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NR4A Nuclear Orphan Receptors: Protective in Vascular Disease?

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Abstract

*Purpose of review* The nuclear orphan receptors Nur77 (NR4A1), Nurr1 (NR4A2) and NOR-1 (NR4A3) are known to be involved in T-cell apoptosis, brain development, and the hypothalamic-pituitary-adrenal axis. Here, we review our current understanding of the NR4A nuclear receptors in processes that are relevant to vascular disease.

*Recent findings* NR4A nuclear receptors have recently been described to play a role in metabolism by regulating gluconeogenesis, lipolysis, energy expenditure, and adipogenesis. The function of NR4A nuclear receptors has also extensively been investigated in cells crucial in vascular lesion formation, such as macrophages, endothelial cells (ECs) and smooth muscle cells (SMCs).

*Summary* The involvement of NR4A nuclear receptors in both metabolism as well as in processes in the vessel wall supports a substantial role for NR4A nuclear receptors in the development of vascular disease.
Introduction

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, Nur77 and NOR-1 are functionally involved in thymocyte selection, and Nur77 has been described in cancer cell apoptosis. Recently, it has been shown that all NR4A nuclear receptors are expressed in human atherosclerotic lesions, fasted liver, skeletal muscle and adipose tissue (Figure 1). Here we review our present understanding of the NR4A nuclear receptors in relation to metabolism and vascular disease.

NR4A nuclear receptors

The nuclear hormone receptor superfamily comprises 49 human receptors, which are classified into 7 subfamilies based on amino-acid homology. Nur77 (also indicated as NR4A1, TR3, NGFI-B), Nurr1 (NR4A2, NOT) and NOR-1 (NR4A3, MINOR) are members of the NR4A subfamily and 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). So far, ligands have not been identified for NR4A nuclear receptors, and therefore these receptors are classified as orphan receptors. Crystallography of the LBD of Nurr1 demonstrated that the ligand binding pocket of this receptor is filled with hydrophobic amino-acid side chains, and revealed an atypical co-activator cleft. It has therefore been proposed that the LBD of Nurr1 is nonfunctional in the view of ligand interactions, 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. NR4A nuclear receptors bind as monomers to the NGFI-B (nerve growth factor-induced clone B) response element (NBRE; AAAGGTCA), and as homodimers to the Nurr1 response element (NurRE; TGATATTTn6AAATGCCA) in promoters of direct target genes. In addition, Nur77 and Nurr1, but not NOR-1, can...
NR4A nuclear receptors in metabolic processes.

Obesity and diabetes involve dysregulated lipid and glucose metabolism and are major risk factors for vascular disease. With regard to the role of NR4A receptors in glucose metabolism, expression of all three NR4A nuclear receptors is induced in cultured mouse hepatocytes by glucagon, and in the liver of fasted mice. 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), enolase3 (Eno3), and glycosylphosphatidylinositol 1 (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 for Fbp1 and Gpi1 by Nur77 remains to be investigated. Furthermore, Pei et al. demonstrated that overexpression of Nur77 in mouse liver results in an increase in gluconeogenesis and an increase in blood glucose after fasting. In addition, expression of a dominant-negative mutant of Nur77, which inhibits transcriptional activity of NR4A nuclear receptors results in both fasting and random fed diabetic mice in reduced expression of Fbp1 and Slc2a2, and these mice have lowered blood glucose levels. The effects of the NR4A nuclear receptors in gluconeogenesis observed in this study are shown to be independent of peroxisome proliferator-activated receptor-γ-coactivator-1α (PGC-1α), which is considered a major transcriptional regulator of hepatic gluconeogenesis. Skeletal muscle is important in lipid and glucose utilization, and is a major...
Introduction to NR4A nuclear receptors

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 2 (UCP2), UCP3, CD36, caveolin 3 (Cav3), and AMP-activated protein kinase γ3 (AMPKγ3). Accordingly, lipolysis is reduced in skeletal muscle cells in which Nur77 was knocked down. These data were further supported by in vivo findings showing that knockdown of Nur77 in mouse tibialis muscle attenuates UCP3 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 is proposed that NOR-1 plays a role in the regulation of skeletal muscle mass. Finally, it has been demonstrated that all three NR4A family members are expressed in murine white and brown adipose tissue, and are induced in the early phase of adipogenesis. In the latter process, Nur77 is involved in clonal expansion of pre-adipocytes through cyclin D1 and cyclin E2. In conclusion, it has been shown that NR4A nuclear receptors play specific roles in distinct metabolic processes (Figure 1). 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.

NR4A nuclear receptors in macrophages and vascular cells

Vascular disease is mainly caused by atherosclerosis and involves predominantly macrophages, T-cells, endothelial cells (ECs), and smooth muscle cells (SMCs) that interact with each other in the vessel wall. Central in atherosclerosis are macrophages that scavenge modified lipoproteins present in dysfunctional arteries. Eventually these macrophages become lipid laden ‘foam cells’ that secrete cytokines resulting in SMC recruitment and more macrophage accumulation. This inflammatory process results in further activation and damage of the endothelial cell layer, which aggravates the progression of atherosclerosis. NR4A nuclear receptors play key roles in macrophages, SMCs, and ECs as described in the next paragraphs.

Macrophages

NR4A nuclear receptors are induced in monocytes and macrophages in response to atherogenic stimuli, such as oxidized low-density lipoprotein (ox-LDL), lipopolysaccharide and tumor necrosis factor-α (TNF-α). Expression of Nur77, Nurr1 and NOR-1 is observed in atherosclerotic lesion macrophages, particularly in areas of plaque activation and remodeling,
suggesting involvement of these transcription factors in plaque progression. In mouse RAW macrophages, it has been shown that Nur77 may function as a pro-inflammatory mediator, which involves induction of inducible I-kappa-B kinase (IKKι/IKKe). This induction of IKKι is mediated through direct binding of Nur77 to the NBRE sequence of the murine promoter region of the latter gene. We have demonstrated in cultured human THP-1 derived macrophages that overexpression of NR4A nuclear receptors decreases inflammatory cytokine expression. Interleukin-1β (IL-1β), IL-6, IL-8, macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and monocyte chemoattractant protein-1 (MCP-1) are downregulated in macrophages overexpressing NR4A nuclear receptors. Moreover, overexpression of NR4A nuclear receptors in macrophages results in decreased ox-LDL loading consistent with reduced expression levels of scavenger receptors SR-A and CD36. In agreement with these data, knockdown of Nur77 or NOR-1 in macrophages using short hairpin (sh) RNA increases inflammatory cytokine expression and enhances ox-LDL uptake in these cells. The latter finding indicates that endogenous NR4A nuclear receptors may be involved in inhibitory feedback mechanisms that modulate macrophage activation. We proposed that NR4A nuclear receptors exert their effect on inflammatory gene expression at least in part by transrepression of NF-κB. The latter hypothesis is in line with reports that Nur77 reduces the transcriptional activity of NF-κB, which could be explained by direct binding of Nur77 to the p65 subunit of NF-κB. In addition to the effects of Nur77 observed on inflammation and lipid loading, it has also been described that Nur77 is involved in macrophage cell death. In conclusion, these transcription factors are involved in macrophage cell death, have an inhibitory role in inflammatory cytokine secretion, and inhibit foam cell formation (Figure 2). We therefore propose that NR4A nuclear receptors inhibit adverse processes in lesion macrophages and consequently may protect against atherogenesis.

**Smooth muscle cells**

SMCs are under ‘normal’ conditions quiescent, contractile cells that regulate blood flow, blood pressure, and vessel wall stability. Upon local inflammation or vascular damage these functional SMCs become activated, and start migrating and proliferating into the intimal compartment of the vessel wall. NR4A nuclear receptors are expressed in activated SMCs in atherosclerotic lesions and other vascular pathologies, such as vein-graft disease and recently we have shown that Nur77 is expressed in the murine vessel wall during cuff-induced SMC-rich lesion formation, already 6 hours after injury up to 7 days later. Silencing of NOR-1 in SMCs using anti-sense oligonucleotides results in attenuated proliferation after activation of these cells with serum. This is in agreement with the finding that SMCs derived from NOR-1-deficient mice show repressed proliferation. This decreased proliferation is accompanied by a decrease in expression of cell cycle proteins cyclin D1 and cyclin D2, although the
precise regulation by NOR-1 of the latter proteins remains to be investigated.\textsuperscript{36} We have shown 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 results in enhanced SMC proliferation. Inhibition of SMC proliferation by Nur77 is accompanied with increased expression of the cell cycle inhibitor p27\textsuperscript{Kip1} and a decrease in cell cycle protein cyclin A.\textsuperscript{32,33,35} Furthermore, overexpression of Nur77 increases expression of calponin and smooth muscle (SM)-\(\alpha\) actin, indicating that Nur77 induces a more differentiated SMC phenotype.\textsuperscript{33} Transgenic mice that overexpress Nur77 in the arterial vessel wall under control of the arterial SMC-specific promoter-fragment of SM22\(\alpha\) show decreased vascular lesion formation, both after carotid artery ligation and upon femoral artery cuff placement.\textsuperscript{32,35} Moreover, mice overexpressing the dominant-negative variant of Nur77 develop larger lesions, indicating that endogenous NR4A nuclear receptors protect against excessive SMC proliferation (Figure 2).

**Endothelial cells**
NR4A nuclear receptors are induced in ECs by several stimuli, such as hypoxia, TNF-\(\alpha\), IL-1\(\beta\), and vascular endothelial growth factor (VEGF).\textsuperscript{37-39} VEGF treatment of endothelial cells has also been shown to decrease phosphorylation of Nur77 at its negative regulatory site serine-350.\textsuperscript{37} At present, the only gene known to be directly regulated by Nur77 in ECs is plasminogen activator inhibitor-1 (PAI-1), which is an important inhibitor of vascular fibrinolysis. Nur77 has been shown to induce 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-\(\alpha\).\textsuperscript{40} Inhibition of NOR-1 expression by anti-sense oligonucleotides decreases EC proliferation and migration upon VEGF stimulation.\textsuperscript{38} We have shown that overexpression of Nur77 using adenoviral vectors arrests the cell cycle of ECs in the G1 phase of the cell cycle upon serum stimulation, and we proposed that Nur77 is involved in maintenance of vascular integrity.\textsuperscript{41} Zeng et al. have demonstrated that silencing of Nur77 decreases VEGF-induced proliferation of ECs.\textsuperscript{39} In line with these data, overexpression of Nur77 was shown to enhance EC survival and proliferation, accompanied with induction of cyclin A, cyclin D1, proliferating cell nuclear antigen (PCNA) and transcription factor E2F. Furthermore, it was shown 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. In summary, it has been shown that Nur77 and NOR-1 modulate EC growth, survival, and angiogenesis (Figure 2).
Enhancement of the activity of NR4A nuclear receptors by 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 after organ transplantation. It has recently been shown that 6-MP enhances the transcriptional activity of NR4A nuclear receptors. 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, which subsequently increase the transcriptional activity of NR4A nuclear receptors. Venous and arterial SMCs show a dose-dependent inhibition of proliferation in response to 6-MP. Knockdown of Nur77 using siRNA in these cells reduces the anti-proliferative effect of 6-MP, demonstrating that the inhibitory effect of 6-MP on proliferation is at least partially mediated through involvement of Nur77. In addition, 6-MP treatment of venous SMCs was shown to increase expression of calponin and SMα-actin, indicating that the cells differentiated to a more quiescent SMC phenotype which is also observed in SMCs overexpressing Nur77. 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 in mice. To evaluate the contribution of Nur77 it was demonstrated that the effect of 6-MP was even stronger in transgenic mice overexpressing Nur77 under control of the SM22α promoter, while 6-MP had no effect on vascular lesion formation in mice expressing a dominant-negative variant of Nur77 in arterial SMCs. Treatment of endothelial cells with 6-MP induces expression and activation of hypoxia inducible factor-1α (HIF-1α), and this is partially mediated through NR4A nuclear receptors. In the latter study, 6-MP enhances VEGF levels and capillary tube formation of endothelial cells. Together, the protective effect of 6-MP on endothelial cell survival and the growth-inhibitory effect of

Figure 2. Schematic representation of hypothesized involvement of nuclear receptor Nur77 in atherosclerosis
this drug on SMCs involving NR4A nuclear receptor activity may support the hypothesis that NR4A nuclear receptors are *bona fide* targets to treat SMC-rich pathologies.

**Conclusion**
The interplay of metabolic processes regulated by NR4A nuclear receptors in liver, muscle and adipose tissue requires more research to establish the eventual effect on metabolic homeostasis. In the vessel wall, NR4A nuclear receptors may have a protective role in vascular lesion formation, involving inhibition of inflammation, reduced foam cell formation, and protection against SMC-rich lesion formation. Although at present the signaling pathways of NR4A function involved in metabolism and vascular disease are not fully unraveled, we propose that local activation of Nur77 is a rational approach to treat vascular lesion formation.

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