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NR4A Orphan Nuclear Receptors: Transcriptional Regulators of Gene Expression in Metabolism and Vascular Biology

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Abstract

Members of the nuclear hormone receptor superfamily, including the peroxisome proliferator-activated receptor (PPAR) and liver X receptor (LXR) subfamilies, orchestrate transcriptional networks involved in the control of metabolism and the development of vascular disease. In addition to these well characterized ligand-activated transcription factors, the nuclear receptor superfamily comprises a large number of orphan receptors, whose ligands and physiological functions remain unknown. Among this group of orphan receptors is the NR4A subfamily including the three members Nur77 (NR4A1), Nurr1 (NR4A2) and NOR1 (NR4A3). These orphan nuclear receptors constitute an evolutionary ancient and highly conserved group of transcription factors. In contrast to other members of the superfamily, NR4A receptors function as ligand-independent transcription factors and immediate/early response genes, which are rapidly induced by a pleiotropy of environmental cues. Early functional studies have pointed to a critical role of NR4A receptors in regulating differentiation, proliferation and apoptosis. More recent research has characterized NR4A receptors as key transcriptional regulators of glucose and lipid homeostasis, adipogenesis, inflammation, and vascular remodeling. In this review, we will summarize recent advances in understanding the molecular biology and physiological functions of NR4A receptors and discuss their role in the transcriptional control of metabolism and vascular remodeling.

Keywords

nuclear receptor; gene expression; metabolism; vascular biology

Introduction

Members of the nuclear hormone receptor superfamily have emerged as a potentially large class of therapeutic targets for the treatment of obesity, diabetes, and atherosclerotic disease [1]. Most signaling pathways in these complex diseases ultimately converge to control networks of gene expression through signal-regulated transcription factors, including nuclear receptors (NR). The understanding of their ability to sense environmental cues and translate endocrine and metabolic signals into specific gene expression programs in metabolism and vascular biology has considerably expanded our knowledge on the pathophysiology of these most prevalent diseases. For example, the adopted orphan NR of

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the peroxisome proliferator-activated receptors (PPAR) and liver X receptors (LXR) subfamilies have been characterized to orchestrate gene expression programs involved in the control of glucose homeostasis, lipid metabolism, as well as inflammation and proliferation in the vascular wall [2, 3]. Pharmacological ligands for these two NR have been developed and their ligand-induced activation improves glucose metabolism and prevents atherosclerosis in murine models, attesting to the importance of these receptors and the approach to develop pharmacologic ligands [4–7]. The human genome contains 48 members of the NR superfamily [8]. In addition to the classical endocrine receptors and the adopted orphan receptors, the NR superfamily comprises an even larger group of orphan receptors [9]. Although the ligands for these orphan receptors remain unknown, considerable progress has been made to identify their regulated target genes and characterize their physiological functions [10]. Among these orphan receptors is the NR4A subfamily including the three members Nur77 (NR4A1) [11], Nurr1 (NR4A2) [12], and NOR1 (NR4A3) [13]. Members of this subfamily function as ligand-independent NR and early-response genes regulating key cellular processes, including inflammation, proliferation, differentiation, and survival [14–16]. In this review will summarize recent progress in understanding the physiological function of NR4A receptors and discuss their role as transcriptional regulators of gene expression in metabolism and vascular biology.

Molecular Biology of NR4A Orphan Nuclear Receptors

Nuclear receptors share a common structure consisting of a ligand-independent AF-1 transactivation domain in the N-terminal region, a highly conserved DNA-binding domain (DBD) composed of two zinc fingers recognizing specific DNA sequences, and a ligand-binding domain (LBD) that contains a ligand-dependent AF-2 transactivation domain in its C-terminal portion [17]. NR4A receptors share this common NR structure, and the three members reveal a high degree of homology in their genomic structure and conservation of their DNA binding domain (degree of conservation > 90%) [18]. However, several lines of evidence indicate that NR4A receptors may represent a distinct group of transcription factors that do not function in a classical manner. Mutational analysis indicated that NR4A receptors function as constitutively-active receptors whose transcriptional activity is independent of the LBD [19, 20]. Instead, their transcriptional activity and coactivator recruitment appear to be dependent on the N-terminal AF-1 domain [20–22], which constitutes a common distinction of ligand-independent transcriptional activation by NR [23,24]. This initial observation was supported by the finding that the LBD of NR4A contains hydrophilic surfaces instead of the classical hydrophobic cleft that mediates coactivator recruitment of other NR [22]. Finally, this unusual structure of the NR4A LBD has been recently confirmed by X-ray crystallography demonstrating that the Nurr1 LBD contains no cavity as a result of hydrophobic residues in the region normally occupied by ligands [25]. Considering these observations, NR4A receptors are currently thought to function as constitutively-active and ligand-independent receptors, whose transcriptional activity is primarily dependent on the expression of the receptor and its posttranslational modification.

NR4A receptors are early immediate response genes, which are induced by a pleiotropy of stimuli including growth factors, inflammatory stimuli, cytokines, peptide hormones, and cellular stress [16]. Once their expression is induced, NR4A receptors activate transcription by binding as monomers or homodimers to canonical DNA target sites, the NGFI-B-responsive element (NBRE) consisting of an octanucleotide AAAGGTCA motif [26, 27]. NR4A homodimers preferentially bind to the Nur-responsive element (NurRE), which constitutes an everted repeat of the NBRE-related sequence (AAAT(G/A)(C/T)CA) found in the pro-opiomelanocortin (POMC) gene promoter [28]. In addition, Nur77 and Nurr1 (but not NOR1) heterodimerize with RXR and activate transcription through a DR-5 element in a 9-cis retinoic acid-dependent manner [29, 30]. This heterodimerization of Nurr1 with RXR is

isotype-specific since Nurr1 interacts only with RXRα and RXRγ but not with RXRβ[31]. Furthermore, different NR4A receptors can form heterodimers to synergistically activate transcription [32]. While it was initially thought that NR4A receptors only activate genes, a recent study has provided first evidence that Nurr1 can also repress inflammatory gene promoters by recruiting corepressor complexes [33].

In addition to the rapid expression as early response genes, the transcriptional activity of NR4A receptors is regulated by posttranslational modification. All three NR4A receptors are phosphorylated at serine residues in response to growth factor-dependent activation of various kinases, including MAPK, PI3K, Akt, JNK, and RSK [31, 34–37]. For example, Nur77 is phosphorylated at Ser-350 and Ser-354 within the DNA binding domain, which inhibits the transactivation activity [38, 39]. Furthermore, phosphorylation of Nur77 at Ser-105 induces nuclear export of the Nur77/RXR heterodimer complex [40], providing an additional mechanism by which phosphorylation may inhibit the transcriptional activity of Nur77. In addition to phosphorylation, all NR4A receptors contain sumoylation consensus sites, and sumoylation of Nurr1 induces or inhibits the transcriptional activity in a sumoylation site-specific manner [41]. Although still in its infancy, these postranslational modifications regulate the transcriptional activity and may represent a major mode of the control of gene expression by NR4A receptors.

NR4A Receptors in Metabolism and Energy Balance

Carbohydrate Metabolism

All three NR4A receptors are potently induced in the liver in response to physiological stimuli, including fasting and glucagon stimulation (Figure 1) [42, 43]. Furthermore, hepatic NR4A receptor expression is increased in diabetic mice as a model of pathologic gluconeogenesis [42]. Functional studies further demonstrated that adenoviral overexpression of Nur77 increases the expression of genes involved in gluconeogenesis and stimulates hepatic glucose production in mice [42]. Interestingly, Nur77 overexpression induces several gluconeogenic genes including G6pc, Fbp1 and Fbp2, and enolase 3, which all contain NBRE consensus sites in their promoters [42]. Therefore, this study has provided the first experimental evidence that NR4A receptors regulate gluconeogenesis and may serve to link hormonal stimulation to downstream metabolic gene expression.

In skeletal muscle, NR4A receptors are induced by growth factors, β-adrenergic signalling, and endurance exercise [44–47]. Maxwell et al. first demonstrated that knock-down of Nur77 in muscle cells results in decreased lipolysis and expression of genes regulating energy expenditure and lipid homeostasis, including AMP-activated protein kinase, UCP3, Glut4, CD36, adiponectin receptor 2, and caveolin-3[45]. Conversely, Cao et al. reported that overexpression of Nur77 in C2C12 muscle cells increases the expression of genes involved in glucose and glycogen metabolism while Nur77 deficiency in mice reduces the expression of genes involved in skeletal muscle glucose utilization *in vivo* [48]. Consistent with this role of Nur77 to promote glucose utilization was the observation that Nur77-deficient mice develop skeletal muscle insulin resistance when fed a high fat diet due to altered insulin signaling and reduced GLUT4 expression [49]. Although glucose metabolism has not been studied in NOR1-deficient mice, knock-down of NOR1 in skeletal muscle cells attenuates the expression of genes that control fatty acid oxidation and pyruvate use (i.e. PGC-1α, PGC-1β, lipin-1α, PDP1r and PDP1c) indicating that NOR1 may be necessary for oxidative metabolism [50]. Finally, NOR1 has recently been demonstrated to also promote insulin-stimulated glucose uptake in adipocytes by augmenting insulin signaling and Glut4 translocation [51]. In concert, these intriguing observations point to a key role of NR4A receptors in the transcriptional control of glucose homeostasis and oxidative metabolism.

Lipid Metabolism

Accumulating evidence indicates that NR4A receptors regulate various aspects of lipid metabolism. As noted earlier, initial experiments by Maxwell et al. demonstrated that Nur77 promotes lipolysis in muscle [45]. Subsequently, Pols et al. revealed that Nur77 modulates plasma lipoprotein profiles and hepatic lipid metabolism in mice [52]. In this study, adenoviral-mediated overexpression of Nur77 increased plasma LDL cholesterol and decreased HDL cholesterol while reducing hepatic triglyceride levels, which was thought to be due to a repression of the lipogenic transcription factor SREBP1c [52]. Consistent with these data, Chao et al. noted hepatic steatosis and increased SREBP1c expression in Nur77-deficient mice fed a high fat diet [49]. However, since Nur77 did not directly affect SREBP1c activity in reporter assays, the authors concluded that the hepatic steatosis in Nur77-deficient mice was likely secondary to the lipogenic effect of hyperinsulinemia [49].

In 3T3-L1 preadipocytes, NR4A receptors expression is induced during adipogenesis and initiating of the differentiation program [53, 54]. Initial studies using siRNA approaches and overexpression of a Nur77 mutant lacking the N-terminal AF-1 transactivation domain indicated that Nur77 is not required for adipocyte differentiation [55]. However, a functional role for NR4A receptors in adipogenesis was suggested by two recent *in vitro* studies, which have demonstrated that constitutive NR4A receptor expression in 3T3-L1 preadipocytes inhibits adipocyte differentiation [56, 57]. One of the mechanisms proposed for this negative regulation of adipogenesis by NR4A receptors has been the inhibition of the mitotic clonal expansion of preadipocytes, [56]. However, considering that the initial mitotic expansion step is primarily a prerequisite for 3T3-L1 preadipocyte differentiation, further studies seem required and there are likely additional mechanisms involved by which NR4A receptors inhibit adipogenesis. These may include a direct regulation of target genes affecting adipogenesis, including extracellular matrix genes [56]. In addition, NR4A receptors may cross-talk with adipogenic signalling and transcriptional programs, particularly since Nurr1 and Nur77 have been reported to interact with Wnt signaling pathways or the glucocorticoid receptor, which both play important roles in adipogenesis [58–60].

Energy Homeostasis

Brown adipose tissue plays a key role in energy balance and is the primary organ involved in thermogenesis through uncoupling of mitochondrial respiration by the action of uncoupling proteins (UCP). Early studies have demonstrated that Nur77 expression is highly induced in response to β-adrenergic stimulation of brown adipocytes while transcript levels of all three NR4A receptors are induced during cold-exposure [61, 62]. Kanzleiter et al. demonstrated a repressive effect of Nur77 on the UCP-1 promoter in brown adipocytes, which was likely indirect since Nur77 did not directly interact with the UCP-1 promoter [61]. Despite this repression of UCP-1, nonshivering thermogenesis was not affected by Nurr77 deficiency in mice [61]. In contrast, Kumar et al. observed that NOR1 transcriptionally up-regulates UCP-1 expression by binding to an NBRE site on the UCP-1 promoter [62]. Furthermore, overexpression of a Nur77 mutant lacking the N-terminal AF-1 transactivation domain prevented UCP-1 transcription induced by β-adrenergic signaling [62]. The reasons underlying these seemingly conflicting two studies remain unclear but are likely due to a differential regulation of UCP-1 by Nur77 and NOR1. Moreover, NR4A receptors may affect the central regulation of energy homeostasis since injection of NOR1 siRNA into the third cerebral ventricle significantly suppresses food intake and body weight in mice [63]. In concert, these intriguing studies characterize NR4A receptors as important regulators of energy balance and food intake, although the underlying mechanisms remain elusive and warrant further studies in gene-targeted mice.

NR4A Receptors in Vascular Biology

An increasing number of studies have demonstrated that all three members of the NR4A subfamily are expressed in the developing neointima and in advanced atherosclerotic lesions [64–70]. Moreover, accumulating evidence indicates that NR4A receptors constitute important transcription factors in the control of vascular gene expression and play critical roles in essentially all aspects of vascular remodeling, including cell viability, proliferation, and inflammation (Figure 2). In the following section, we will briefly summarize these studies pointing to a previously unrecognized function of NR4A receptors in vascular biology.

Cell Viability and Proliferation

Endothelial cell injury followed by the expression of adhesion molecules and the subsequent recruitment of circulating monocytes constitute critical events for the initiation of atherosclerosis [71]. All three NR4A receptors are potently induced by a variety of pro-atherogenic stimuli in endothelial cells, including atherogenic lipoproteins, inflammatory cytokines, growth factors, and hypoxia (Figure 2) [65, 72–78]. The transcriptional mechanisms governing this inducible expression have been primarily studied in the context of growth factor and hypoxia-induced NOR1 expression. While the former mechanisms involve a cAMP response element binding protein (CREB)-dependent activation of the NOR1 promoter [74, 78], NOR1 expression in response to hypoxia is dependent on hypoxia-inducible factor 1 (HIF-1) binding to a hypoxia response element in the promoter [76]. Arkenbout et al. performed the first functional experiments in endothelial cells and demonstrated that adenoviral overexpression of Nur77 inhibits proliferation of this cell type by upregulating $p27^{Kip1}$ and downregulating cyclin A [65]. However, the role of Nur77 for endothelial cell proliferation remains controversial since Zeng et al. reported that Nur77 induces proliferation and cell cycle gene expression [75]. Moreover, this report noted that angiogenesis is induced by overexpression of Nur77 and decreased in Nur77−/− mice [75]. With respect to the sibling NOR1, Rius et al. identified a mitogenic role for this receptor by demonstrating that antisense oligonucleotides against NOR1 inhibit endothelial cell growth and wound repair after injury [74]. Consistent with these observations, NOR1 has recently been characterized as a pro-survival gene in endothelial cells exposed to hypoxia by inducing the expression of cellular inhibitor of apoptosis protein 2[76]. Collectively, these studies establish a role for Nur77 and NOR1 in regulating endothelial cell survival and proliferation; however, little is known about the transcriptional target genes and molecular mechanisms. At present, only two direct NR4A target genes have been identified in endothelial cells. Gruber et al. characterized PAI-1 as a Nur77 target gene, which is activated by the receptor through direct binding to an NBRE site in the promoter [72]. In addition, You et al. demonstrated that Nur77 overexpression prevents NF-κB nuclear translocation in endothelial cells by enhancing the expression of IκBα, which is mediated through a direct transactivation of a NBRE site in the IκBα promoter [77]. Interestingly, the functional relevance of Nur77-dependent IκBα expression was confirmed by the finding that Nur77 inhibited the expression of VCAM-1 and ICAM-1 in endothelial cells [77].

Similarly as in endothelial cells, NR4A receptor expression is rapidly induced in response to atherogenic stimulation of smooth muscle cells (SMC), including lipoproteins, cyclic stretch, and mitogenic stimuli [64, 66–68, 79]. The transcriptional induction of NOR1 in SMC is mediated through mitogen-induced CREB binding to CRE sites in the NOR1 promoter and can be pharmacologically inhibited by simvastatin [66, 67, 79, 80]. Consistent with the earlier described growth-inhibitory function of Nur77 in endothelial cells, Nur77 overexpression inhibits SMC proliferation *in vitro* by stabilizing of $p27^{Kip1}$ [64, 81] and reduces neointima formation *in vivo* [64]. Interestingly, data from the same group has further recently suggested that SMC-specific overexpression of Nur77 inhibits pathological outward remodeling in response to carotid artery ligation, which was associated with decreased macrophage

accumulation and MMP expression [82]. While these studies clearly indicate that Nur77 prevents SMC proliferation, NOR1 has been reported to act mitogenic suggesting a function that is distinct from that of Nur77. A proliferative role of NOR1 was first reported by Martínez-González et al. using antisense NOR1 oligonucleotides [66]. Consistent with these initial observations, data from our group has demonstrated a proliferative defect and an increased propensity for apoptosis in SMC isolated from NOR1-deficient mice [67, 68]. *In vivo*, the proliferative response and neointima formation following endovascular femoral artery guide wire injry was decreased in NOR1-deficient mice [68]. At a molecular level, this mitogenic activity of NOR1 was at least in part mediated by a transactivation of a canonical NBRE site in the cyclin D1 promoter, characterizing cyclin D1 as a *bona fide* NOR1 target gene in SMC [68]. Furthermore, DNA microarray profiling revealed a lower expression of NOR1 in elongated SMC while NOR1 knock-down suppressed DNA synthesis, further supporting the mitogenic function of NOR1 and pointing to a potential role of NOR1 in regulating cell shape [83]. In concert, these studies establish not only an important but also distinct role for Nur77 and NOR1 in the control of vascular cell proliferation and remodeling. Continued investigation will be required to define the transcriptional target genes and the molecular basis underlying the differential function of NOR1 and Nur77 in SMC biology.

Inflammation

The first evidence linking NR4A expression with inflammatory signaling was reported by Woronicz et al. and Liu et al., who noted that Nur77 is induced in apoptotic T-cells and that inhibition of Nur77 function prevented apoptosis [84, 85]. However, mice deficient in Nur77 exhibit unimpaired T-cell apoptosis, and functional redundancy of Nur77 and NOR1 in T-cell apoptosis has been suggested [86, 87]. Similarly to T-cells, Nur77 expression is increased in apoptotic macrophages and, in contrast to the experiments performed in T-cells, peritoneal macrophages isolated from Nur77-deficient mice reveal a phenotype of reduced cell death [88]. In response to inflammatory activation, all three NR4A receptors are potently induced in macrophages [69, 70]. This inducible expression of NR4A receptors in macrophages depends on the activation of NF-κB signaling, as exemplified by the recruitment of NF-κB to response elements in the Nur77 promoter [69]. Functional studies have indicated that NR4A receptors both activate and repress inflammatory genes in macrophages [33, 70, 89]. An initial microarray analysis by Pei et al. discovered that NR4A overexpression in macrophages induces proinflammatory gene expression [89]. Interestingly, among the identified direct Nur77 target genes was the inducible kinase IKKi/IKKepsilon, which functions as a NF-κB activating kinase, providing a potential mechanism for the activation of inflammatory gene expression by Nur77 in macrophages [89]. In contrast to these studies, Bonta et al. revealed that lentiviral overexpression of each NR4A member reduces certain inflammatory genes (i.e. IL-1β, IL-6, IL-8, MIP1α and 1β and MCP-1) and the uptake of oxidized LDL [70]. Finally, a recent study by Saijo et al. identified that Nurr1 transcriptionally represses the inflammatory genes TNFα, iNOS, and IL-1β in microglia and the murine RAW264.7 cell line [33]. This transrepression was mediated through a Nurr1-dependent recruitment of the corepressor for element-1-silencing transcription factor (CoREST) complex to the target promoter and the subsequent clearance of NF-κB [33]. While these studies indicate that NR4A receptors function as important transcriptional regulators of inflammatory gene expression, further *in vivo* evidence using animal models deficient for either of the NR4A receptors seems required, particularly with respect to the development of atherosclerosis.

Concluding Remarks

In conclusion, the ligand-independent NR4A orphan nuclear receptors are immediate early response genes, whose protein products are rapidly induced in metabolic and vascular

tissues in response to a pleiotropy of stimuli. Emerging evidence indicates that NR4A receptors regulate the transcription of genes involved in glucose homeostasis, lipid metabolism, and energy balance. Moreover, the initial characterization of their function in vascular biology has implicated these transcription factors in the control of inflammation, proliferation, apoptosis, thrombosis, and angiogenesis. Despite recent advances in understanding the role of NR4A receptor function in physiological and pathological processes, important questions remain for future research. For example, future effort will require further validation of NR4A receptor function in murine models and rely on various gene-targeting approaches. In particular, it seems essential to determine whether the three different NR4A receptors exhibit similar or distinct functions in various tissues. Furthermore, at present, few NR4A receptor-regulated genes have been identified, and it will not only be important to characterize target genes but also to define the detailed transcriptional mechanisms underlying this regulation. Finally, a possibility to modulate the expression and/or transcriptional activity of NR4A receptors may provide pharmacological applications. Considering the lack of a classical ligand-binding pocket, such approach might involve the modulation of cofactor recruitment and/or posttranslational modifications. Continued investigation of these questions and identification of NR4A-regulated target genes will provide new insights into how these orphan nuclear receptors participate in the development of physiology and disease.

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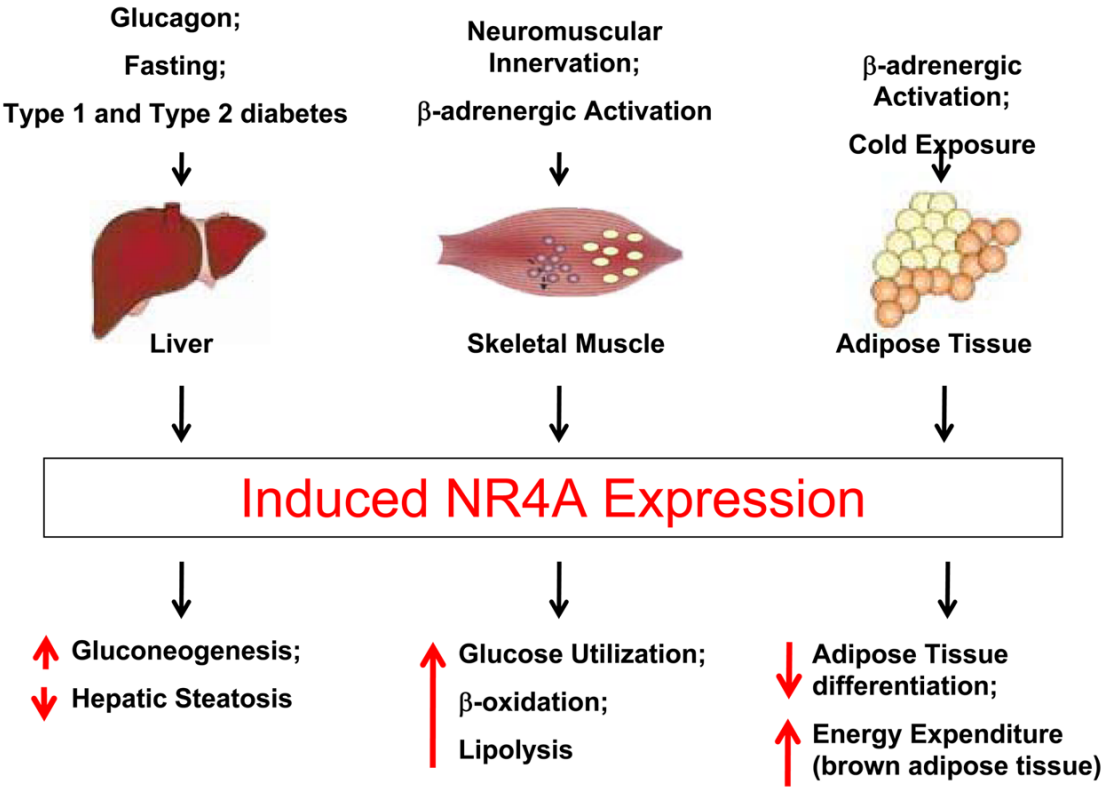

Figure 1.
NR4A Receptor Function in Metabolism and Energy Balance. NR4A orphan nuclear receptors are potently induced by physiological and pathological stimuli in liver, muscle and adipose tissue. In these tissues, NR4A orphan receptors function as transcriptional regulators of gene expression programs involved in the control of glucose homeostasis, lipid metabolism, and energy expenditure (see text for details).

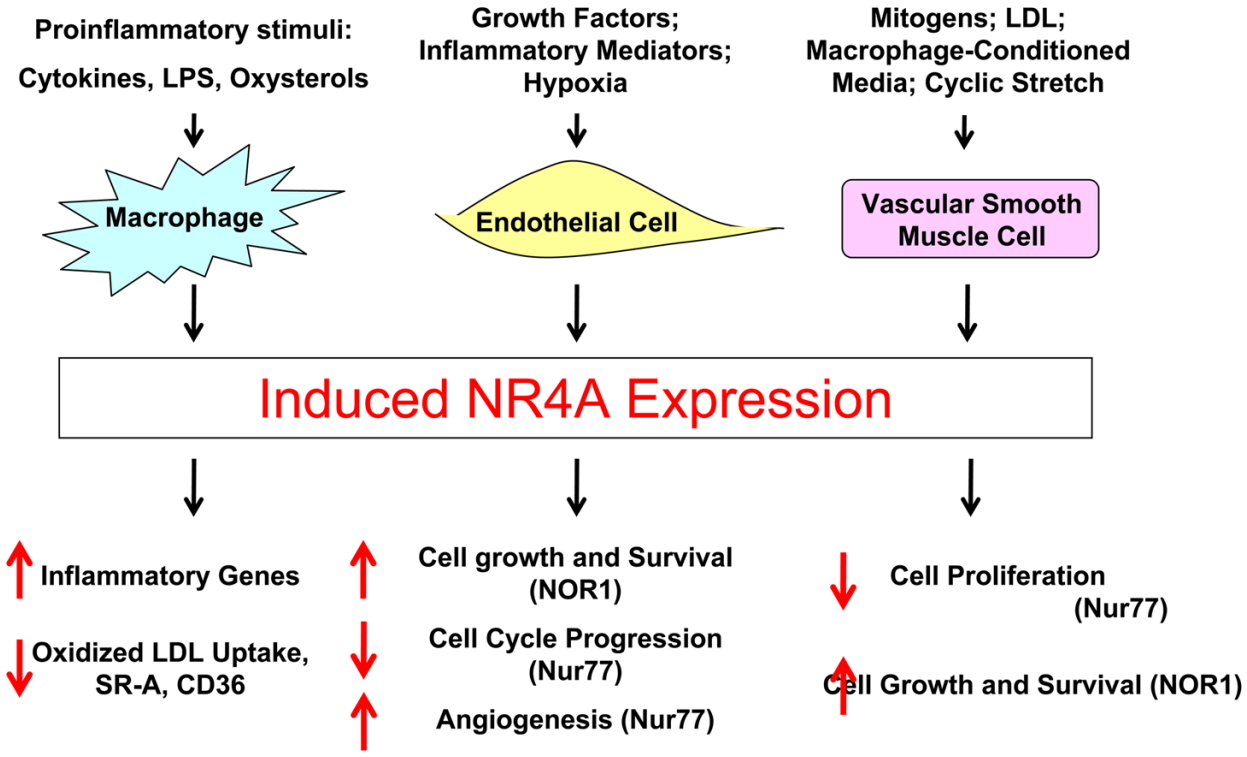

Figure 2.

Expression and Function of NR4A Receptors in Vascular Cells. NR4A orphan nuclear receptors are induced in vascular cells by a variety of stimuli, including inflammatory mediators, cytokines, hypoxia, and growth factors. In response to these pathophysiological environmental cues, NR4A receptors modulate gene expression leading to cell-specific processes (see text for details).