NR4A nuclear receptors in atherosclerosis
Pols, T.W.H.

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§1.1 Vascular pathologies

§1.1.1 Atherosclerosis

Blood vessels are vital in maintaining blood circulation to transport oxygen and nutrients to organs and to remove carbon dioxide and other waste products from the body. The most inner layer of a blood vessel is the intima, which mainly comprises a monolayer of endothelial cells (ECs) that form the physical barrier with the lumen through which the blood flows. The intima is covered by the media, which is composed of multiple layers of smooth muscle cells (SMCs) and extracellular matrix components. SMCs in the media provide stability to the vessel and are important in the regulation of blood flow. The border between the intima and the media is marked by the elastica interna. The adventitia is the most outer layer of the blood vessel and covers the media (Figure 1). The adventitia amongst others contains fibroblasts and capillaries that feed the vessel wall.

Atherosclerosis is a chronic inflammatory process in arteries, and is the major underlying cause of cardiovascular disease (CVD) of which morbidity and mortality is high. Systemic risk factors of atherosclerosis are well-described and amongst others include hypertension, smoking, chronic inflammatory diseases, and diabetes (Lusis, 2000). Next to systemic risk factors, also local circumstances are important, since atherosclerotic lesions have the tendency to arise at bifurcations and other locations where local blood flow is turbulent.

The first sign of atherosclerosis is a so-called fatty streak. Fatty streaks mostly consist of lipid-laden macrophages, so-called foam cells, which are located beneath the EC layer. Fatty streaks are prevalent in young people, and do not cause symptoms. Eventually, fatty streaks may progress into atherosclerotic lesions, which is a silent process that usually takes decades. A typical advanced atherosclerotic lesion is characterized by a lipid-rich necrotic core, calcifications, activated proliferating smooth muscle cells (SMCs), the presence of inflammatory cells such as T cells and, predominantly, monocyte-derived macrophages, and activated endothelial cells (Lusis,
The most critical event in atherosclerosis is the rupture of lesions, after which blood clots may fully obstruct blood flow through the artery. Depending on the location of the obstruction, this may cause an acute myocardial infarction or an ischemic stroke, which in turn have serious consequences, most severely immediate death.

§1.1.2 Vascular pathologies related to the treatment of atherosclerosis

In addition to myocardial infarction and stroke, atherosclerosis may also result in angina pectoris, which is a partial obstruction of the blood flow through a coronary artery. This causes temporary insufficient oxygen supply to the heart, giving rise to chest pain. Patients with angina pectoris may be treated with different methods, such as percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass grafting (CABG). CABG concerns the placement of bypass vessels that reroute the blood around the obstructed part of the artery, thereby restoring the blood flow. The choice of vessels used for CABG depends on the availability of the vessels, and are usually mammary arteries or saphenous veins. Saphenous veins are not accustomed to the higher arterial blood pressures, which may result in vein-graft disease. This pathology is characterized by excessive SMC proliferation causing obstruction of the lumen of the venous bypass.

A less invasive intervention to treat angina pectoris is PTCA. This procedure involves a catheter equipped with a deflated balloon that is guided into the narrowed coronary artery. Inflation of the balloon, with optional placement of a stent, may restore the blood flow. A major drawback of this treatment is (in-stent) restenosis which, like vein-graft disease, is a SMC-rich lesion that re-narrows the coronary artery. Now a days, stents may be used that are coated with cytostatic drugs (so-called drug-eluting stents) that inhibit cell proliferation and thereby decrease the incidence of in-stent restenosis. This thesis focuses on the role of the NR4A nuclear receptors in atherosclerosis and pathologies related to atherosclerosis, like (in-stent) restenosis.

§1.2 Introduction on NR4A nuclear receptors

§1.2.1 NR4A subfamily of nuclear receptors

The nuclear hormone receptor superfamily comprises 49 human receptors, which are classified into 7 subfamilies based on amino-acid homology. This nuclear receptor superfamily comprises amongst others the estrogen receptors (ERs), peroxisome proliferator activated receptors (PPARs), and liver X receptors (LXRs). Next to the latter ligand-activated nuclear receptors, the nuclear receptor superfamily also includes ‘orphan’ nuclear receptors, for which no ligands have been discovered up till now (A unified nomenclature system for the nuclear receptor superfamily, 1999).
Nur77 (also indicated as NR4A1, TR3, NGFI-B, or NAK-1), Nurr1 (NR4A2, NOT) and NOR-1 (NR4A3, MINOR) are ‘orphan’ nuclear receptors within the NR4A subfamily. Although the human NR4A nuclear receptors are officially designated TR3, NOT and MINOR, we use the names of the mouse homologues (i.e. Nur77, Nurr1, and NOR-1) in this thesis also to indicate the human NR4A nuclear receptors for reasons of convenience. Nur77 was first identified as an ‘early response gene’ expressed in PC12 cells upon nerve growth factor stimulation (Nakai et al., 1990), and thereafter, Nurr1 and NOR-1 were documented (Law et al., 1992; Ohkura et al., 1994). NR4A nuclear receptors are expressed in several tissues throughout the human body and are involved in specific processes. For example, Nurr1 plays a key role in development of the brain (Zetterstrom et al., 1997), Nur77 and NOR-1 are functionally involved in thymocyte selection (Winoto and Littman, 2002), and Nur77 has been described in cancer cell apoptosis (Luciano et al., 2007; Lin et al., 2004).

§1.2.2 Regulation of NR4A nuclear receptors

NR4A nuclear receptors are ‘early response genes’ and are induced by a wide variety of stimuli, like growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor-BB (PDGF-BB), epidermal growth factor (EGF) and thrombin, as well as inflammatory cytokines, lipopolysacharide (LPS), lipoproteins including oxidized low density lipoprotein (ox-LDL), fatty acids, prostaglandins and physical stimuli such as stretch (Bonta et al., 2006; de Waard et al., 2006; Martinez-Gonzalez and Badimon, 2005; Maxwell and Muscat, 2006; Pei et al., 2005). Signaling pathways described in regulation of NR4A receptor expression are protein kinase A, protein kinase C, calcium-calciurein, and mitogen activated pathway kinases (MAPK) leading to activation of transcription factors including cyclic AMP responsive element binding protein (CREB), activator protein 1 (AP-1), nuclear factor kappa B (NF-κB), and myocyte enhancer factor 2 (MEF-2), which have been described to induce NR4A expression (Hsu et al., 2004; Martinez-Gonzalez and Badimon, 2005; Pei et al., 2005). In addition to tightly regulated expression, post-translational modifications are important in modulating NR4A transcriptional activity. For example, mouse Nur77 protein is phosphorylated by protein kinase B/Akt at serine-350 (serine-351 in human Nur77 protein), which strongly decreases its DNA-binding capacity (Pekarsky et al., 2001; Liu et al., 2003), and sumoylation of Nurr1 by protein inhibitor of activated STATγ (PIASγ) represses the transcriptional activity of this nuclear receptor (Galleguillos et al., 2004). Furthermore, Nur77 is antagonized by the glucocorticoid receptor (NR3C1) most likely involving a direct protein-protein interaction (Martens et al., 2005).
§1.2.3 Gene regulation by NR4A nuclear receptors

The NR4A nuclear receptors consist, like other nuclear receptors, of an N-terminal activating function-1 (AF-1) domain, a central two zinc-finger DNA binding domain (DBD), and a C-terminal ligand binding domain (LBD), of which the latter is 58-65% homologous among the NR4A members (Mangelsdorf et al., 1995; Martinez-Gonzalez and Badimon, 2005). The N-terminal domain diverges among the NR4A members in amino-acid composition (approximately 26-28% homology) and is involved in ligand-independent co-activator recruitment (Wansa et al., 2002; Maira et al., 2003). Their DNA-binding domain is well-conserved (over 90% homology) and consists of two zinc-fingers that interact in promoters of specific target genes as monomers with the consensus NGFI-B responsive element (NBRE; AAAGGTCA) as monomers and as homo- and/or heterodimers with the Nur-responsive element (NurRE; TGATATTXX6AAAGTCCA) in promoters of specific target genes (Wilson et al., 1991). Furthermore, Nur77 and Nurr1 can form heterodimers with retinoid X receptors and bind to the DR5 response element (AGGTTCAACGAAAGGTCA) (Perlmann and Jansson, 1995). A schematic representation of the NR4A nuclear receptors is shown in Figure 2. NR4A nuclear receptors also influence transcription through transrepression mechanisms, as has recently been demonstrated for Nurr1, which inhibits matrix metalloproteinase (MMP)-1 expression through repression of the activity of E26 transformation-specific sequence (ETS)-1, a transcription factor involved in MMP-1 expression (Mix et al., 2007). Similarly, NR4A nuclear receptors have been demonstrated to be involved in repression of NF-κB (Hong et al., 2004; Harant and Lindley, 2004; Diatchenko et al., 2005).

Based on structural analyses, the C-terminal ligand-binding domain of the NR4A receptors is considered atypical, since the classical ligand-binding cavity is filled with hydrophobic and aromatic amino-acid side chains and the characteristic co-activator recruitment cleft is absent (Wang et al., 2003). It has therefore been proposed that the LBD of Nurr1 is nonfunctional in the view of ligand interactions, inhibiting matrix metalloproteinase (MMP)-1 expression through repression of the activity of E26 transformation-specific sequence (ETS)-1, a transcription factor involved in MMP-1 expression (Mix et al., 2007). Similarly, NR4A nuclear receptors have been demonstrated to be involved in repression of NF-κB (Hong et al., 2004; Harant and Lindley, 2004; Diatchenko et al., 2005).

**Figure 2. Schematic representation of a nuclear receptor and its structure domains.** Nuclear receptors consist of an amino-terminal (N-terminal) A/B region which includes the activation function-1 (AF-1) domain. The C region encodes the DNA binding domain (DBD) comprising two zinc-fingers. The D domain is a hinge region that connects the DBD with the ligand binding domain (LBD), the E region contains the LBD and the AF-2 domain, and the F region encodes the carboxy-terminal (C-terminal) region.
although an induced fit of (small) unknown ligands may not be excluded. Since the LBD of Nurr1 is highly homologous to the LBD of Nur77 and NOR-1, it seems likely that the LBDs of all NR4A nuclear receptors function similarly. Accordingly, classical ligands have not been identified for the NR4A receptors, emphasizing the importance of expression levels, post-translational modifications, transrepression and co-activator as well as co-repressor recruitment for the functional activity of these nuclear receptors. Recently, a novel hydrophobic co-activator-binding surface has been identified in the LBD (Flaig et al., 2005; Volakakis et al., 2006). For Nurr1 it is demonstrated that binding of peptides derived from the co-repressors NCoR and silencing mediator of retinoid and thyroid hormone receptors (SMRT), bind to this surface and reduce Nurr1 activity (Codina et al., 2004). Furthermore, several ‘small molecule’ activators, enhancing the NR4A activity, have been described. Among these NR4A activators are 6-mercaptopurine (6-MP, a metabolite of the immunosuppressive drug azathioprine), prostaglandin A2 (PGA2), certain diindolylmethane-C compounds and benzimidazole derivatives (Chintharlapalli et al., 2005; Dubois et al., 2006; Kagaya et al., 2005; Ordentlich et al., 2003; Wansa et al., 2003). Except for PGA2, none of these compounds has been shown to interact directly with NR4A proteins.

Little is know about direct downstream target genes of the NR4A receptors. The only genes that have thus far been documented to be directly regulated by the NR4A receptors are E2F1 (encoding a transcription factor involved in cell-cycle regulation and apoptosis), several genes involved in the hypothalamus-pituitary-adrenal-axis and, most recently, some genes implicated in glucose metabolism in the liver (Bassett et al., 2004; Maxwell and Muscat, 2006; Kagaya et al., 2005; Pei et al., 2006b). In vascular cells, plasminogen activator inhibitor-1 (PAI-1) is the only described direct target of Nur77 in ECs (Gruber et al., 2003).

§1.3 NR4A nuclear receptors in vascular disease and metabolism

§1.3.1 NR4A nuclear receptors in metabolism

Obesity and diabetes involve dysregulated lipid and glucose metabolism and are major risk factors for vascular disease (Van Gaal et al., 2006). With regard to the role of NR4A receptors in glucose metabolism, it was demonstrated that expression of all three NR4A nuclear receptors is induced in cultured mouse hepatocytes by glucagon, as well as in the livers of fasted mice (Pei et al., 2006b). This induction of Nur77, Nurr1, and NOR-1 is mediated through activation of CREB, a transcription factor important in hepatic gluconeogenesis. Nur77 subsequently induces expression of the gluconeogenic enzymes glucose-6-phosphatase (G6pc) and fructose biphosphatase 1
(Fbp1). Other proteins involved in glucose metabolism and found to be induced by Nur77 are Fbp2, glucose transporter Glut2 (Slc2a2), enolase 3 (Eno3), and glucose phosphate isomerase (Gpi1). Nur77 induces G6pc, Slc2a2, and Eno3 by direct binding to functional NBREs in the promoter region of these murine genes, whereas the mechanism for induction of Fbp1 and Gpi1 by Nur77 remains to be investigated. Furthermore, Pei et al. demonstrated that overexpression of Nur77 in mouse liver results in enhanced gluconeogenesis and in increased blood glucose levels after fasting (Pei et al., 2006b). In addition, expression of a dominant-negative mutant of Nur77, which inhibits the transcriptional activity of the NR4A nuclear receptors, results in reduced expression of Fbp1 and Slc2a2 in both fasting and random-fed diabetic mice, and these mice have reduced blood glucose levels. The effects of the NR4A nuclear receptors in gluconeogenesis observed in the latter study are independent of PGC-1α, which is considered a major transcriptional regulator of hepatic gluconeogenesis (Herzig et al., 2001).

Skeletal muscle is important in lipid and glucose utilization, and is a major contributor to energy expenditure in the human body. Activation of adrenergic receptors (AR) by β-AR agonists in skeletal muscle cells induces lipolysis and increases energy expenditure. β-AR agonists also induce expression of NOR-1 and Nur77 protein in C2C12 skeletal muscle cells. Small interfering (si) RNA-mediated knockdown of Nur77 in skeletal muscle cells reduces expression of several genes involved in lipolysis and energy expenditure, most importantly glucose transporter 4 (Glut4), uncoupling protein (UCP)-2, UCP-3, CD36, caveolin 3 (CAV3), and AMP-activated protein kinase γ3 (AMPKγ3). Accordingly, lipolysis is reduced in skeletal muscle cells in which Nur77 is silenced. These data were further supported by in vivo observations that silencing Nur77 in mouse tibialis cranialis muscle attenuates UCP-3 expression. Furthermore, Nur77 and NOR-1 are expressed during myogenesis of C2C12 cells and knockdown of NOR-1 in skeletal muscle cells using siRNA increases myostatin mRNA expression. Consequently, it has been proposed that NOR-1 plays a role in the regulation of skeletal muscle mass (Pearen et al., 2006; Maxwell et al., 2005).

**Figure 3.** Schematic representation of hypothesized involvement of NR4A nuclear receptors in vascular disease and metabolism.
β-AR agonists induce expression of Nur77 in cultured murine brown adipocytes, and it is demonstrated that cold-exposed mice display a transient increase in Nur77 protein, predominantly in the brown adipose tissue (Kanzleiter et al., 2005). Overexpression of Nur77 in brown adipocytes induces UCP-1 expression, a protein important in thermogenesis and believed to play a role in energy expenditure. Cold-acclimated Nur77-deficient mice, however, did not reveal a distinct phenotype compared to cold-acclimated wild-type mice. Kanzleiter and colleagues explain this latter finding by redundancy between Nur77 and NOR-1 in regard to thermogenesis, since Nur77-deficient mice exhibit a super-induction of NOR-1 in response to cold exposure. Finally, it has been demonstrated that all three NR4A subfamily members are expressed in murine white and brown adipose tissue, and are induced in the early phase of adipogenesis (Bookout et al., 2006; Yang et al., 2006; Fu et al., 2005). In the latter process, Nur77 is involved in clonal expansion of preadipocytes through cyclin D1 and cyclin E2 (Fumoto et al., 2007).

In conclusion, NR4A nuclear receptors play specific roles in distinct metabolic processes (Figure 3). Since dysregulation of lipid and glucose metabolism is important in development of vascular disease, it will be crucial to further dissect the relevance of NR4A nuclear receptors in metabolism of liver, skeletal muscle, and adipose tissue.

§1.3.2 NR4A nuclear receptors in endothelial cells

NR4A nuclear receptors are induced in ECs by several stimuli, such as hypoxia, TNF-α, IL-1β, and vascular endothelial growth factor (VEGF) (Rius et al., 2006; Liu et al., 2003; Zeng et al., 2006). VEGF treatment of ECs also decreases phosphorylation of Nur77 at its negative regulatory site serine-350 (Liu et al., 2003). At present, the only gene known to be directly regulated by Nur77 in ECs is PAI-1, which is an important inhibitor of fibrinolysis. Nur77 induces PAI-1 expression through directing binding to an NBRE in the promoter region of the PAI-1 gene, whereas ECs that overexpress a dominant-negative variant of Nur77 show abrogated PAI-1 expression in response to TNF-α (Gruber et al., 2003).

Inhibition of NOR-1 expression by anti-sense oligonucleotides decreases EC proliferation and migration upon VEGF stimulation (Rius et al., 2006). We have demonstrated that overexpression of Nur77, using adenoviral vectors, arrests the cell cycle of ECs in the G1 phase upon serum stimulation, and we proposed that Nur77 is involved in the maintenance of vascular integrity (Arkenbout et al., 2003). Zeng et al. have demonstrated that silencing of Nur77 decreases VEGF-induced proliferation of ECs (Zeng et al., 2006). In line with these data, overexpression of
Nur77 enhances EC survival and proliferation, accompanied with an induction of cyclin A, cyclin D1, proliferating cell nuclear antigen (PCNA) and the transcription factor E2F. Furthermore, it is demonstrated that Nur77 is induced in ECs during angiogenesis and that overexpression of Nur77 enhances this process. Finally, angiogenesis is decreased in Nur77-deficient mice both upon VEGF stimulation as well as in transplanted melanoma tumors, leading to an inhibition of tumor growth.

§1.3.3 NR4A nuclear receptors in macrophages
Recently, we and others revealed expression of Nur77, Nurr1 and NOR-1 in human atherosclerotic lesion macrophages (Bonta et al., 2006; Pei et al., 2005). We demonstrated that NR4A receptors are expressed in macrophages present at areas of plaque activation and progression such as the shoulder region, where plaque rupture frequently occurs. In these macrophages NR4A proteins localize predominantly to the nucleus, suggesting that these transcription factors are active in this cellular compartment.

In cultured macrophages, NR4A receptor expression is regulated by stimuli relevant to atherogenesis. Robust and early transient expression of all NR4A members was demonstrated in human and murine primary and cell line-derived macrophages in response to LPS and tumor necrosis factor-α (TNF-α), interferon-γ, phorbol 12-myristate 13-acetate (PMA), granulocyte-macrophage colony stimulating factor (GM-CSF) and various modified lipids including ox-LDL (Barish et al., 2005; Bonta et al., 2006; Pei et al., 2005). Promoter studies revealed direct involvement of NF-κB in the expression of Nur77 in macrophages in response to LPS (Pei et al., 2005). Macrophage apoptosis is a relevant process in atherogenesis (Tabas, 2005), and both Nur77 and NOR-1 promote apoptosis in T cells (Cheng et al., 1997). Nur77 is also involved in pan-caspase inhibitor zVAD-mediated apoptosis in LPS-stimulated murine macrophages (Kim et al., 2003). In the latter study, zVAD inhibits proteasomal degradation of the transcription factor MEF-2, resulting in enhanced MEF-2 binding to the Nur77 promoter and increased Nur77 expression. The pro-apoptotic effect of zVAD was mimicked by overexpression of a Nur77 dominant-active variant lacking the DNA-binding domain, which is consistent with data obtained on Nur77-mediated apoptosis in cancer cells involving translocation of Nur77 from the nucleus to mitochondria (Li et al., 2000; Lin et al., 2004).

Nur77 may function as a pro-inflammatory mediator in mouse RAW macrophages, which involves induction of inducible I-kappa-B kinase (IKKi/IKKε) (Pei et al., 2006a). This induction of IKKi is mediated through direct binding of Nur77 to the NBRE sequence of the murine promotor region of the latter gene. We have
demonstrated that overexpression of Nur77, Nurr1 or NOR-1 in human THP-1 macrophages results in a substantial down-regulation of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-6 and chemokines IL-8, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α and MIP-1β. Furthermore, overexpression of the NR4A receptors results in reduced ox-LDL loading. In agreement with these latter data, short hairpin (sh)RNA-mediated knockdown of Nur77 or NOR-1 results in augmented inflammatory responses and increased lipid loading in these cells. The latter findings indicate that endogenous NR4A nuclear receptors are involved in inhibitory feedback mechanisms operational in these processes. The underlying mechanism of inhibition of human macrophage foam-cell formation and inflammatory responses by NR4A receptors may involve reduced monocyte to macrophage differentiation, which is consistent with reduced expression of scavenger receptor A (SR-A), CD36, and CD11b in NR4A gain of function experiments in these cells. Another explanation for the NR4A-mediated reduction of inflammatory responses in macrophages may be inhibition of the NF-κB pathway. This is in line with Hong and colleagues who have demonstrated that Nur77 directly binds to the p65 subunit of NF-κB through the C-terminal domain of Nur77 (Hong et al., 2004). In addition to the C-terminal domain of Nur77, the N-terminal domain has also been shown to repress NF-κB (Harant and Lindley, 2004). In line with these data, Diatchenko et al. reported that Nur77 inhibits NF-κB activation (Diatchenko et al., 2005). Based on this knowledge, we propose that our data may at least in part be clarified by NR4A nuclear receptor transrepression of the NF-κB pathway.

In summary, we propose that, even though NR4A receptors are expressed in atherosclerotic lesion macrophages and are induced by atherogenic stimuli, these nuclear receptors inhibit human macrophage foam-cell formation and pro-inflammatory cytokine as well as chemokine production. Although the latter conclusion seems paradoxical, comparable atheroprotective functions crucially involved in controlling vascular pathologies have been described for other nuclear receptors like the PPARs and the LXRs (Castrillo and Tontonoz, 2004; Barish, 2006).

§1.3.4 NR4A nuclear receptors in smooth muscle cells

We and others have demonstrated that Nur77, Nurr1, and NOR-1 are induced in human SMCs following activation by inflammatory cytokines and growth factors (de Vries et al., 2000; Martinez-Gonzalez et al., 2003; Nomiyama et al., 2006). In line with these in vitro data, NR4A nuclear receptors are expressed in human vascular,
atherosclerotic lesions (Arkenbout et al., 2002; Arkenbout et al., 2003; Martinez-Gonzalez et al., 2003; Nomiyama et al., 2006), and NOR-1 is transiently expressed in porcine coronary SMCs in response to balloon dilatation (Martinez-Gonzalez et al., 2003). In addition, mechanical stretching of venous SMCs, an important pathological stimulus at the onset of vein-graft disease, results in increased Nur77 expression levels. Accordingly, SMCs in vein segments exposed to perfusion under arterial blood pressure also show enhanced Nur77 expression, predominantly in the outer, circularly oriented SMC layer (de Waard et al., 2006). Recently we have demonstrated that Nur77 is expressed in the femoral artery during cuff-induced SMC-rich lesion formation, already 6 hours after injury up to 7 days later (Pires et al., 2007).

The NOR-1 promoter region contains three functional CREs that are essential for NOR-1 expression in SMCs in response to growth stimuli (Martinez-Gonzalez et al., 2003; Martinez-Gonzalez and Badimon, 2005; Rius et al., 2006). Reduced proliferation of human coronary SMCs is observed upon silencing of NOR-1, using antisense oligonucleotides (Martinez-Gonzalez et al., 2003). Moreover, SMCs isolated from NOR-1-deficient mice show repressed proliferation after PDGF stimulation, whereas reconstitution of these cells with NOR-1 partly rescues the proliferation rate of these cells (Nomiyama et al., 2006). In the latter study, cell-cycle proteins cyclin D1 and cyclin D2 were decreased in SMCs isolated from NOR-1-deficient mice, which is in line with effects of NOR-1 on proliferation.

We have demonstrated that Nur77, unlike NOR-1, plays an inhibitory role in SMC proliferation, since Nur77 overexpression in both venous and arterial SMCs results in reduced proliferation. Inhibition of the activity of all three NR4A nuclear receptors by overexpression of a dominant-negative variant of Nur77 or silencing of Nur77 using small interfering RNA (siRNA) results in enhanced SMC proliferation in response to mitogenic stimuli. The inhibition of SMC proliferation by Nur77 is accompanied with increased expression of the cell-cycle inhibitor p27^Kip1 and with a decrease in cell-cycle protein cyclin A (Arkenbout et al., 2002; de Waard et al., 2006; Pires et al., 2007). Furthermore, overexpression of Nur77 increases expression of calponin and smooth muscle (SM)-α actin, indicating that Nur77 induces a more differentiated SMC phenotype (de Waard et al., 2006). Transgenic mice that overexpress Nur77 in the arterial vessel wall under control of the arterial SMC-specific promoter-fragment of SM22α show decreased vascular lesion formation, both after carotid artery ligation and upon femoral artery cuff placement (Arkenbout et al., 2002; Pires et al., 2007). Moreover, mice overexpressing the dominant-negative variant of Nur77 develop larger lesions, indicating that endogenous NR4A nuclear receptors protect against excessive SMC proliferation. Thus, although Nur77 is expressed in atherosclerotic
lesion SMCs, this nuclear receptor inhibits proliferation and increases expression of SMC markers and as such promotes a quiescent SMC phenotype.

§1.3.5 NR4A Nuclear receptors and 6-Mercaptopurine
Azathioprine is the pro-drug of 6-Mercaptopurine (6-MP) and is used as an immunosuppressive drug in autoimmune conditions, such as inflammatory bowel disease and in transplant recipients (Cara et al., 2004). It has recently been shown that 6-MP enhances the transcriptional activity of the NR4A nuclear receptors (Wansa et al., 2003; Ordentlich et al., 2003). This effect of 6-MP is mediated through the N-terminal AF-1 domain and most probably involves changed recruitment of co-activators, such as TRAP220 (Wansa and Muscat, 2005), which subsequently increase the transcriptional activity of NR4A nuclear receptors.

It has been demonstrated that treatment of stretch-activated venous SMCs with increasing concentrations of 6-MP results in a dose-dependent inhibition of proliferation (de Waard et al., 2006). De waard et al. have subsequently demonstrated that this effect of 6-MP on SMC growth is fully diminished when Nur77 was knocked down in these cells with siRNA, indicating crucial involvement of Nur77 in the 6-MP-mediated inhibition of venous SMC proliferation. In addition to the 6-MP-mediated inhibition of venous SMC proliferation, it was observed that 6-MP enhances expression levels of the SMC-markers calponin and SMα-actin, and cell-cycle inhibitor p27$^{Kip1}$, similar as observed in Nur77-overexpressing SMCs.

To study the effect of 6-MP in vascular lesion formation in vivo, we applied 6-MP locally, using a perivascular drug-eluting cuff around the femoral artery, and observed that 6-MP inhibits SMC-rich lesion formation. We next demonstrated that the effect of 6-MP was even stronger in transgenic mice overexpressing Nur77 under control of the SM22α promotor, while 6-MP had no effect on vascular lesion formation in mice expressing a dominant-negative variant of Nur77 in arterial SMCs, suggesting involvement of Nur77 in the 6-MP-mediated inhibition of lesion formation (Pires et al., 2007). In addition to effects of 6-MP in SMCs, treatment of ECs with 6-MP induces expression of hypoxia inducible factor-1α (HIF-1α), and this is partially mediated through the NR4A nuclear receptors. In the latter study, it was also demonstrated that 6-MP enhances VEGF levels and capillary tube formation of ECs (Yoo et al., 2006).

§1.4 Thesis aim
The goal of the research described in my thesis was to explore the role and therapeutic potential of the NR4A nuclear receptors in atherosclerosis and processes closely related to atherosclerosis, such as in-stent restenosis and lipid homeostasis.
The liver plays a major role in lipid homeostasis, and it has been demonstrated that NR4A nuclear receptors are expressed in this organ. We therefore aimed to identify a potential function of the NR4A nuclear receptors in liver lipid metabolism, which is highly relevant to atherosclerosis, and describe our results in Chapter 2. Furthermore, we investigated the role of NR4A nuclear receptors in inflammation and lipid loading in cultured macrophages and the data obtained from this study are presented in Chapter 3. In Chapter 4, we report on further insight that was gained on the function of the NR4A subfamily in the inflammatory and proliferative response of SMCs with respect to (in-stent) restenosis.

Another aim of the research described in my thesis was derived from the knowledge that 6-MP, a metabolite of the immunosuppressive pro-drug Azathioprine, enhances the transcriptional activity of the NR4A nuclear receptors. We therefore investigated 6-MP both in atherosclerosis as well as in (in-stent) restenosis using dedicated animal models and in vitro approaches, in which we focused on involvement of the NR4A nuclear receptors. The results of these studies are reported in Chapter 5 and Chapter 6 of this thesis. Finally, we discuss in Chapter 7 our novel findings in relation to relevant literature and propose directions for future research.
§1.5 References


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