NR4A nuclear receptors in atherosclerosis
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§7.1 Thesis outline

The research described in my thesis focuses on the three nuclear receptors of the NR4A subfamily: Nur77, Nurr1, and NOR-1, as potential novel molecular targets to intervene in atherosclerosis and related (vascular) pathologies. We aimed to gather further knowledge on mechanisms of actions of these transcription factors in processes relevant to vascular pathologies. 6-mercaptopurine (6-MP), derived from the immunosuppressive pro-drug Azathioprine has been reported to enhance the transcriptional activity of the NR4A nuclear receptors. We therefore also investigated 6-MP with respect to vascular pathologies.

I introduce the NR4A nuclear receptors and describe their documented actions in processes relevant to cardiovascular disease in Chapter 1. Next, I demonstrate in Chapter 2 that hepatic expression of Nur77 reduces hepatic triglyceride levels and modulates the plasma lipid profile, which is at least partly caused by a potent suppression of the transcription factor sterol regulatory element binding protein (SREBP)-1c by Nur77. We subsequently show in Chapter 3 that the three NR4A nuclear receptors are expressed in atherosclerotic lesion macrophages, and inhibit cytokine secretion and reduce foam cell formation of cultured THP-1 macrophages. We demonstrate in Chapter 4 that the NR4A nuclear receptors Nur77, Nurr1 and NOR-1 are expressed in human in-stent restenosis. We report that Nurr1 inhibits inflammation and proliferation of primary human smooth muscle cells (SMCs), and inhibits SMC-rich lesion formation in vivo. In Chapter 5 we report that 6-MP inhibits SMC proliferation and SMC-rich lesion formation with involvement of Nur77. We show in Chapter 6 that locally applied 6-MP inhibits atherosclerosis, which is at least partially mediated through anti-atherogenic effects of 6-MP on monocytes/macrophages, involving adhesion, apoptosis, and inhibition of monocyte chemoattractant protein (MCP)-1 secretion.

In the current chapter (Chapter 7), we discuss our results and other issues of the studies that have not been discussed in the preceding chapters. I also describe the potential prospective of the NR4A nuclear receptors and of 6-MP as potential targets/drugs for intervention in vascular pathologies, and generate considerations and recommendations for future research.

§7.2 Nur77 in hepatic lipid metabolism

In Chapter 2, we investigated the role of nuclear receptor Nur77 in hepatic lipid metabolism by an adenoviral gain-of-function approach, and made several novel observations. Briefly, even though no effects of Nur77 on total plasma cholesterol and triglycerides were induced, we observed that Nur77 increased both plasma
low-density lipoprotein (LDL)-triglycerides as well as plasma LDL-cholesterol. Furthermore, we observed that Nur77 reduces hepatic triglyceride levels. We propose that these physiological changes are at least partially caused by a strong inhibition of expression of the transcription factor SREBP-1c by Nur77 (Figure 1). Although SREBP-1c is also regulated at post-transcriptional level, involving binding to SREBP cleavage-activating protein (SCAP), it is conceivable that the strong inhibition of expression of SREBP-1c by Nur77 influences SREBP-1c activity. In line with this, we indeed observed suppression of a number of SREBP-1c-target genes (Figure 1). Of note, a similar gain-of-function approach for nuclear receptor Nurr1 gave comparable results regarding the physiological changes observed in response to Nur77, as well as in terms of changes in hepatic gene expression (unpublished data; not shown in this thesis). In line with our findings that overexpression of Nur77 and Nurr1 inhibit SREBP-1c expression, Maxwell et al. reported that silencing of Nur77 in mouse C2C12 skeletal muscle cells increases SREBP-1c expression (Maxwell et al., 2005), indicating that endogenous levels of Nur77 are physiologically relevant in regulating SREBP-1c expression levels.

The observed increase in plasma LDL-cholesterol and LDL-triglycerides by Nur77 may at least partially be explained by decreased uptake of LDL particles via the LDL-receptor (LDLR), of which expression is inhibited in response to Nur77. Decreased expression of the LDLR is most probably caused by Nur77-mediated suppression of SREBP-1c that directly regulates LDLR expression. A primary event in atherosclerosis is the accumulation of modified LDL particles in the intimal compartment of arteries, and higher LDL levels are correlated with increased deposition of LDL particles (Lusis, 2000). The observation that expression of Nur77 and Nurr1 in murine livers results in increased plasma LDL levels is obviously undesirable in the treatment of atherosclerosis, and indicates that precautions must be taken to avoid this effect in response to enhancement of the expression or activity of Nur77 and/or Nurr1.

Figure 1. Hypothetical schematic representation of the inhibition of SREBP-1c by Nur77. Nur77, downstream of the glucagon-cAMP axis in hepatocytes, inhibits expression of SREBP-1c. This results in decreased SREBP-1c activity, and reduced expression of the SREBP-1c downstream genes: the LDL receptor and several lipogenic enzymes.
Several intriguing questions are still remaining. We observed that a relatively large number of genes are changed in response to Nur77. It will be of interest to determine the full effect induced by Nur77 on the hepatic transcriptome and the potential connection between modulated genes. This may perhaps also provide an explanation for the finding that total plasma lipids are unchanged in response to Nur77, despite increased LDL lipid levels. Plasma lipid profiles of wild-type mice are different from those of humans. It will therefore be of interest to investigate Nur77 also in ApoE*3-Leiden.cholesteryl ester transfer protein (CETP) transgenic mice, which have plasma lipid profiles that are more comparable to that of humans (Zadelaar et al., 2007). This approach will give even better insight into potential effects of hepatic expression of Nur77 in humans. In addition, studies in primary hepatocytes could be performed to dissect the precise mechanism by which Nur77 modulates SREBP-1c expression.

SREBP-1c has been proposed to play a key role in hepatic steatosis, a condition of the liver characterized by excessive hepatic lipid accumulation, which may result in fibrosis and cirrhosis of the liver (Shimomura et al., 1999; Ahmed and Byrne, 2007). Since Nur77 and Nurr1 inhibit expression of SREBP-1c, enhancing the activity or expression of Nur77 and/or Nurr1 may have a beneficial outcome in hepatic steatosis, although potential increases in plasma LDL levels should be taken into consideration. Further insight into potential beneficial effects of Nur77 and Nurr1 in hepatic steatosis may be obtained by using dedicated animal models. For example, Obese/Obese (Ob/Ob) mice, that are deficient in the appetite-suppressing hormone leptin, develop fatty livers, and as such provide a tool to investigate a potential beneficial role of Nur77 and Nurr1 in the formation of hepatic steatosis (Yang et al., 1997).

§7.3 Mouse models of in-stent restenosis and atherosclerosis
In addition to extensive experiments in cultured cells, we applied various animal models to gain insight into the function of Nur77-like nuclear receptors and 6-MP in atherosclerosis and in (in-stent) restenosis. In our studies concerning (in-stent) restenosis, we used two different mouse models of SMC-rich lesion formation. Notably, the mouse carotid artery guide wire-injury model (used in the study described in Chapter 4) and the mouse femoral artery cuff model (used in the study described in Chapter 5). The rational to choose between the various animal models of vascular lesion formation was based on the specific properties of each of these animal models. An advantage of the femoral artery cuff model is that this model allows placement of drug-eluting cuffs that deliver certain drugs, like 6-MP, locally to the perivascular space around the artery (Pires et al., 2005). Clearly, this mouse femoral artery cuff
model also has its weaknesses. For example, the precise mechanism responsible for the induction of vascular lesion formation in the mouse femoral artery cuff model is not exactly known, and may involve a disturbance in the adventitial blood flow, vascular damage and/or production of cytokines by the granulation tissue (Pires et al., 2006). The mechanism responsible for the cuff-induced SMC-rich lesion formation thereby does not mimic the initial pathogenic stimulus as it occurs in the pathogenesis of human in-stent restenosis. Human restenotic lesions are believed to be initiated by de-endothelialization, crush of the plaque (often with dissection into the media and occasionally adventitia), and stretch of the entire artery by the balloon angioplasty procedure (Costa and Simon, 2005). An advantage of the mouse carotid artery guide wire-injury model is therefore that this lesion formation is evoked by damage to the endothelial cell layer, and as such may more closely resemble this aspect of human restenosis development, although also this model still considerably differs from human in-stent restenosis in multiple other facets, e.g. stretch of the artery, which occurs in balloon angioplasty, is not mimicked in this model.

In Chapter 6, we applied a variation of the femoral artery cuff model to study atherosclerotic lesion formation. This model, described by Lardenoye et al., involves the placement of cuffs around the femoral artery of ApoE*3-Leiden transgenic mice that are fed a high fat diet, resulting in atherosclerotic lesion formation (Lardenoye et al., 2000). It should be noted that a large difference exists between human atherosclerosis and animal models of atherosclerosis regarding the time-span of atherosclerosis development. In humans, the development of atherosclerosis may take decades, whereas in animal models, such as in the latter mouse femoral artery cuff model, lesions are generated in 1-4 weeks. Despite the large difference in time-span of lesion development between human atherosclerosis and the lesions in animal models, a typical atherosclerotic lesion phenotype is observed in the latter mouse femoral artery cuff model, exemplified by the presence of foam cell-like macrophages, and a fibrous cap containing SMCs (Lardenoye et al., 2000).

In conclusion, none of the used animal models is ‘ideal’ and each model holds several deviations from human vascular pathologies, such as differences in genetics, vessel size dimensions, initiation stimuli, and time-span of lesion development. Data obtained from the animal models described in this thesis should therefore be interpreted with care. Despite these disadvantages, the animal models share basic aspects of human vascular lesion formation, such as inflammation, foam cell formation, and SMC proliferation, involving complex cell-cell interactions in an environment that can not be mimicked with cultured cells. As such, animal models provide a first step into identification of roles of specific proteins in vascular
pathologies and may provide useful insight into the efficacy and safety of potential novel treatments/drugs.

§7.4 Nur77-like nuclear receptors in atherosclerotic lesion macrophages and inflammation

Monocyte-derived macrophages are key players in atherosclerosis in which they act throughout the three well-defined stages of the disease: initiation, progression, and rupture. In Chapter 3 of this thesis, we demonstrate that the NR4A nuclear receptors are expressed in macrophages in atherosclerotic lesions and inhibit oxidized-LDL loading and inflammatory responses of these cells.

Our findings demonstrating that the NR4A nuclear receptors reduce oxidized-LDL uptake in macrophages, and thereby potentially prevent macrophage foam-cell formation, may be considered anti-atherogenic. This reduction in oxidized-LDL loading in macrophages by the NR4A nuclear receptors is at least partially caused by abrogated expression levels of the scavenger receptors SR-A and CD36, involved in the uptake of foreign particles like oxidized-LDL and other modified forms of LDL present in atherosclerotic lesions. The precise mechanism responsible for the decrease in SR-A and CD36 in macrophages by the NR4A nuclear receptors is unknown, but likely involves effects of the NR4A nuclear receptors on macrophage differentiation. This is further underlined by the finding that the NR4A nuclear receptors decrease expression of CD11b, a gene representative for macrophage differentiation. It remains of great interest to investigate whether and how Nur77 drives macrophages into a potential anti-atherogenic macrophage phenotype.

Figure 2. Hypothetical schematic representation of Nur77 in the negative regulation of NF-κB.

Activation of the TNF receptor results in activation of NF-κB by releasing it from its IκB inhibitor molecules. This results in nuclear localization of NF-κB and, amongst others, the transcription of several inflammatory cytokines. Nur77 is also induced by NF-κB, and Nur77 protein inhibits NF-κB activity, providing a negative feedback loop that limits the duration and intensity of NF-κB signaling.
potential approach to gain additional insight in the latter phenomenon may involve
gene expression profiling of the Nur77-induced phenotype and compare this with
other macrophage phenotypes, of which many are documented (e.g. M1, M2a, M2b, M2c) (Mantovani et al., 2004).

We also demonstrated that NR4A nuclear receptors inhibit the inflammatory
response of activated macrophages. Since atherosclerosis is a chronic inflammatory
process, anti-inflammatory properties of the NR4A nuclear receptors in macrophages
are clearly beneficial in atherosclerosis. We believe that the anti-inflammatory
properties of the NR4A nuclear receptors, in addition to potential effects that are
derived via a change in macrophage phenotype, are at least partially mediated through
inhibition of NF-κB (extensively discussed in Chapter 1). In line with the anti-
inflammatory actions of the NR4A nuclear receptors in macrophages, we describe
in Chapter 4 anti-inflammatory properties of Nurr1 in SMCs, which may also be
derived from inhibition of NF-κB. The mouse promoter region of the Nur77 gene
contains two NF-κB response elements that are also present in the human Nur77
promoter. It has been demonstrated that these NF-κB binding sites are involved in the
upregulation of Nur77 in response to the pro-inflammatory stimulus lipopolysaccharide
(Pei et al., 2005). The data that NR4A nuclear receptors are driven by NF-κB and
expressed in atherosclerosis, while reducing inflammation may be explained by the
hypothesis that Nur77 is part of a negative feedback loop that controls the intensity
and duration of NF-κB activity (Figure 2). This hypothesis may point towards a
means to counterbalance NF-κB signaling via enhancing expression of the NR4A
nuclear receptors, which may have a beneficial outcome in atherosclerosis.

§7.5 Nuclear receptors Nurr1 and Nur77 in smooth muscle cells: beneficial
or detrimental in atherosclerosis?
SMCs in the media of normal healthy vessels are quiescent differentiated cells that are
crucial in regulating blood pressure and vessel wall stability. SMCs are not terminally
differentiated, and have retained their ability to differentiate into activated SMC
phenotypes. This phenotypic plasticity of SMCs is important in repair of damaged
vessels and allows the vessel wall to adapt to specific, altered, circumstances. SMCs
needed for these processes are not only derived from the media, but may also be
recruited from other sources, such as from circulating SMC progenitor cells, which
are originating from the bone marrow (Kawai-Kowase and Owens, 2007). In case
of percutaneous balloon dilatation angioplasty or bypass operations the vessel is
damaged (also described in Chapter 1 and in paragraph 7.3), and a repair reaction of
SMCs is initiated. This repair response of SMCs to damage is sometimes excessive,
giving rise to a SMC-rich vascular pathology that obstructs the blood flow. The inhibition of SMC proliferation by the NR4A nuclear receptors Nur77 and Nurr1 is therefore evidently beneficial in SMC-rich vascular pathologies (described in Chapter 4 and Chapter 5).

The role of SMC proliferation is more complex with regard to atherosclerosis. Although SMCs greatly contribute to atherosclerotic lesion size, SMCs and their secreted matrix molecules also provide stability to plaques. In this way, SMCs protect against acute myocardial infarction and ischemic stroke that result from rupture of unstable/vulnerable atherosclerotic lesions. The role of Nur77 and Nurr1, and their inhibition of SMC proliferation, is therefore also more intricate in atherosclerosis. It can not be excluded that expression or activation of Nur77 and/or Nurr1 makes atherosclerotic plaques more prone to rupture. I speculate that the adverse effect of Nur77 and Nurr1 in SMCs regarding plaque vulnerability is compensated by a strong beneficial outcome of the NR4A nuclear receptors on inflammation (described in Chapter 3 and Chapter 4), which is also important in the rupture of atherosclerotic lesions. Atherosclerosis models on NR4A-deficient mice may provide more insight into how and to what extent the NR4A nuclear receptors modulate plaque composition and stability.

§7.6 6-Mercaptopurine is anti-atherogenic through both NR4A-dependent and NR4A-independent pathways

In an attempt to identify compounds that enhance the transcriptional activity of Nurr1, Ordentlich et al. screened 800 prototypic compounds, and observed that 6-MP enhanced the transcriptional activity of Nurr1 in CV-1 cells. An additional screening of 340,000 compounds did not result in any other hits, underlining this unique property of 6-MP (Ordentlich et al., 2003). The same authors observed that 6-MP enhances the transcriptional activity of NOR-1 in a similar screening, and in line with the previous study, Wansa et al. reported that 6-MP enhances the transcriptional activity of Nur77, Nurr1 and NOR-1 in C2C12 cells (Wansa et al., 2003). The primary mechanism by which 6-MP modulates the transcriptional activity of the NR4A nuclear receptors likely involves interference of 6-MP in purine biosynthesis and/or genotoxic stress, since simultaneous addition of certain compounds of the purine synthesis (e.g. adenine, adenosine, guanosine, hypoxanthine, and inosine) inhibited Nurr1 activation by 6-MP (Ordentlich et al., 2003).

We 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. In line with the study from de Waard et
al. demonstrating that effects of 6-MP on venous SMC proliferation is fully dependent on Nur77 (de Waard et al., 2006), we observed that 6-MP inhibited proliferation of arterial SMCs at least partially through involvement of Nur77, which is described in Chapter 5 (Figure 3). We subsequently describe in Chapter 6 novel unrecognized anti-atherogenic actions of 6-MP in monocytes/macrophages (Figure 3). In contrast to the effects of 6-MP on SMCs, we observed that the anti-atherogenic effects of 6-MP on monocytes/macrophages with respect to apoptosis and MCP-1 secretion were independent of the NR4A nuclear receptors. In addition to effects of 6-MP on apoptosis and MCP-1 secretion, 6-MP also inhibited expression of the monocyte adhesion integrins VLA-4 and platelet cell adhesion molecule-1 (PECAM-1), and inhibits monocyte adhesion (Figure 3). It remains to be resolved whether this action of 6-MP involves NR4A transcriptional activity. A potential approach to identify changes of 6-MP on gene expression which are mediated through enhanced activity of the NR4A nuclear receptors may involve comparison of the transcriptome of 6-MP treated monocytes/macrophages in which NR4A function is manipulated and control cells, for example by using micro-arrays.

§7.7 Perspective of the NR4A nuclear receptors as targets for intervention in vascular pathologies
NR4A nuclear receptors are expressed in the diseased vessel wall as well as in other tissues in which these nuclear receptors have specific more or less well-defined functions. In my thesis, several novel facets of the NR4A nuclear receptors and of 6-MP are described that are highly relevant to the potential utilization of these receptors as targets for intervention in vascular pathologies.
We demonstrate novel athero-protective actions of NR4A nuclear receptor Nurr1 in cultured SMCs and in a mouse model of (in-stent) restenosis. Furthermore, we report that 6-MP could be promising as a potential novel anti-restenotic compound, exerting its protective effects with involvement of Nur77. In addition to a protective effect of the NR4A nuclear receptors in in-stent restenosis, we report beneficial properties of the NR4A nuclear receptors Nur77, Nurr1 and NOR-1 in cultured macrophages with respect to atherosclerosis. Furthermore, we demonstrate novel, NR4A-independent, anti-atherogenic actions of 6-MP in cultured monocytes/macrophages, and demonstrate that 6-MP inhibits atherosclerosis development in mice. These data clearly demonstrate that NR4A nuclear receptors and 6-MP are promising tools to treat in (in-stent) restenosis and atherosclerosis.

Our results showing that Nur77 interferes with the transcription factor SREBP-1c in the liver may suggest that local targeting of the NR4A nuclear receptors, e.g. using drug-eluting stents, may have advantages over systemic targeting. However, more research is required to fully elucidate all effects of NR4A nuclear receptors in metabolism and vascular biology. For example, the effect of the NR4A nuclear receptors on endothelial activation is still unknown. In conclusion, the results described in this thesis provide further knowledge on the NR4A nuclear receptors with respect to vascular pathologies and brings us more close to the identification of the full prospective of these receptors as potential targets for intervention in vascular pathologies.
§7.8 References


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