NR4A nuclear receptors in vascular disease
Bonta, P.I.

Citation for published version (APA):

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General Discussion
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The aim of this thesis is to increase our understanding of fundamental pathways critical in atherosclerosis and restenosis. To achieve this aim, we performed a number of detailed studies on the role of NR4A nuclear receptors Nur77, Nurr1 and NOR-1 in vascular smooth muscle cells (SMC) and macrophages, two cell types that are extremely relevant to these pathologies. To bridge ‘bench and bedside’, the specific expression of NR4A nuclear receptors in human atherosclerotic arteries and in human in-stent restenosis lesions is further substantiated. The function of these transcription factors in vascular disease is studied in gain and loss of function experiments in SMCs or macrophages in both in vitro and in vivo models using (lentiviral and transgenic) overexpression and short-hairpin (sh)RNA-mediated knockdown strategies. We found that although NR4A nuclear receptors are only expressed in human vascular lesions, and not in healthy arteries, they have anti-atherogenic characteristics in human SMCs and macrophages as they reduce inflammation, proliferation and foam-cell formation, critical cellular processes in vascular lesion formation. To enhance the transcriptional activity of NR4A nuclear receptors we applied the immunosuppressive drug 6-mercaptopurine (6-MP) in mouse models relevant to atherosclerosis and restenosis. Finally, in patients, the association with clinical in-stent restenosis and/or target lesion revascularization risk is described for Nurr1 genetic haplotypes and a single nucleotide polymorphism located in the promoter of the p27kip1 gene, a downstream target of Nurr1 and Nur77. In this general discussion of my thesis, I will discuss specific subjects concerning NR4A nuclear receptors with regard to vascular disease not mentioned in the individual chapters and give an indication of what I believe are the most important research areas for the NR4As in the near (and further) future.

NR4A nuclear receptors in monocytes/macrophages in vascular disease

Monocytes/macrophages are crucially involved in atherosclerosis and restenosis. In Chapter 4 we describe the expression and function of all three NR4A nuclear receptors in monocytes/macrophages in inflammation and foam-cell formation. Consistent with the NR4A expression observed in macrophages in human atherosclerosis lesions, we and others demonstrated that their expression in vitro is dependent on signaling pathways active during atherosclerotic lesion formation, including toll-like receptor-4 (TLR-4) and tumor necrosis factor-α (TNF-α) receptor-mediated and phorbol 12-myristat 13-acetate (PMA)-induced responses. Both TLR-4, which can be activated by LPS or oxidized low-density lipoprotein cholesterol (ox-LDL) and TNF-α receptors, activate downstream signaling pathways that integrate at the nuclear factor kappa B (NF-κB) level to drive pro-inflammatory gene expression. Indeed, promoter analysis of the Nur77 gene revealed that NF-κB is involved in Nur77 expression regulation.
At the same time, cumulative evidence exists that NR4A nuclear receptors suppress NF-κB transactivation. We have shown that Nur77, Nurr1 and NOR-1 reduce inflammatory responses in macrophages (Chapter 4) and that Nurr1 inhibits cytokine synthesis in SMCs (Chapter 7). Similar anti-inflammatory properties have been described for Nur77 in HEK293 cells, T-cells and endothelial cells (EC) and for Nurr1 in neuronal microglia. The anti-inflammatory function of NR4A nuclear receptors involves inhibition of the NF-κB pathway in two ways (Figure 1). First, Nur77 has been described to increase expression of the inhibitor of NF-κB α (IκB-α) in ECs by direct binding to the promoter of this gene. Second, in microglia, largely similar to the anti-inflammatory function of PPARs and LXR, Nurr1 has been shown to bind to the p65 subunit of the p50/p65 NF-κB complex bound to promoters of inflammatory genes, which results in transrepression of NF-κB activity. In monocytes/macrophages the exact mechanism by which inflammatory responses are inhibited by NR4A nuclear receptors is unknown. We hypothesize that both direct binding to and subsequent transrepression of NF-κB and IκB-α induction are involved. Since NF-κB expression is regulated and its activity repressed by NR4A nuclear receptors in multiple cell-types, it seems that these ‘orphan’ receptors are part of a negative feedback loop in the NF-κB pathway and as such stabilize inflammatory responses.
In addition to the involvement of NR4A nuclear receptors in inflammatory responses, we investigated the function of these nuclear receptors in foam-cell formation by exposing PMA-differentiated THP-1 macrophages to ox-LDL in both NR4A overexpression and shRNA-mediated knockdown experiments. We revealed that Nur77, Nurr1 and NOR-1 reduce ox-LDL loading, which is explained by reduced expression levels of scavenger receptor-A (SR-A) and CD36. The modulation of these macrophage surface markers by NR4A nuclear receptors, the induction of NR4A nuclear receptor expression in THP-1 monocytes exposed to PMA (unpublished data) and in primary monocytes upon adherence may point towards a specific role of these nuclear receptors in macrophage differentiation. Indeed, CD11b, which is considered a marker of macrophage differentiation, is also repressed by NR4A nuclear receptors. Myeloid-derived cells, in particular circulating monocytes and macrophages, are well-known for their plasticity and many different, tissue-specific macrophage populations have been described. A possible approach to further characterize the effect of NR4A nuclear receptors on macrophage differentiation would be to perform micro-array expression analysis in similar NR4A overexpression and knockdown experiments and compare them to well described monocyte/macrophage subpopulations (e.g. M1/M2).

Based on the observation that NR4A nuclear receptors have anti-inflammatory and anti-foam-cell properties in macrophages, we hypothesize a protective function for these nuclear receptors in atherogenesis. This hypothesis needs to be verified in dedicated in vivo models and several approaches may be considered and ideally should be tested simultaneously. Bone-marrow transplantation experiments, in which bone marrow cells of Nur77−/− or NOR-1−/− mice (Nurr1−/− mice are not viable because of dopaminergic agenesis), are transplanted into atherosclerosis-prone mice (e.g. low-density lipoprotein receptor (LDL-R)−/− or ApoE−/− mice) are a reasonable approach. However, it should be noted that redundancy and compensatory expression of NR4A nuclear receptors have been described in experiments in which one of the three NR4A nuclear receptors is deficient. For this reason Nur77/NOR-1 double −/− mice were generated, however, unexpectedly these mice develop lethal acute myeloid leukemia. In agreement with these data, we observed that ex-vivo Nur77 or Nurr1 lentiviral transduction of bone-marrow cells, and subsequent transplantation of these transduced cells in lethally irradiated LDL-R−/− recipient mice, results in an unusually low percentage of bone-marrow-derived cells that express these genes (unpublished data). To avoid such bone-marrow suppression and the problem of redundancy, the generation of transgenic mice that overexpress NR4A nuclear receptors late in the monocyte-macrophage lineage, using well-described CD68 or SR-A promoter sequences in the transgene constructs and subsequent bone marrow transplantation to or back-crossing of these transgenic mice in atherosclerosis prone mice, could be an alternative approach.
NR4A nuclear receptors in SMCs in vascular disease

SMCs form a substantial part of human atherosclerotic lesions and vascular lesions of so-called SMC-rich vascular pathologies including restenosis, pulmonary hypertension, transplant arteriosclerosis and vein graft disease. In this thesis SMC-specific expression of NR4A nuclear receptors in human vascular pathologies and the function of these nuclear receptors in SMCs in several animal models relevant to atherosclerosis and restenosis are studied. In mice, SMC-specific modulation of Nur77 activity was achieved by using transgenic mice, in which overexpression of Nur77 or its dominant negative variant ∆TA, is driven by an arterial SMC-specific promoter sequence.

In Chapter 5 it is demonstrated that overexpression of Nur77 in vascular SMCs inhibits flow-induced carotid artery remodeling in mice. To understand the underlying mechanism we investigated the effect of Nur77 on vascular tone and inflammatory cell recruitment, which are considered critical determinants of vascular remodeling. Myograph-based vasoconstriction and vasorelaxation studies revealed that Nur77 does not modulate vascular tone. Alternatively, the inhibitory function of Nur77 in vascular remodeling in these transgenic mice is explained by reduced macrophage recruitment and diminished matrix metalloproteinase-1 (MMP-1) and MMP-9 expression. Nur77-driven downregulation of chemokine expression in activated SMCs may be a plausible explanation for the reduced macrophage recruitment observed and deserves further exploration. It should be emphasized that macrophages are considered the most important source of MMPs in vascular disease. However, in the remodeling model used in this study the number of macrophages is relatively low and alternative to an effect of Nur77 in SMCs on macrophage recruitment, we speculate that a direct inhibitory effect of Nur77 on MMP-1 and/or MMP-9 expression in SMCs might be involved. Indeed, in vascular SMCs MMP-9 expression has been shown to be dependent on NF-κB and similar Nur77-mediated inhibitory mechanisms of NF-κB signaling as described above for macrophages, but now in SMCs, may provide an additional explanation. Previously, our group has discovered that Nur77 is anti-proliferative in SMCs and protects against SMC-rich lesion formation in mice. We postulate that in addition to the mechanisms described above, also reduced SMC proliferation contributes to the inhibitory function of Nur77 in arterial remodeling. Since remodeling is of importance in tissue engineering and several vascular- and non-vascular diseases, including (pulmonary) hypertension, aneurysm formation and airway remodeling, it may be of interest to investigate the role of NR4A nuclear receptors in these pathologies.

In Chapter 7 we show Nurr1 expression in human in-stent restenosis lesions and demonstrate that Nurr1 inhibits inflammatory responses and, like Nur77, also inhibits SMC proliferation in vitro. This anti-proliferative function of Nurr1 involves increased expression of p27kip1, a key
cell-cycle inhibitor described protective in restenosis. Finally, it is demonstrated that Nurrl is protective in wire-injury-induced restenotic lesion formation in mice. The modulation of SMC proliferation and activation by Nur77 or Nurrl may be explained by a comprehensive modulation of the arterial SMC phenotype, consistent with the earlier described regulation of SMC-markers calponin and smooth muscle α-actin by Nur77 in venous SMCs.¹⁶

Since SMC proliferation, activation and SMC-mediated monocyte/macrophage recruitment and remodeling are all pivotal in vascular pathologies, these data provide evidence that Nur77 and Nurrl in SMCs, next to a beneficial role in restenosis, may also have a protective function in atherosclerosis and atherosclerosis-related pathologies like for example aneurysm formation. As far as atherosclerosis is concerned, we have preliminary data indicating that selective overexpression of Nur77 in SMCs in ApoE⁻⁻ mice reduces atherosclerotic lesion formation as compared to wild-type ApoE⁻⁻ mice (Figure 2A, B). The observation that enhanced expression of the transcription factor Nur77 in SMCs results in reduced atherosclerosis in mice, is a remarkable finding that may further substantiate a key role of SMCs in atherosclerosis.

**NR4A nuclear receptor function in human atherosclerosis and restenosis**

Multiple experimental studies have been performed in dedicated mouse models to delineate functional involvement of Nur77, Nurrl and NOR-1 in vascular disease. The exact function of these transcription factors in clinical atherosclerosis and restenosis remains a major challenge.

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**Figure 2. SMC-specific expression of Nur77 reduces atherosclerotic lesion formation in ApoE⁻⁻ mice.** ApoE⁻⁻ mice and ApoE⁻⁻ mice overexpressing Nur77 specifically in their arterial SMCs (ApoE⁻⁻xSMC-Nur77) were exposed to a high-fat diet (HFD) for 8-24 weeks. (A) Typical examples of aortic root atherosclerotic lesions seen in ApoE⁻⁻ and ApoE⁻⁻xSMC-Nur77 mice after 20 weeks of HFD. As can be appreciated of these examples ApoE⁻⁻xSMC-Nur77 mice have reduced atherosclerotic lesion surface area as compared to their ApoE⁻⁻ controls. Lipid droplets in red (oil-red-o) and nuclei in blue (hematoxylin). (B) Quantification of atherosclerotic lesion surface area at the aortic root at different time points reveals that ApoE⁻⁻xSMC-Nur77 develop significantly less atherosclerosis at 20 and 24 weeks of HFD as compared to their ApoE⁻⁻ controls.
for the future, however data provided in this thesis already contribute to the hypothesis that NR4A nuclear receptors are functionally active and relevant to human vascular disease. First, the differential expression in diseased, atherosclerosis and restenosis arteries versus healthy human arteries is a key observation. Second, critical signaling pathways and cellular processes active in atherosclerosis and restenosis are modulated by NR4A nuclear receptors in cultured human SMCs and human macrophages. Third, the association of Nurr1 genetic haplotypes (Chapter 7) and a single nucleotide polymorphism in p27kip1 (Chapter 9), a downstream target of Nurr1 and Nur77, with clinical in-stent restenosis, target lesion/vessel revascularization and/or the combined endpoint major cardiovascular events (MACE) risk provides evidence for a functional role of these transcription factors in human vascular disease. The impact of the Nurr1 haplotype association detected will be enhanced once we know that specific haplotypes that associate with reduced in-stent restenosis risk correlate with increased expression levels of Nurr1.

Differences exist between human atherosclerotic lesions and atherosclerotic lesions in mice and these differences may modulate NR4A nuclear receptor function described in experimental studies as compared to human atherosclerosis. A key difference is that human atherosclerotic lesions, as opposed to the lesions in mice, are largely composed of SMCs and their secreted extracellular matrix molecules largely contribute to lesion size and luminal narrowing. Therefore the anti-proliferative function of Nur77 and/or Nurr1 in SMCs can contribute to reduced lesion formation in human atherosclerosis. However, clinical relevant acute coronary syndromes are mainly caused by atherosclerotic plaque rupture, resulting in sudden thrombus formation and subsequent obstruction of arterial blood flow. These ruptured, culprit plaques are characterized by large lipid cores, increased number of macrophages and diminished SMCs and SMC-derived extracellular matrix content. Consequently, the anti-proliferative function of Nur77 and Nurr1 in SMCs may, at least in theory, result in a more unstable, plaque phenotype. Overall however, I envision that the combination of anti-inflammatory and anti-foam cell properties of Nur77 and/or Nurr1 in monocytes/macrophages and the anti-proliferative function of these transcription factors in SMCs might result in a beneficial profile in atherosclerosis.

**NR4A nuclear receptors and 6-mercaptopurine in vascular disease**

NR4A nuclear receptors have proven to be multi-potential transcription factors that modulate critical pathways in atherosclerosis and SMC-rich vascular pathologies including restenosis. The data provided in this thesis contribute significantly to this knowledge and may become relevant for clinical intervention since recently, several compounds that enhance NR4A activity have been identified, among which 6-mercaptopurine (6-MP) (See Chapter 2, Table
1 for a complete list). In Chapter 6 we demonstrate that 6-MP is protective in atherosclerotic lesion formation in ApoE*3Leiden mice involving induction of apoptosis in monocytes and to a lesser extent in macrophages, reduced monocyte adhesion and inhibition of macrophage MCP-1 expression. In Chapter 8 6-MP is applied in a drug-eluting cuff model in wild-type mice to study its effect on SMC-rich lesion formation, a process which relates to human (in-stent) restenosis. The involvement of Nur77 in the inhibitory function of 6-MP in this model is determined by using transgenic mice overexpressing Nur77 and appropriate controls, including its dominant-negative variant ΔTA. The protective function of 6-MP in restenosis in vivo, is explained by inhibition of SMC proliferation. In addition to effects of 6-MP described above, it might well be that other NR4A-dependent and/or -independent effects of 6-MP, like for example inhibition of inflammatory responses, contribute to the protective potential of this drug in restenotic lesion formation.

In clinical practice, in-stent restenosis remains the major drawback of percutaneous intervention although the application of sirolimus or paclitaxel drug-eluting stents has considerably reduced the incidence of in-stent restenosis. These drugs effectively inhibit SMC proliferation, but also hinder beneficial re-endothelialization and delay arterial healing, pathophysiological processes implicated in (late) in-stent thrombosis, a potentially fatal complication. Consequently, novel targets that support cell-specific intervention in in-stent restenosis, need to be identified. More specifically, such drugs ideally inhibit SMC proliferation, have anti-inflammatory effects on SMCs and macrophages and at the same time enhance re-endothelialization of the stented vascular luminal surface. We have demonstrated beneficial NR4A-dependent and -independent effects of 6-MP on SMC and monocytes/macrophage function. In addition, the effect of 6-MP on ECs has been studied, which revealed that 6-MP promotes angiogenesis, which is explained by Nur77-dependent stabilization of hypoxia-inducible factor-1α (HIF-1α) and increased VEGF expression levels. Taken together, these characteristics of 6-MP provide a combination of biological effects, which determine this compound as a candidate drug to prevent restenosis. Ideally, drug-eluting stents loaded with 6-MP will be developed, which should be tested head-to-head with currently available drug-eluting stents in pre-clinical animal models for their efficacy on restenosis inhibition and re-endothelialization. Extrapolated from the potential application of 6-MP in treatment of in-stent restenosis, perivascular application of 6-MP-loaded pluronic gels to saphenous vein grafts during bypass surgery to prevent graft failure could be an alternative application, which has already been successfully tested in pre-clinical animal models by others. As opposed to atherosclerosis, the local nature of both in-stent restenosis and vein graft failure presumably make these latter vascular pathologies particularly suitable for treatment with 6-MP. Systemic application of 6-MP or the 6-MP pro-drug azathioprine
to treat atherosclerosis is highly unlikely, since long-term usage has been associated with significant adverse effects. Patients that require long-term immunosuppressive agents, for example after renal transplantation, may however benefit from azathioprine as compared to other immunosuppressive drugs, because of the anti-atherogenic effects and presumed favorable cardiovascular risk profile of this drug described. \textsuperscript{23} However, whether this holds true in clinical practice remains unclear and needs further investigation, especially since recently mycophenolate mofetil (MMF), another frequently used immunosuppressive drug used in transplantation patients, has been put forward as a more atheroprotective drug as compared to azathioprine. \textsuperscript{24}

**Future perspectives**

The studies described in this thesis substantially contribute to the understanding of the role of NR4A nuclear receptors Nur77 and Nurrol in atherosclerosis and restenosis. However, they elicit novel important research questions of which several have been raised already in the paragraphs above. A central, remaining question concerns the mechanism by which NR4A nuclear receptors actually determine their downstream effects. In addition to transrepression of other transcription factors such as NF-κB or ETS-1, it is likely that direct binding to the promoter of target genes is of critical importance, although only a limited number of direct target genes have been described so far. Genome-wide approaches that combine bioinformatics with a biological relevant research design will be crucial to elucidate direct target genes. Chromatin immunoprecipitation sequence (ChIP-seq), is a recently developed technique that allows detection of direct binding of the transcription factor of interest with the DNA and can be combined with detection of RNA-polymerase binding/activity and as such identifies direct target genes that are transcribed on a genome-wide basis. Alternatively, micro-array-based genome-wide approaches could be used to unravel direct and indirect gene targets modulated by NR4A nuclear receptors. These approaches when applied to relevant vascular disease models in combination with NR4A gain and loss of function strategies might ultimately provide a systems biology understanding, including temporal and possibly spatial dimensions, of these nuclear receptors.

Another important, remaining question is whether NR4A nuclear receptors can be selectively targeted with small molecules. Several lines of evidence suggest that this will be difficult. First, crystal structure analysis of Nurrol has revealed that the classical ligand-binding pocket in the ligand-binding domain, which is highly homologous among the NR4A nuclear receptors, is filled with hydrophobic amino-acid side-chains and therefore is not available for a classical ligand. Second, since these nuclear receptors are constitutively active, and therefore do not need ligand-induced activation, their downstream effects are highly dependent on their expression levels, post-translational modifications as well as interaction with other proteins, including
other transcription factors, co-repressors and co-activators and localization within the cell. It is therefore surprising that 6-MP and several other compounds have been discovered that enhance the transcriptional activity of NR4A nuclear receptors. The mechanism by which these compounds activate NR4A nuclear receptors is at present unknown and their effects are far from specific since these compounds influence multiple other NR4A-independent pathways. Detailed mechanistic insight in the way NR4A nuclear receptors are regulated and exert their downstream effects in general and how this is modulated by these NR4A activating compounds, may allow the development of more selective NR4A agonists to further increase the potential of these transcription factors as future targets for clinical intervention.

As described and discussed NR4A nuclear receptors have a fundamental impact on monocyte/macrophage and SMC biology and the relevance for atherosclerosis and restenosis is described in this thesis. However, in many other disorders, SMCs and/or macrophages and even more relevant the signaling pathways modulated by NR4A nuclear receptors play a crucial role. Therefore, it is conceivable that NR4A nuclear receptors are expressed and functionally active in other vascular and/or inflammation-driven pathologies, like pulmonary hypertension, transplant arteriosclerosis, asthma, inflammatory bowel disease or sepsis. Indeed in human volunteers exposed to LPS expression of all three NR4A nuclear receptors is strongly induced in whole blood samples with optimal levels about 1 hour after LPS administration (unpublished data) and in histopathological samples of patients with Crohn’s disease NR4A nuclear receptors are expressed in submucosal SMCs (unpublished data). Finally, NR4A nuclear receptor function has already been related to cancer, notably acute myeloid leukemia and Nurr1 to Parkinson’s disease.

Conclusions
This thesis contributes to the understanding of NR4A nuclear receptors in vascular disease. For the first time we have described expression of NR4A nuclear receptors in an array of vascular pathologies, including human and mouse atherosclerosis and restenosis and mouse vascular remodeling. Nur77 and Nurr1 are discovered anti-inflammatory, anti-proliferative, anti-foam cell transcription factors in monocytes/macrophages and/or SMCs, which are key cell-types and key cellular functions in vascular disease. In addition to NR4A-independent atheroprotective characteristics of 6-MP in monocytes/macrophages, 6-MP can target Nur77 in SMCs, which is shown protective in neointima formation in mice. The relevance for human vascular disease is illustrated by the association of Nurr1 haplotypes with in-stent restenosis, target revascularization, repeat PCI and MACE risk. The characterization of NR4A nuclear receptor expression and function in vascular disease could well contribute to new diagnostic and therapeutic strategies in patients with cardiovascular disease.
References

