and 72 hours, and 7 days), with 4 mice per group. Femoral arteries were isolated, harvested, and snap-frozen. Total RNA was isolated with the RNeasy Fibrous Tissue Mini-Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer’s protocol. cDNA was made from all RNA samples with Ready-To-Go
reverse transcription PCR beads (Amersham Biosciences, Uppsala, Sweden). Intron-spanning primers and probes were designed to hybridize with murine Nur77 cDNA (sense, 5′-GGGCATGTTGAA-GGAAGTTGTG-3′; antisense, 5′-AAGCTGCTGGTGGTTTGAG-3′; probe, 5′-CCGCTTITTATGGCTGTCCG-3′) with Primer Express 1.5 (Applied Biosystems, Foster City, Calif). Hypoxanthine phosphoribosyltransferase was assayed to correct for cDNA input. For each time point, reverse transcription PCR was performed in duplicate. Data are presented as fold-induction of Nur77 mRNA expression in injured over noninjured vessels.

Quantification and Histological and Immunohistochemical Analysis of Lesions in Cuffed Femoral Arteries

Mice were euthanized at indicated time points after cuff placement. The thorax was opened and a mild pressure-perfusion (100 mm Hg) with 4% formaldehyde in 0.9% NaCl (v/v) was performed for 5 minutes by cardiac puncture. After perfusion, femoral arteries were harvested, fixed overnight in 4% formaldehyde, dehydrated, and embedded in paraffin. Serial cross sections (5 μm thick) for histological analysis were used throughout the entire length of the cuffed femoral artery. All samples were routinely stained with hematoxylin-phloxine-saffron. Weigelt’s elastin staining was used to visualize elastic laminae. Ten equally spaced cross sections were used in all mice to quantify intimal lesions. Image analysis software (Leica Qwin, Wetzlar, Germany) was used to measure the total cross-sectional medial area between the internal and external elastic lamina; total cross-sectional intimal area was measured between the endothelial cell monolayer and the internal elastic lamina.13 Standard biotin-streptavidin procedures were used for immunohistochemistry to detect mouse leukocytes (CD45, 1:200; BD Pharmingen, San Diego, Calif), proliferating cell nuclear antigen (1:100; Calbiochem, San Diego, Calif), or p27kip1 (1:100; Novocastra, Newcastle on Tyne, UK). Biotin was detected with streptavidin–horseradish peroxidase conjugates (DAKO), and sections were subsequently developed with diaminobenzidine and hydrogen peroxide. Apoptotic cells were detected by terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick-end labeling (TUNEL) with an in situ cell death detection kit (Roche). The relative number of positive cells was counted.

Statistical Analysis

Data are reported as mean±SEM and were analyzed with the nonparametric Mann-Whitney U test (SPSS 14.0 for Windows, SPSS Inc, Chicago, Ill). Two-sided probability values were tested against adjusted type I error rates with Bonferroni-Holm step-down of the family-wise error. Statistical significance is indicated with an asterisk (*) in the table and figures.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

6-MP Enhances Nur77 Activity in Cultured SMCs

It has been shown that 6-MP enhances the transcriptional activity of Nur77 in C212 mouse skeletal muscle cells, whereas 6-MP does not affect Nur77 activity in CV-1 monkey kidney fibroblast cells.11,19 To investigate whether 6-MP increases Nur77 transcriptional activity in vascular cells, human SMCs were transduced with lentivirus encoding Nur77, which resulted in expression in 85% to 90% of the infected cells and increased Nur77 mRNA levels compared with mock-infected SMCs (data not shown). Immunofluorescence of transduced SMCs revealed Nur77 protein overexpression located in the nucleus (data not shown). SMCs that overexpressed Nur77 were subsequently electroporated with the firefly luciferase reporter construct, which contains the palindromic Nur response element (Nur77 response element from the POMC promoter) sequence (TGATATTtAAA-TGCCA)17 to monitor Nur77 transcriptional activity in combination with the thymidine kinase–renilla luciferase construct as a control for transfection efficiency. Incubation of SMCs for 24 hours with 50 μmol/L 6-MP resulted in a 10-fold increase in Nur77 activity (P=0.021, Figure 1), comparable with the induction observed in C212 cells. Our data indicate that 6-MP robustly enhances Nur77 activity in cultured SMCs.

Figure 1. 6-MP enhances Nur77 activity in cultured SMCs. The transcriptional activity of Nur77 was monitored by measuring luciferase activity in SMCs that expressed Nur77 transfected with a Nur77 reporter-luciferase construct, which contained the POMC-derived Nur response element. Cells were cultured in the absence of (C) or for 24 hours in the presence of 6-MP (n=4, mean±SEM). *Statistically significant.

6-MP Inhibits Proliferation of SMCs: Involvement of Nur77

To study whether 6-MP modulates SMC proliferation, we investigated DNA synthesis of cultured human SMCs in the presence of increasing concentrations of 6-MP. As expected, 6-MP inhibits DNA synthesis in SMCs (P=0.001 for 25 μmol/L and P=0.001 for 50 μmol/L 6-MP; Figure 2A). To assess the specific contribution of Nur77 in this process, Nur77 expression was knocked down by siRNA in injured over noninjured vessels. DNA synthesis is inhibited by 6-MP for 61% when SMCs are transfected with control siRNA, whereas the effect of 6-MP is significantly less in SMCs transfected with Nur77-specific siRNA because only 41% inhibition of DNA synthesis is observed (P=0.021 at 25 μmol/L and P=0.021 at 50 μmol/L 6-MP). These data clearly demonstrate that 6-MP inhibits DNA synthesis in SMCs at least partly through activation of Nur77.
6-MP Is Not Cytotoxic to SMCs and Does Not Induce Apoptosis

To verify whether 6-MP is cytotoxic, quiescent SMCs were incubated for 24 hours with increasing concentrations of 6-MP, and viability of the cells was determined with a standard MTT assay. The number of viable cells was reduced in response to staurosporine (35% reduction, P<0.021), whereas 6-MP did not affect cellular viability (Figure 3A), which indicates that under these conditions 6-MP has no cytotoxic effect on human SMCs.

To investigate whether 6-MP induces apoptosis in SMCs, the cells were cultured for 24 hours with increasing concentrations of 6-MP. As a positive control, staurosporine was shown to induce apoptosis in SMCs (50±2%). Clearly, no evidence was found that 6-MP affects cell death under these conditions (3±1%) in comparison to control cells (4±1%) (Figure 3B).

Nur77 Is Expressed During the Process of Neointima Formation

To assess the potential inhibitory effect of 6-MP on SMC-rich lesion formation in vivo, the well-established murine model of cuff-induced neointima formation was applied. We studied Nur77 mRNA expression during neointima formation and observed that Nur77 mRNA expression is upregulated after cuff placement, with optimal expression 6 hours after vascular injury (189±26-fold increase; Figure 4). Nur77 mRNA expression is enhanced up to 7 days after surgery in comparison to noncuffed vessels (13.4±1.1-fold increase). Given that Nur77 mRNA transcripts are regulated on vascular injury strictly dependent on conditions and time, it is conceivable that Nur77 plays a role in the process of neointima formation.
6-MP Inhibits Proliferation of SMCs in Early Cuff-Induced Lesions

To evaluate the effect of 6-MP on cuff-induced neointima formation in vivo, we used a drug-eluting cuff loaded with increasing concentrations of 6-MP, which allowed restricted, local, perivascular delivery of compounds to the cuffed vessel segment.18 The effect of 6-MP was initially evaluated in wild-type animals, and we compared lesions induced by control cuffs with lesions in cuffs containing 0.5% or 1% 6-MP within 7 days. Morphometric analysis of the lesions revealed that the intimal area was significantly reduced in 1% 6-MP cuffs (see the Table). No changes were observed in total vessel area or medial surface; consequently, the intima-media ratio was also reduced in response to local 6-MP delivery. To understand the mechanism that underlies the effect of 6-MP, we characterized the effect of 6-MP on inflammation, apoptosis, and cellular proliferation. Immunohistochemistry with anti-CD45 antibodies was performed to quantify leukocyte accumulation in the media and neointima. No differences were observed in relative leukocyte numbers within the cuffed vessel segments on perivascular delivery of increasing 6-MP concentrations as compared with control-cuffed femoral arteries. TUNEL staining showed that local 6-MP delivery has no significant effect on the number of apoptotic cells in the media or in the intimal region as compared with arteries treated with control cuffs (Table). It has been shown, especially in early lesions 5 to 7 days after vascular injury, that the number of proliferating cells in the vessel wall is relatively high.20 For that reason, we used proliferating cell nuclear antigen staining to quantify cellular proliferation in these early lesions and observed in femoral arteries with a control cuff that 22.3±4.5% of the cells were positive in the medial and 21.3±1.8% in the intimal regions. In line with the data on lesion size, 1% 6-MP–eluting cuffs led to a profound inhibition of medial (68.6%) and intimal (71.3%) cellular proliferation (Table).

Effect of 6-MP on Cuff-Induced Neointima Formation in WT, Nur77-Transgenic, and ΔTA-Transgenic Mice

To reveal involvement of Nur77 in the 6-MP–mediated effects after vessel injury, we analyzed lesion formation in wild-type and transgenic mice that expressed full-length Nur77 cDNA in the arterial wall. Microscopic analysis of cuffed femoral artery segments revealed that after 4 weeks a concentric neointima was formed in mice administered an empty control drug-eluting cuff in both wild-type and Nur77-transgenic mice. Animals administered a 6-MP–eluting cuff showed reduced lesion formation (Figure 5A). Morphometric analyses revealed significant inhibition of cuff-induced neointima formation in vessel segments, locally treated with the 1% 6-MP concentration, in both wild-type (P=0.016) and Nur77-transgenic mice (P=0.007, Figure 5B). Wild-type animals treated with 0.5% 6-MP–eluting cuffs did not show a decrease in neointima formation (P=0.32), whereas the same 6-MP concentration substantially reduced neointima formation in Nur77-transgenic mice (P=0.015). No changes were observed in medial areas of the cuffed femoral arteries (data not shown). Consequently, a similar dose-dependent decrease was seen in intima-media ratios of 6-MP–treated Nur77-transgenic mice: control cuff, 0.75±0.11; 0.5% 6-MP, 0.47±0.05 (P=0.04); 1% 6-MP, 0.30±0.06 (P=0.003). Again, intima-media ratios of cuffed arteries in wild-type mice were only significantly decreased in the 1% 6-MP cuffs: control cuff, 1.10±0.16; 0.5%, 0.75±0.05 (P=0.15); 1%, 0.51±0.03 (P=0.008).

To further establish functional involvement of Nur77 in 6-MP–mediated effects on neointima formation, 6-MP–elut-
Figure 5. The effect of local 6-MP delivery on neointima formation. A, Representative cross sections of femoral arteries of wild-type mice, transgenic mice that expressed full-length Nur77 cDNA (Nur77), or mice that expressed ΔTA, with cuffs that contained different amounts of 6-MP. The cuffed vessel segments of wild-type and Nur77-transgenic mice were analyzed by hematoxylin-phloxine-saffron staining after 4 weeks and of ΔTA-transgenic mice after 2 weeks (magnification ×400; arrows indicate the internal elastic lamina). B, Morphometric analyses of cuffed vessel segments revealed total intimal area in wild-type and Nur77-transgenic mice 4 weeks after placement of cuffs. Cuffs contained either no (control), 0.5%, or 1% 6-MP. C, Total intimal area in cuffed femoral arteries in ΔTA transgenic mice 2 weeks after placement of cuffs that contained either no, 0.5%, or 1% 6-MP (mean±SEM, n=6). *Statistically significant.

Discussion

In the present study, we investigated whether 6-MP protects against restenosis through activation of the nuclear receptor Nur77. Our data clearly demonstrate that activation of Nur77 by 6-MP inhibits SMC proliferation, a hallmark of restenosis. In addition, with the use of a murine model of neointima formation, we provide evidence that supports the hypothesis that activation of Nur77 by 6-MP inhibits neointima formation.

Wansa and colleagues have shown that 6-MP activates Nur77 in murine myoblast C2C12 cells, whereas Ordentlich et al did not observe enhanced Nur77 activity in response to 6-MP in CV-1 cells. These data suggest that Nur77 is not activated by 6-MP in all cell types, which may be explained by the fact that 6-MP does not affect the activity of Nur77 through direct interaction but rather influences interaction of (unknown) coactivators and/or corepressors with Nur77-like factors. Consequently, the relative expression levels of such presumed cofactors in distinct cell types may determine the effect of 6-MP on Nur77-like factor activation. In this report, we show that 6-MP does enhance Nur77 activity in human SMCs. Moreover, SMCs transfected with siRNA against Nur77 show less effect of 6-MP on proliferation in comparison to SMCs transfected with control siRNA.
clearly demonstrate involvement of Nur77 in 6-MP–mediated inhibition of human SMC proliferation.

We substantiated these in vitro observations in a well-defined mouse model, in which we showed that perivascular delivery of 6-MP inhibits neointima formation. Moreover, mice that overexpressed Nur77 in the arterial vessel wall and received 6-MP locally showed additional inhibition of lesion formation in comparison to 6-MP–treated wild-type mice. In addition, transgenic mice that overexpressed the dominant-negative form of Nur77 in the vessel wall, which inhibits transcriptional activity of all Nur77-like factors, did not show an effect of 6-MP on neointima formation. The latter experiments unambiguously demonstrated that 6-MP inhibits neointima formation through activation of Nur77-like factors.

We and others have shown that not only Nur77 expression but also Nur1 and NOR-1 expression are enhanced in activated SMCs. For NOR-1, it has been demonstrated that gene-specific anti-sense oligonucleotides decrease SMC proliferation. These data support a proliferative function of that gene-specific anti-sense oligonucleotides decrease SMC proliferation. In the present study, we observed a decrease in DNA synthesis of SMCs in the presence of 6-MP. Nur77 knockdown by a specific siRNA does not abolish the effect of 6-MP entirely, which indicates that the remaining effect of 6-MP may be attributed to residual Nur77 activity and/or to 6-MP–mediated Nur1 activation.

Azathioprine was one of the first immunosuppressive drugs applied for prophylaxis of acute organ transplant rejection, inflammatory bowel disease, and rheumatoid arthritis. Azathioprine is the pro-drug that is converted in vivo into 6-MP and subsequently into thioguanosine nucleotides that, at high doses, may interfere with nucleotide metabolism in proliferating cells. The dose at which azathioprine is applied chronically in transplantation protocols and inflammatory bowel disease does not invoke systemic cytostatic effects and is even compatible with normal pregnancy. Over the years, cyclosporin A has replaced azathioprine as a drug in these pathologies; however, patients treated with cyclosporin A are at relatively high risk for cardiovascular disease. Consequently, the prescription of azathioprine currently is undergoing a revival in clinical practice, with beneficial effects on lipid profiles, extent of plasma low-density lipoprotein oxidation, and fibrinolytic parameters. Our study demonstrates that 6-MP also has a favorable effect directly on the vessel wall, notably at sites of local injury when Nur77-like factors are induced. At this point, we assume that in the drug-eluting cuff experiments, the amount of 6-MP, which is locally released, does not provoke any systemic effects on lipid profiles and fibrinolytic markers. This is supported by the observation that 6-MP does not affect neointima formation in transgenic ΔTA mice. In conclusion, we have shown that activation of Nur77 by local 6-MP delivery in a mouse restenosis model does not affect inflammatory cell influx or apoptosis of vascular cells. Significantly, 6-MP–mediated Nur77 activation reduces SMC proliferation and protects against neointima formation. We therefore propose that activation of the nuclear receptor Nur77 by 6-MP or by other activators/agonists is a rational approach to treat (in-stent) restenosis.

Sources of Funding
N.M.M. Pires, T.W.H. Pols, and Dr Jukema are supported by the Netherlands Heart Foundation grants 2001-T-32, 2003B199, and 2001-D0-32, respectively. Drs Quax, de Vries, Arkenbou, and Bonta are supported by the Molecular Cardiology Program of the Netherlands Heart Foundation (M 93.001, 93.007), and Dr van Tiel is supported by NWO-Research Institute Diseases of the Elderly grant 948-00-006. This work emanates from the European Vascular Genomics Network, which is supported by the European Union FP6 Network of Excellence LSHM-CT-2003-503254.

Disclosures
None.

References


---

**CLINICAL PERSPECTIVE**

Percutaneous coronary intervention is a routine intervention for treating coronary artery disease. A complication of this procedure is restenosis, which is caused by excessive proliferation of smooth muscle cells in response to the local vascular injury provoked by percutaneous coronary intervention. Drug-eluting stents that contain sirolimus or paclitaxel have been proven to prevent in-stent restenosis; however, these drugs may adversely affect vascular tissue histology. Other strategies to locally and selectively inhibit proliferation of intimal smooth muscle cells may be favorable. In previous studies, it has been shown that the nuclear receptor Nur77 inhibits formation of smooth muscle cell–rich lesions and promotes endothelial cell survival. Moreover, the activity of the transcription factor Nur77 is enhanced by 6-mercaptopurine. On the basis of this knowledge, we hypothesized that 6-mercaptopurine may inhibit in-stent restenosis in a Nur77-dependent manner. The data presented in the present study demonstrate that enhancement of the activity of Nur77 with 6-mercaptopurine protects against smooth muscle cell proliferation and prevents lesion formation in a mouse model. These data support consideration of Nur77 as a target for the prevention and treatment of in-stent restenosis.
Activation of Nuclear Receptor Nur77 by 6-Mercaptopurine Protects Against Neointima Formation

_Circulation_. 2007;115:493-500; originally published online January 22, 2007;
doi: 10.1161/CIRCULATIONAHA.106.626838
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/115/4/493

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/