TR3 nuclear orphan receptor in cardiovascular disease
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Chapter 2

General Introduction
2.1 The superfamily of nuclear receptors

Genes transcribed within the first few hours of cell activation, and which do not require de novo protein synthesis, represent the immediate-early response genes. Typically, such genes are induced rapidly, have a short half-life and are primarily transcribed in the first phase of cell activation. Immediate response genes are often coding for transcription factors that are involved in the activation of secondary genes. One group of transcription factors is the superfamily of steroid/thyroid hormone nuclear receptors, which includes over 83 proteins. These nuclear receptors are subdivided in 7 subfamilies comprising, among others, receptors for steroid hormones, vitamin D, ecdysone, retinoic acids and thyroid hormones (www.ens-lyon.fr/LBMC/index-uk.htm). Also oxysterols and prostaglandin J$_2$ are known ligands of the superfamily and act by binding nuclear receptors such as Liver X receptor (LXR) and peroxisome proliferator-activated receptors (PPARs), respectively. In addition to these receptors with known ligands, an increasing number of orphan receptors has been described. The ligands of these receptors have not been identified yet and it may be considered that some of the orphan receptors are regulated in a ligand-independent way. In general, ligands of nuclear receptors are low molecular weight, neutral, lipophilic, non-protein compounds and, consequently, nuclear receptors are becoming increasingly attractive targets for potential therapeutic interventions. In this Chapter, we will focus on the NGFI-B or NR4A subfamily of transcription factors and present an overview of the different family members. We will mainly concentrate on TR3 (NR4A1) and mention a number of important facts with respect to NOT (NR4A2) and MINOR (NR4A3).

2.2 The NGFI-B (NR4A) subfamily of nuclear receptors

2.2.1 Introduction

A growing number of transcription factor proteins have been identified that regulate changes in gene expression during the pathogenesis of atherosclerosis. By using an RT-PCR/differential display approach TR3 orphan receptor (also known as TR3, Nur77, NGFI-B, NR4A1) and Mitogen induced nuclear orphan receptor (MINOR, NOR-1, NR4A3) were identified in smooth muscle cells (SMCs) upon activation by a cocktail of pro-inflammatory, cytokines secreted by oxidized low-density lipoprotein (ox-LDL)-stimulated macrophages. TR3 and MINOR comprise, together with Nuclear orphan receptor of T-cells (NOT, Nurr1, NR4A2), the NGFI-B (Nerve Growth Factor Inducible gene B) subfamily of the superfamily of nuclear hormone receptors and are orphan receptors. For reasons of clarity, names for the human transcription factors will be used throughout this thesis, although a substantial amount of research has been performed with the murine homologues of this subfamily (Nur77, Nurr1 and NOR-1). The NGFI-B family is defined as a subfamily within the
steroid/thyroid hormone superfamily based on high sequence similarities between the subfamily members and their ability to bind DNA as a monomer.

All nuclear receptors share the same modular structure (see Figure 1). An amino-terminal transactivation domain comprising the activation function-1 (AF-1) domain, two Zinc-finger motifs (see Figure 2) forming the DNA-binding domain and a carboxyl-terminal domain which is involved in ligand binding, dimerization and transactivation (AF-2). The DNA-binding domains of TR3, MINOR and NOT are remarkably homologous (>91%), an observation that is also reflected by the fact that these receptors bind the same response elements. The carboxyl-terminal domains of the TR3-like factors share 54-67% homology, whereas the amino-terminal domains are less homologous (21-27%). Together these data may implicate that TR3, MINOR and NOT transactivate the same genes. However, as especially the regulatory domains of the TR3-like factors are divergent, these factors will have distinct interactions with co-activators and co-repressors, resulting in unique patterns of regulation of downstream genes.

![Figure 1. Schematic comparison of the amino acid sequence of TR3, MINOR, NOT and the dominant-negative variant of TR3. The borders for the N-terminal transactivation domain, the DNA-binding domain and the ligand binding domain are shown. Sequences were aligned and percent identity of other proteins to TR3 is indicated within each domain.](image)

TR3 was originally identified by virtue of its rapid induction by nerve growth factor (NGF) in PC12 cells and by serum in fibroblasts. In addition, it has been demonstrated that TR3 expression is induced in apoptosis of T-cell hybridomas. MINOR was originally identified in cultured rat fetal forebrain cells. NOT was isolated from a neonatal mouse brain cDNA library, using low stringency hybridization conditions with a probe encoding the DNA-binding domain of COUP-TF, an additional orphan member of the nuclear receptor superfamily.
2.2.2 Homo- and heterodimerization of TR3-like factors

Members of the superfamily of nuclear receptors regulate transcription by binding to a specific DNA targets, originally named hormone response element (HRE). Most nuclear receptors bind as homodimers or heterodimers to their HREs, which consist of the following consensus hexameric halfsites 5'-\( (A/G)G(G/T)TCA-3' \), 5'-AAGTCA-3' or 5'-AGAAACA-3'. Specificity is determined by the exact half-site sequence, the relative orientation of the half-sites (inverted, everted or direct repeats) and the number of spacer nucleotides between the half-sites.\(^7\) As a unique feature all three members of the NGFI-B subfamily have been shown to bind DNA as monomers by using the target DNA as a positive allosteric factor. The TR3 subfamily members bind to an estrogen receptor half-site element, containing two additional adenosine-residues at the 5' end the so-called NGFI-B response element (NBRE) 5'-AAAGGTCA-3'.\(^{10,11}\) Detailed analysis of the crystal structure of the DNA-binding domain of TR3 revealed that the 'A-box', which is located adjacent to the Zinc-fingers, is required for recognition of these two additional nucleotides in the NBRE.\(^{12}\) (Fig. 2).

**Figure 2.** a) Domain structure of TR3. Amino acid sequence of rat NGFI-B. Depicted are the two zinc-fingers in the DNA-binding domain. b) The estrogen response element (ERE) half-site and the required 5' AAA sequence (adapted from Meinke et al.\(^{12}\))

NGFI-B subfamily members can also bind as homodimers to a palindromic DNA binding motif; the NurRE, TGATATTNN\(_6\) AATGCCA (Fig. 3).\(^{13}\) The non-consensus dimeric NGFI-B response element may be essential for certain aspects of the function of the receptors \textit{in vivo}. Physiologic processes that have been shown to depend on NGFI-B
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Nur response elements

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<td>NurRE&lt;sub&gt;POMC&lt;/sub&gt;</td>
<td>TGATATTACCCTCAATGCCA</td>
<td>M1 M2 M3</td>
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<td>NurRE&lt;sub&gt;gon&lt;/sub&gt;</td>
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<td>NBRE</td>
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Figure 3. DNA sequences of various TR3 response elements. The POMC NurRE sequence is present in the rat POMC promoter. The NurRE<sub>gon</sub> is composed of two canonical sites organized as in the NurRE<sub>POMC</sub>. The NBRE sequence was described by Wilson et al. and the Nur-responsive DR-5% was described by Perlmann and Jansson. (adapted from Maira et al.)

activation, such as the induction of T-cell apoptosis and the response of pituitary cells to corticotrophin-releasing hormone (CRH), result in increased activity of NurRE-regulated but not NBRE-regulated synthetic promoters. Often, more than a single NGFI-B subfamily member is induced in response to extracellular signals. Maira et al. found that the NR4A members cooperate to synergistically activate NurRE-dependent transcription and that this is likely to result from heterodimerization between subfamily members. Different dimers exhibit sequence preference and may be responsible for the activation of subsets of target genes. In addition to heterodimer formation among subfamily members, heterodimer formation with other steroid receptors has been reported. Both TR3 and NOT, but not MINOR, can heterodimerize with RXR and bind to a direct repeat element of estrogen receptor half-sites separated by five nucleotides. RXR can also function as a non-DNA-binding cofactor for TR3 or NOT orphan receptors, because heterodimerization of RXR with constitutively active TR3 or NOT creates a novel hormone-dependent complex. MINOR is a functionally distinct member of the NGFI-B subfamily, because it does not form heterodimers with RXR and consequently does not promote ligand activation of RXR from its target DR5 response elements. TR3 and NOT heterodimerization involves specific helices in the carboxyl-terminal domain. However, MINOR lacks three essential amino acids in its corresponding domain, which observation may explain the lack of dimerization. In conclusion, more than one member of the NGFI-B family can be induced in response to extracellular signals that can specifically interact with each other or other nuclear receptors. It should be emphasized that NGFI-B family members have also transactivation properties when bound as monomers to their cognate response element. This may point to a dual role for the NGFI-B type of receptors both as transcriptional activators and as regulators of other nuclear receptors.
2.2.3 NOT

NOT was identified in T-cells upon mitogenic activation. In vivo, high expression of NOT mRNA expression was detected in the brain. The phenotype of the NOT -/- knockout-mouse indeed lacks expression in this organ. The mice are hypoactive and die postpartum due to lack of development of dopaminergic neurons. It has been shown that NOT is essential for differentiation of ventral mesencephalic late dopaminergic precursor neurons and for induction of a full dopaminergic phenotype of these cells. NOT is a candidate gene for neurological and psychiatric disorders with an involvement of the dopaminergic neuron system, such as Parkinson’s disease, schizophrenia, and manic depression. Indeed, missense mutations were found in these patient groups in the coding region of NOT, leading to a reduction in transcriptional activity. Furthermore, decreased expression of NOT in dopaminergic neurons of cocaine abusers was reported. Recently, Wagner et al. showed induction of a dopaminergic phenotype in type 1 astrocytes upon overexpression of NOT. This procedure yields cells that may be a useful source for neuronal replacement in Parkinson’s disease. In addition to the physiological expression of NOT in the brain we and others have reported that NOT is also involved in inflammatory pathologies, such as atherosclerosis (in this thesis), glomerulonephritis, and rheumatoid arthritis. In the study on rheumatoid arthritis, it was shown that proinflammatory mediators induced NOT transcription in synovial tissue, whereas NF-kB and CREB bind to the promoter of the NOT gene. In this thesis, we show induction of NOT with proinflammatory mediators in SMCs and endothelial cells (EC) as well as expression of NOT mRNA in atherosclerotic plaques.

2.2.4 MINOR

Rat MINOR cDNA was cloned from primary cultured rat forebrain cells that were undergoing apoptosis. The human homologue was identified from fetal brain and from human T-cells activated with phytohemagglutinin (PHA) and phorbol 12-myristate 13-acetate (PMA). Expression of MINOR is induced by mechanical agitation of leukemic cell lines and, recently, it was reported that the members of the NGFI-B family are induced in liver generation after hepatectomy. This fact was already established for NOT, which was named regenerating liver nuclear receptor (RNR-1). The induction of MINOR was also detected during cell death in a human breast cancer cell line. There are two alternative splice variants identified in MINOR mRNA in human skeletal muscles. Two groups independently generated MINOR knockout mice with completely different phenotypes. The group of Conneely demonstrated that interruption of the MINOR gene resulted in a mild phenotype with mice showing an “ear defect”. In contrast, the MINOR -/- mice
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generated by in the laboratory of Winoto were embryonic lethal, suggesting that MINOR plays a crucial role in gastrulation. The explanation proposed to explain this difference in phenotype was that in the embryonic lethal mice the entire coding sequence of MINOR was disrupted, whereas part of the coding sequence of MINOR was maintained in the mice with the mild phenotype. The embryonic defects observed in the MINOR knockout mice demonstrate that MINOR exhibits a crucial function in embryonic development, which is cannot be replaced by its homologues TR3 or NOT.

2.3 TR3

2.3.1 Modulation of TR3 activity by phosphorylation

Unphosphorylated TR3 is distributed equally among the cytoplasm and the nucleus, whereas its highly phosphorylated form is predominantly present in the cytoplasm. The amino acid serine at position 350 (Ser-350) in TR3 is a phosphorylation site that is located in the ‘A-box’, the protein motif required for selective DNA binding. Ser-350 phosphorylation interferes with the DNA-binding ability of TR3 and decreases its transcriptional activity by 50-80%. This serine residue can be phosphorylated by Akt (PKB) kinase, which is a key player in the transduction of anti-apoptotic and proliferative signals in T-cells.

Nuclear export signal sequences (NES) are leucine-rich motifs that are critical to transport proteins out of the nucleus. Recently, three NES sequences were identified within the ligand-binding domain of TR3. It was shown that, in addition to these NES sequences, phosphorylation of the serine residue at position 105 in TR3 is also required to translocate RXR-TR3 heterodimeric complexes from the nucleus to the cytoplasm. As a consequence of this cellular relocalization of RXR-TR3 complexes, the transcriptional activity of RXR-RAR heterodimers is reduced. The interaction of TR3 with RXR has been reported to result on the one hand in a rexinoid-responsive transcription complex and on the other hand in a protein complex that is transcriptionally inactive due to export from the nucleus. These divergent effects of TR3 on RXR function and its activity on gene expression as a monomer, indicate that TR3 may interconnect multiple signalling pathways.

2.3.2 Regulation of TR3 transcription

TR3 expression is induced in numerous cell types by a variety of stimuli. The promoter sequence of the TR3 gene comprises consensus response-elements for multiple transcription factors. The mechanism underlying the induction of TR3 mRNA expression in T cell apoptosis, involving changes in intracellular calcium (Ca^{2+}), has recently been described in detail. Two Ca^{2+}-responsive DNA elements in the TR3 promoter were identified as consensus binding sites for the transcription factor myocyte enhancer factor 2 (MEF2). In the inactive
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state, MEF2 is bound to Cabin1, which is also an endogenous inhibitor of the protein phosphatase calcineurin. In response to an increase in intracellular calcium, calmodulin becomes activated, binds in a competitive way to Cabin1 and releases MEF2, which subsequently transactivates TR3 expression. In accordance with these data, overexpression of Cabin1 has been shown to inhibit both TR3 expression and T-cell receptor (TCR)-mediated apoptosis.\textsuperscript{39} Interestingly, MEF2 was originally discovered as a transcription factor for muscle-specific gene expression. It was postulated that a specific co-activator was required for the extensive increase in MEF2 activity in response to antigen receptor signaling. Indeed, MEF2 was shown to bind ERK5, which contains a transactivation domain to recruit the basal transcriptional machinery. In this way, ERK5 functions as a transcriptional co-activator of MEF2 activity in response to calcium fluxes and is essential to activate TR3 expression.\textsuperscript{40}

2.3.3 Involvement of TR3 in T-cell apoptosis

The process of programmed cell death (apoptosis) plays a central role in regulation of lymphocyte development and homeostasis. In the thymus, maturing T cells develop self-recognition and thymocytes that lack this ability are deleted by apoptosis. TR3 has been implicated in this process as an important transcription factor and indeed TR3 expression was found to correlate with TCR-mediated apoptosis.\textsuperscript{5,6} Blocking TR3 activity by a dominant-negative construct (which lacks the transactivation domain), or inhibition of TR3 protein expression with anti-sense oligonucleotide approaches, prevents TCR-mediated apoptosis in T-cell hybridomas.\textsuperscript{5,6} Interestingly, mice with a loss of function mutation of the TR3 gene have normal thymic and peripheral T-cell death. This lack of phenotype suggests redundancy by the related members of the NGFI-B family, MINOR and NOT.\textsuperscript{41} It has been shown that NOT and MINOR can transactivate genes through the same DNA elements as TR3, however, functional redundancy for TR3 in T-cell apoptosis was only established for MINOR.\textsuperscript{42} MINOR is induced upon TCR stimulation with the same kinetics as TR3 and constitutive expression of MINOR in developing thymocytes in transgenic mice leads to massive apoptosis.

Li et al. revealed another mechanism by which TR3 is capable to induce apoptosis.\textsuperscript{43} In prostate cancer cells TR3 is, in response to apoptotic stimuli, translocated from the nucleus to the mitochondria to induce cytochrome C release, which event initiates apoptosis. Interestingly, DNA binding and transactivation are not required in this process.\textsuperscript{43} In contrast, Rajpal et al. showed that TR3-mediated apoptosis in thymocytes does not involve cytoplasmic cytochrome C release and cannot be rescued by Bcl-2.\textsuperscript{44} In this study, micro-array analyses revealed that TR3 induces expression of many genes in thymocytes, including two novel
genes 'NDG1' and 'NDG2'. Of interest, NDG1 initiates a novel apoptotic pathway in a Bcl2-independent manner. Together these data point in the direction that TR3 may regulate apoptosis via transcription-dependent as well as via transcriptional activity independent pathways.

2.3.4 Downstream target genes of TR3, MINOR and NOT

So far, a limited number of target genes downstream of TR3 have been identified that are related to inflammation and steroidogenesis. The expression of TR3 in cells throughout the hypothalamic-pituitary-adrenal (HPA)-axis suggests that this transcription factor performs signaling functions at all levels of the HPA-axis. Indeed, TR3 induces expression of corticotropin-releasing hormone (CRH) in the hypothalamus and mediates the effect of CRH on transcription of the pituitary pro-opiomelanocortin (POMC) gene. The NGFI-B family regulates transcription of genes encoding the steroidogenic cytochrome P-450 enzymes in the adrenal cortex as illustrated by direct interaction with promoter sequences of the steroid-21-hydroxylase (21-OHase) gene upon adrenocorticotropic hormone (ACTH) activation of cells. Furthermore, TR3 induces expression of 20-a-hydroxysteroid dehydrogenase, a steroidogenic enzyme involved in the catabolism of progesterone in response to prostaglandin F2a. MINOR was shown to be induced by ACTH and angiotensin II treatment of adrenal fasciculata cells and also induces the expression of genes encoding steroidogenic enzymes. However, MINOR does not affect POMC expression. Studies with TR3 knockout mice have shown that the basal regulation of hypothalamic and pituitary function as well as the adrenocortical steroidogenesis is indistinguishable from wild-type mice. This lack of phenotype, and the knowledge that expression of NOT and MINOR is enhanced in these mice, illustrates that there is a clear redundancy between the family members in the NGFI-B family. Finally, Gruber et al. recently identified TR3 as an inducible DNA-binding protein that binds specifically to the promoter of the plasminogen activator inhibitor 1 (PAI-1) gene to drive its expression.

2.4 Outline of the thesis

Vascular smooth muscle cells (SMCs) can undergo relatively rapid and reversible phenotypical changes in response to local environmental alterations, which occur during atherogenesis. Transcription factors may be considered key regulators in phenotypic changes of SMCs. Upon activation of human SMCs with an atherogenic stimulus, we found that the mRNA expression of all three members of the Nerve Growth Factor Induced gene-B (NGFI-B) subfamily of nuclear hormone receptors, TR3, MINOR and NOT, are induced rapidly and
transiently. At the onset of this study, no reports were available on expression of these immediate-early response genes in atherogenesis.

In this thesis we investigated whether TR3, MINOR and NOT are functionally involved in atherosclerosis and other vascular pathologies involving SMCs, like vein-graft disease and in-stent restenosis.

In Chapter 1 and the current Chapter, an overview is presented regarding atherosclerosis and the NGFI-B subfamily of transcription factors, respectively. In Chapter 3 we report that cultured SMCs express TR3, MINOR and NOT in response to an atherogenic stimulus. In line with these data, we show that mRNA encoding these transcription factors is absent in normal, unaffected vascular tissue, whereas each of these genes is expressed in neointimal SMCs in human atherosclerotic lesions. To reveal functional involvement of these nuclear receptors in atherogenesis, we applied a variant of TR3, named ΔTA, which lacks the transactivation domain, but exhibits normal DNA binding, and consequently functions as a dominant-negative inhibitor of all three subfamily members. Transgenic mice were generated that overexpress TR3 or ΔTA in arterial SMCs and these mice were challenged by carotid artery ligation. These experiments clearly demonstrated that TR3 protects against excessive SMC hyperplasia in vivo (Chapter 3). Furthermore, expression of these transcription factors was observed in other vascular cells such as lesion macrophages and endothelial cells. In Chapter 4 we demonstrate that TR3 overexpression in cultured endothelial cells results in growth inhibition of the cells by maintaining them in the G1 stage of the cell cycle. We also studied the expression and function of TR3 in vein-graft disease (Chapter 5). Both in vein segments exposed to high-pressure flow and in cultured venous SMCs, exposed to mechanical strain induction, of TR3 expression was observed. In this study we show that overexpression of TR3 prevents stretch-induced proliferation of venous SMCs. In Chapter 6 expression of TR3, MINOR and NOT was explored in in-stent restenosis lesions and downstream target genes of TR3 in SMCs were identified. Finally, we demonstrated that TR3 is not involved in arterial responsiveness to vasoactive compounds and vascular remodelling during collateral artery formation as assessed in the transgenic mice (Chapter 7).
Chapter 2

References


30. Searce L.M., Laz T.M., Hazel T.G. et al. RNR-1, a nuclear receptor in the NGFI-B/Nur77 family that is rapidly induced in regenerating liver. J. Biol. Chem. 1993; 268: 8855-8861.


