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Review

Regulation of the expression of inducible nitric oxide synthase

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Abstract

The role of nitric oxide (NO) generated by the inducible isoform of nitric oxide synthase (iNOS) is very complex. Induction of iNOS expression and hence NO production has been described to have beneficial antiviral, antiparasital, microbicidal, immunomodulatory, and antitumoral effects. However, induced at the wrong place or at the wrong time, iNOS has detrimental consequences and seems to be involved in the pathophysiology of different human diseases. The pathways regulating iNOS expression seem to vary in different cells or different species. In general, activation of the transcription factors nuclear factor (NF)-κB and signal transducer and activator of transcription (STAT)-1α and thereby activation of the iNOS promoter seems to be an essential step in the regulation of iNOS expression in most cells. Also, post-transcriptional mechanisms are critically involved in the regulation of iNOS expression.

Keywords: iNOS (inducible nitric oxide synthase); Expression; Signal transduction; Pathway; Promoter; Transcription factor; mRNA stability; RNA binding; Protein

Contents

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1. Introduction

In mammals, three distinct isoforms of nitric oxide synthase (NOS) exist (neuronal (n)NOS, inducible (i)NOS and endothelial (e)NOS) (Forstermann et al., 2003). nNOS and eNOS are primarily expressed in neurons and endothelial cells, respectively. They are low output, Ca^{2+}-dependent enzymes producing nitric oxide (NO) in a pulsative manner. In contrast, iNOS is a high output, Ca^{2+}-independent enzyme whose expression can be induced in a wide range of cells and tissues by cytokines and other agents. After induction, iNOS produces continuously NO until the enzyme is degraded (MacMicking et al., 1997).

NO produced by iNOS has been described to have beneficial microbicidal, antiviral, antiparasital, and antitumoral effects (Bogdan, 2001; MacMicking et al., 1997). However, aberrant iNOS induction seems to be involved in the pathophysiology of human diseases such as asthma, arthritis, multiple sclerosis, colitis, psoriasis, neurodegenerative diseases, tumor development, transplant rejection or septic shock (Bogdan, 2001; Kröncke et al., 1998).

There are some reports describing regulation of iNOS activity by interacting proteins (Daniliuc et al., 2003; Ratovitski et al., 1999a,b), reduction of arginine supply through arginase (Bruch-Gerharz et al., 2003; Mori and Gotoh, 2000) or by modulating arginine uptake (Closs et al., 2000) as well as by modulation of the intracellular concentration of the essential cofactor tetrahydrobiopterin (BH_4; (Muhl and Pfeilschifter, 1994)). However, it is believed generally that modulation of iNOS expression is the most important component of iNOS regulation.

2. Signaling pathways regulating iNOS expression

iNOS expression in murine and rat cells is induced by incubation with bacterial lipopolysaccharide (LPS), stimulatory cytokines such as interferon-γ (IFN-γ), interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) or other compounds. In contrast, the majority of human cells requires a complex cytokine combination including IFN-γ, IL-1β and TNF-α for iNOS induction (Kleinert et al., 2003).

The different inducers of iNOS expression have been shown to activate different signaling pathways (for a detailed description, see Kleinert et al., 2003). Many different compounds (more or less specific) and also molecular activators or inhibitors (permanent active or dominant negative isoforms of kinases, etc.) have been shown to induce, enhance or inhibit iNOS expression by activating or blocking a wide variety of signal transduction pathways (Kleinert et al., 2003). Analyzing the effects of these compounds or molecular tools on iNOS expression in different cell types and species often resulted in contradictory data. Despite possible subtle differences in the usage of the different activators/inhibitors for one specific signal pathway, this may be explained by the cell type and species specificity of iNOS induction. However, in general, it seems that activation or inhibition of the janus kinase/signal transducer and activator of transcription (JAK/STAT)- and/or the nuclear factor (NF)-κB-pathway is the central mechanism explaining the effects of many different compounds.

3. iNOS promoter sequences

The published sequences of cloned iNOS promoters of different species exhibit homologies to binding sites for numerous transcription factors (such as activating protein-1, AP-1; CCAAT-enhancer box binding protein, C/EBP; cAMP-responsive element binding protein, CREB; interferon regulatory factor-1, IRF-1; NF-κB; nuclear factor-IL6, NF-IL6; octamer factor-1, Oct-1; serum response factor, SRF; and STAT-1α) known to be involved in the LPS/cytokine-mediated induction of transcription (Lin et al., 1996); GenBank AF333248; (Chu et al., 1998); GenBank NT_010808 and AC005697; (Lowenstein et al., 1993; Wang et al., 2001; Xie et al., 1993; Zhang et al., 1998). iNOS promoters of all species investigated contain a TATA box about 30 bp from the transcription start site. Near the TATA box, all mammalian promoters possess binding sites for the transcription factors NF-κB, NF-IL6 and octamer factors, and for transcription factors induced by TNF-α. At position −900 bp, the rat, murine and human promoters display binding sites for transcription factors induced by IFN-γ (γ-interferon activated site, GAS; γ-interferon responsible element, γIRE; interferon stimulated response element, ISRE).

In transfection experiments using homologous or heterologous cell systems, fragments containing up to 3200 bp of the murine (Kleinert et al., 1996; Lowenstein et al., 1993; Xie et al., 1993) or the rat iNOS promoter (Darville and Eizirik, 1998; Guo et al., 2002; Kuo et al., 1997; Zhang et al., 1998) showed marked inducibility by LPS or cytokines.

The human 1000-bp iNOS promoter fragment showed low, but significant basal promoter activity, but no inducibility with cytokines in most human cells analyzed (Chu et al., 1998; de Vera et al., 1996b; Linn et al., 1997). Interestingly, when the same human 1000-bp promoter fragment was transfected into heterologous murine RAW 264.7 macrophages, it became markedly inducible with LPS, IFN-γ and IL-1β (Kolyada et al., 1996). In human AKN, A549, DLD1 or HepG2 cells, only iNOS promoter fragments larger than 3.8 kb showed any significant induction with cytokines (Chu et al., 1998; de Vera et al., 1996b; Linn et al., 1997; Majano et al., 2004; Taylor and Geller, 2000). Thus, transcription factor binding sites relevant for cytokine induction seem to be located upstream of −3.8 kb of the 5′-flanking sequence

of the human iNOS gene. A marked induction (8–10-fold) was found with a 16-kb human iNOS promoter fragment in A549 or AKN cells (de Vera et al., 1996b; Hausding et al., 2000; Rodriguez-Pascual et al., 2000). Surprisingly, another group (Chu et al., 1998; Kristof et al., 2001; Marks-Konczalik et al., 1998) showed a much higher inducibility (up to 50-fold) of the human 8.3-kb iNOS promoter fragment in A549 cells. The reason for this marked difference in promoter inducibility is not clear at this time.

4. Transcription factors involved in iNOS expression

4.1. Nuclear factor-κB (NF-κB)

The transcription factor NF-κB (Ghosh et al., 1998) seems to be a central target for activators or inhibitors of iNOS expression. LPS, IL-1β, TNF-α and oxidative stress for instance have been shown to induce iNOS expression in different cell types by activating NF-κB. Also, inhibition of iNOS expression by numerous agents, such as glucocorticoids, transforming growth factor-β1 (TGF-β1), antioxidants (e.g. pyrrolidine dithiocarbamate, PDTC) and inhibitors of phosphatidylcholine-specific phospholipase (PC-PLC) has been shown to be mediated by inhibition of NF-κB activation. This inhibition can result from direct capture of NF-κB by protein–protein interactions (Kleinert et al., 1996; Mukaida et al., 1994), inhibition of nuclear translocation of NF-κB (Jeon et al., 1998), inhibition of NF-κB transactivation activity (Yu et al., 2002) or from enhancement of the expression of the specific inhibitor of NF-κB, I-κB (de Vera et al., 1997; Saura et al., 1998).

An important role of NF-κB binding sites for the induction of iNOS promoter activity has been shown in murine (Goldring et al., 1996; Lowenstein et al., 1993; Xie et al., 1994), rat (Eberhardt et al., 1998; Spink et al., 1995) and human (Marks-Konczalik et al., 1998; Taylor et al., 1998) cells. For the location of the important NF-κB binding sites in the human iNOS promoter, conflicting results have been published. Taylor et al. (1998) reported involvement of multiple NF-κB binding sites between positions −5.2 and −6.5 kb in the induction of the iNOS promoter. Other groups, however, reported on the importance of a downstream NF-κB binding site (positions −115 to −106 bp, near the TATA box) for human iNOS promoter activity (Marks-Konczalik et al., 1998; Nunokawa et al., 1996; Sakitani et al., 1998). Feng et al. (2002) described the inhibition of the human iNOS promoter by the NF-κB-repressing factor (NRF). Mutation of a negative regulatory element (NRE) at position −6.7 kb resulted in an enhanced basal (but not cytokine-induced) promoter activity in transient transfection experiments. Overexpression of NRF inhibited basal and cytokine-induced iNOS promoter activity. Interestingly, downregulation of NRF expression in HeLa cells resulted in basal iNOS mRNA expression (Feng et al., 2002).

4.2. Interferon regulatory factor-1 (IRF-1)

The essential role of the IRF-1 binding site (positions −913 to −923 bp) for the induction of the murine iNOS promoter has been demonstrated exemplarily in RAW 264.7 macrophages. Mutations of this binding site blocked the IFN-γ-mediated enhancement of LPS-induced iNOS promoter activity (Martin et al., 1994; Spink and Evans, 1997). Supershift experiments showed that IRF-1 is a component of the protein complexes bound to this binding site after IFN-γ incubation (Martin et al., 1994). In vivo footprint experiments with LPS-incubated RAW 264.7 macrophages supported this data (Goldring et al., 1996). In cooperation with IRF-1, the IFN consensus sequence-binding protein (ICSBP or IRF-8) was found to bind to this sequence (Xiong et al., 2003). Overexpression of ICSBP enhanced IFN-γ-induced iNOS promoter activation in RAW 264.7 cells. IFN-γ-induced iNOS promoter activation was markedly reduced in ICSBP$^{-/-}$ macrophages as in macrophages and glial cells from IRF-1$^{-/-}$ mice (Blair et al., 2002; Fujimura et al., 1997; Kamijo et al., 1994; Shiraishi et al., 1997). Therefore, complex formation of ICSBP with IRF-1 seems to be essential for iNOS expression in murine macrophages (Xiong et al., 2003).

By overexpression of wild type murine IRF-1 in human embryonic kidney (HEK-293) cells, Upreti et al. (2004) demonstrated cytokine-independent activation of the transcription of the endogenous human iNOS gene. Overexpression of a mutant murine IRF1 containing a six-amino-acid replacement (human IPVEVV instead of murine MQMDII) in HEK cells did not result in iNOS gene activation (Upreti et al., 2004). Therefore, the slight change in the amino acid sequence of the IRF-1 protein seems to result in a major change in the regulation of IRF-1-mediated iNOS gene expression.

4.3. Signal transducer and activator of transcription-1α (STAT-1α)

All mammalian iNOS promoters contain several homologies with the IFN-γ-regulated transcription factor STAT-1α binding sites (GAS). Exposure of cells to IFN-γ results in IFN-γ receptor dimerization and activation of cytoplasmatic janus kinases (JAKs) that tyrosine-phosphorylate themselves, the IFN-γ receptor and the latent cytoplasmatic STAT transcription factors. After tyrosine-phosphorylation, the STATs dimerize, translocate to the nucleus and activate STAT-dependent gene expression (Schindler and Darnell, 1995). An essential involvement of the IFN-γ-JAK2-STAT-1α pathway in iNOS induction had been shown for murine (Meraz et al., 1996; Nishiya et al., 1997; Singh et al., 1996), rat (Doi et al., 2002; Kitamura et al., 1996) and human cells

(Ganster et al., 2001; Kleinert et al., 1998a). In rat vascular smooth muscle cells (VSMC), a dominant-negative JAK2 isoform inhibited cytokine-induced iNOS expression, again underscoring the important role of this tyrosine kinase in iNOS induction (Doi et al., 2002). Also several compounds were described to inhibit iNOS expression by blocking cytokine-induced activation of STAT-1α (Tedeschi et al., 2003, 2004; Yao et al., 2003).

Gao et al. (1997) reported binding of STAT-1α to the GAS of the murine iNOS promoter (positions −934 to −942 bp) to be required for optimal induction of the iNOS gene by IFN-γ and LPS. Also, the IFN-γ-mediated enhancement of IL-1β-induced promoter activity in rat RINm5F cells or rat aortic smooth muscle cells was dependent on the GAS and ISRE sites around position −900 bp of the rat iNOS promoter (Darville and Eizirik, 1998; Teng et al., 2002b). In human A549 or DLD1 cells, inhibition of the IFN-γ-activated tyrosine kinase JAK2 by tyrphostin B42 (AG 490) reduced STAT-1α DNA-binding activity and iNOS expression (Kleinert et al., 1998a). Using specific signaling inhibitors, Blanchette et al. (2003) were able to show that JAK2/STAT-1α- and extracellular regulated kinase-(ERK1/ERK2)-dependent pathways are the main players in IFN-γ-inducible iNOS induction in murine J774 macrophages. ERK1/ERK2 are responsible for STAT-1α Ser727 residue phosphorylation in IFN-γ-stimulated cells, thus contributing to full activation of STAT-1α (Blanchette et al., 2003). Expression of a dominant-negative mutant of STAT-1α inhibited cytokine-induced human iNOS promoter activity (Ganster et al., 2001). Site-directed mutagenesis of the human iNOS promoter identified a bifunctional NF-κB/STAT-1α motif at −5.8 kb, and a STAT-1α-specific responsive element at −5.2 kb (Ganster et al., 2001).

In contrast to macrophages, pancreatic islets cells, chondrocytes and hepatocytes from IRF-$1^{-/-}$ mice showed normal iNOS induction in response to LPS/IFN-γ (Blair et al., 2002; Shiraishi et al., 1997). In these cell types, IRF-1 seems to be non-essential for iNOS induction. These in vivo data again underline the cell specificity of the regulation of iNOS expression. Furthermore, iNOS induction was blocked in macrophages from mice with a disrupted STAT-1α gene (Meraz et al., 1996).

Therefore, STAT-1α is likely to be involved essentially in the stimulation of iNOS induction, either directly by binding to the iNOS promoter, or indirectly by inducing IRF-1 activity.

4.4. cAMP-induced transcription factors; cAMP-responsive element binding protein (CREB), CCAAT-enhancer box binding protein (C/EBP)

A CCAAT box (C/EBP binding site, positions −155 to −163 bp) was reported to be essential for cAMP-mediated (but not IL-1β-mediated) induction of the rat iNOS promoter (Eberhardt et al., 1998). Supershift experiments showed the involvement of C/EBPβ and C/EBPδ in the cAMP-mediated regulation of this promoter. In rat C6 glial cells, transfection with dominant-negative (dn) forms of CREB and C/EBP resulted in opposite effects, i.e., dnCREB enhanced while dnC/EBP inhibited the rat iNOS promoter activity. Also, overexpression of wild-type activating transcription factor 2 (ATF2) enhanced, while a phosphorylation-defective form of ATF2 suppressed rat iNOS promoter activity. Therefore, in rat glial cells, the cAMP-activated transcription factors CREB, C/EBP and ATF2 seem to be involved in the regulation of iNOS expression (Bhat et al., 2002). Exposure of rats to hypoxia caused pulmonary hypertension and induced expression of both iNOS and C/EBPβ in rat lung and rat pulmonary microvascular smooth muscle cells (rPMSMC). Hypoxia also enhanced rat iNOS promoter activity in transfected rPMSMCs. This hypoxia-induced promoter activity was abolished by the mutation of a C/EBP motif at −910 bp of rat iNOS promoter. Thus, C/EBPβ mediates, at least in part, hypoxia-induced iNOS expression in rPMSMCs (Teng et al., 2002a).

In a heterologous transfection system using primary rat hepatocytes and fragments of the human iNOS promoter, Sakitani et al. (1998) showed inducibility of a short human iNOS promoter fragment (position −365 bp to the start site). When a C/EBP binding site (or an NF-κB binding site) within this region was mutated, promoter activity was markedly reduced. Gelshift/supershift experiments demonstrated the existence of C/EBPβ in the DNA/protein complexes, which may stimulate iNOS gene expression synergistically with NF-κB. In rat pulmonary microvascular endothelial cells, IL-1β was able to induce the activity of a transient transfected human −1034/+88-bp iNOS promoter fragment. C/EBP sites within the −205/+88-bp region of the human iNOS promoter (along with a NF-κB site at −115/−106 bp) were shown to be responsible for this induction. Overexpression of C/EBPα, C/EBPδ, and liver-enriched activator protein (LAP, an active form of C/EBPβ) activated the cotransfected human −1034/+88-bp iNOS promoter fragment, whereas overexpression of liver-enriched inhibitory protein (LIP) strongly suppressed it (Kolyada and Madias, 2001).

Finally, a mutation of an A-activator binding site (AABS) located at −192 nucleotides in the human iNOS promoter region in the context of a −7.2-kb human iNOS promoter construct inhibited basal iNOS promoter activity in primary rat hepatocytes and two human liver cell lines, but had no significant effect on basal iNOS promoter activity in three non-hepatic human cell types (Guo et al., 2003b). This mutation of AABS significantly abrogated cytokine-induced promoter activity in all cell types. By gel shifts assays, C/EBPβ was shown to bind to AABS site. Overexpression of an active form of C/EBPβ (LAP)

increased sixfold human iNOS basal promoter activity in liver (but not non-liver) cells, whereas overexpression of the transcriptional inhibitor (LIP) strongly suppressed both basal and cytokine-inducible promoter activity (Guo et al., 2003b).

These data show that C/EBP isoforms (especially C/EBPβ) may be important for human iNOS promoter activity. C/EBPβ seems to enhance human iNOS promoter activity in a liver-specific fashion.

4.5. Activating protein-1 (AP-1)

There are controversial reports about the role of the transcription factor AP-1 in the regulation of iNOS expression. Mutation of an AP-1-like site of the murine iNOS promoter at position −1125 resulted in a significant increase in LPS-induced promoter activity in J774A.1 cells (Kizaki et al., 2001). In human DLD1- and A549/8 cells, overexpression of AP-1 by cotransfection of c-Jun and c-Fos expression vectors inhibited the activity of a 7-kb human iNOS promoter fragment (Kleinert et al., 1998b). Similarly, agents like calyculin A, okadaic acid, phenylarsine oxide or anisomycin that markedly enhanced c-jun and c-fos mRNA expression and AP-1 binding activity, inhibited cytokine-induced iNOS expression in human DLD1- and A549/8 cells (Kleinert et al., 1998b, 1999). In stably transfected A549/8 cells containing a 16-kb iNOS promoter-luciferase construct, all of these compounds also reduced cytokine-induced iNOS promoter activity (Kleinert et al., 1999). In sharp contrast, Marks-Konczalik et al. (1998) described a marked inhibition of cytokine-induced activity of an 8.3-kb iNOS promoter fragment transfected into A549 cells after site-directed mutagenesis of an AP-1 binding sequence located 5301 bp upstream of the transcription start site. In supershift experiments, this group detected Jun D and Fra-2 as components of the cytokine-induced AP-1–DNA–protein complexes (Kristof et al., 2001; Marks-Konczalik et al., 1998). Extending these analyses, Kristof et al. showed that both pharmacological and molecular inhibitors of ERK and the p38 mitogen activated protein kinase (p38MAPK) pathways reduced cytokine- and LPS/IFN-γ-induced activation of an 8.3-kb human iNOS promoter fragment in A549 cells (Kristof et al., 2001; Marks-Konczalik et al., 1998). The addition of MAP/ERK kinase-1 and p38MAPK inhibitors significantly diminished AP-1 binding in both cytokine- and LPS/IFN-γ-stimulated cells. Therefore, the authors concluded that p38MAPK- and ERK-dependent pathways activate the human iNOS promoter through effects on the AP-1 complex (Kristof et al., 2001). In a recent study, Xu et al. (2003) showed that IFN-γ activation of the human iNOS promoter requires functional AP-1 regulatory region(s). After IFN-γ activation c-Fos interacts with STAT-1α and the c-Fos/STAT-1α complex binds to a GAS element in close proximity to AP-1 sites located at 4.9 kb upstream of the transcription start site in the human iNOS promoter (Xu et al., 2003). These findings support a model in which a physical interaction between c-Fos and STAT-1α participates in the activation of the human iNOS promoter.

The discrepancies between the above-mentioned findings may result from different AP-1 expression levels. The marked overexpression of AP-1 induced by calyculin A, okadaic acid, phenylarsine oxide or anisomycin may result in squelching of transcription cofactors like CREB binding protein (CBP)/p300, which may be essential for STAT-1α- or NF-κB-mediated transcription.

4.6. Other transcription factors regulating iNOS promoter activity

Several other transcription factors (like peroxisome proliferator activated receptor, PPAR; hypoxia inducible factor, HIF-1; STAT3) have been described to regulate human, rat or murine iNOS promoter activity in different cell systems (see Table 1).

5. Regulation of iNOS mRNA stability

In contrast to the murine system, there are marked differences between promoter activity and iNOS mRNA expression in human cells. Although there was no detectable iNOS mRNA in noninduced AKN hepatocytes or DLD1 cells, nuclear run-on experiments demonstrated a significant basal activity of the human iNOS promoter in these cells. This activity was only induced two- to five-fold by cytokines (de Vera et al., 1996a; Rodriguez-Pascual et al., 2000). This paralleled the moderate inducibility seen in transfection experiments with 11- or 16-kb human iNOS promoter fragments (de Vera et al., 1996a; Rodriguez-Pascual et al., 2000). In addition, inhibition of p38MAPK by [4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole] (SB203580) in A549/8 cells stably transfected with a 16-kb human iNOS promoter fragment in front of a luciferase reporter gene, resulted in a marked reduction of cytokine-induced iNOS mRNA expression, but did not change the activity of the transfected iNOS promoter fragment (Linker et al., 2004). Therefore, inhibition of p38MAPK in A549/8 cells seems to regulate specifically iNOS mRNA stability but not iNOS promoter activity.

These findings suggest that—especially in human cells—regulation of iNOS mRNA stability plays an important role in iNOS induction. Sequence analyses of the human iNOS mRNA (Geller et al., 1993) reveal four sequence motifs (AUUUA) in the 3′-untranslated region (3′-UTR) that have been shown to confer destabilization to cytokine- and oncogene mRNAs (Caput et al., 1986). The same sequence motifs are found twice in the 3′-UTR of the murine iNOS

Table 1
Additional transcription factors involved in the regulation of the activity of the iNOS promoter

Transcription factor	Promoter activity	Cell(s)/species	Mechanism	Refs.
Octamer factor (Oct)	⇑	murine and rat cells	binding and activation of iNOS promoter by Oct-family of transcription factors (Oct-1, Oct-2, Brn-3a or Brn-3b)	(Goldring et al., 1996; Kim et al., 1999; Xie, 1997)
Non-histone high mobility group protein I(Y) (HMG-I(Y))	⇑	rat vascular smooth muscle cells	binding to Oct site in iNOS promoter	(Pellacani et al., 2001)
Peroxisome proliferator-activated receptors (PPAR)	⇓	human astrocytes and chondrocytes, rat neonatal myocytes, murine RAW macrophages	reduction of iNOS promoter activity by inhibition of NF-κb and/or AP-1 activity; inhibition may take place by competition for CBP/p300	(Boyault et al., 2001; Fahmi et al., 2001; Li et al., 2000)
STAT3	⇓	murine mesangial cells	inhibition of murine iNOS promoter by inhibition of NF-κB transactivation activity	(Yu et al., 2002)
Tumor suppressor p53	⇓	human epithelial cells, $p53^{-/-}$ mice	transcriptional trans-repression of the iNOS promoter	(Forrester et al., 1996; Ambs et al., 1998)
Hypoxia-induced factor-1 (HIF-1)	⇑	murine macrophages, rat cardiomyocytes, bovine pulmonary endothelial cells	activation of the promoter activity by binding to binding sites in the murine iNOS promoter	(Jung et al., 2000; Melillo et al., 1995)
Retinoic acid receptor-α (RAR-α,), retinoic X receptor (RXR)	⇓	rat vascular smooth muscle cells	reduction of murine iNOS promoter activity; may also take place by RXR-PPAR-γ heterodimers	(Sirsjo et al., 2000; Uchimura et al., 2001)
Estrogen receptor-β (ER-β)	⇑	African green monkey COS-7 cells, neonatal rat cardiomyocytes and peritoneal macrophages	activation of iNOS promoter	(Nuedling et al., 2001; You et al., 2003)
Androstane receptor (CAR)	⇓	human epithelial DLD1 cells	DR4-mediated inhibition of human iNOS promoter activity	(Toell et al., 2002)
Pregnane X receptor (PXR)	⇑	human DLD1 cells	DR4-mediated activation of human iNOS promoter activity	(Toell et al., 2002)
Epithelium-specific Ets transcription factor (ESE-1)	⇑	murine RAW 264.7 macrophages	synergistic activation of the murine iNOS promoter by interaction of ESE-1 and NF-κB p65/p50	(Rudders et al., 2001)
Ets-like transcription factor Elk-3	⇓	murine RAW 264.7 macrophages	inhibition of murine iNOS promoter activity	(Chen et al., 2003)
Antioxidant-responsive element (ARE) binding protein	⇑	rat mesangial cells, primary hepatocytes	activation of the rat iNOS promoter	(Beck et al., 1998; Kuo et al., 2000)
Homology to Sma and MAD proteins (Smad)	⇓	murine RAW 264.7 macrophages	capture of transcriptional coactivator p300 inhibits murine iNOS promoter	(Werner et al., 2000)
Nuclear factor-IL6 (NF-IL6)	⇑	murine NIH-3T3 fibroblasts, J774.A1 macrophages	binding to the murine iNOS promoter	(Dlaska and Weiss, 1999)
Kruppel-like factor 6 (KLF6)	⇑	human Jurkat cells, African green monkey COS-7 cells	binding to CACCC binding sites activates human iNOS promoter	(Warke et al., 2003)
Hepatocyte nuclear factor-4α (HNF-4α)	⇑	rat hepatocytes	binding to and activation of the rat iNOS promoter	(Guo et al., 2002, 2003a)
Transcription cofactor/histone acetylase (HAT) p300	⇑	murine RAW264.7 macrophages	acetylation of the p50 subunit of NF-κB and increase in NF-κB-mediated transactivation	(Deng and Wu, 2003)
Wilms' tumor suppressor gene 1 (Wt-1)	⇑	rat pancreatic RIN cells	binding to and activation of the rat iNOS promoter	(Johannesen et al., 2003)

⇑=upregulation, ⇓=downregulation.

mRNA (Lyons et al., 1992), and four times in the rat iNOS mRNA (Galea et al., 1994).

In murine peritoneal macrophages, TGF-β1 diminished IFN-γ-induced iNOS expression due to a reduction of iNOS mRNA stability, an inhibition of iNOS mRNA translation and a decrease in iNOS protein stability (Vodovotz et al., 1993). In murine J774 macrophages, inhibition of the Jun N-terminal kinase (JNK) by anthra[1,9-*cd*]pyrazol-6-(2*H*)-one (SP600125) reduced LPS-induced iNOS expression by destabilization of the mRNA (Lahti et al., 2003). In rat VSMCs and human mesangial cells, BH_4 has been shown to modulate iNOS mRNA stability (Linscheid et al., 1998; Saura et al., 1996). Finally, Soderberg et al. (2002) described specific binding of the heterogeneous nuclear ribonucleoproteins (hnRNP) L and hnRNP I (PTB) to the 3′-UTR of the murine iNOS mRNA. The complex between hnRNP L/I and the murine iNOS mRNA was detected in liver extracts of mice treated with LPS and D-galactosamine. These authors suggested an involvement of hnRNP L and I in the post-transcriptional regulation of murine iNOS expression.

In transfection experiments using human A549 or DLD1 cells, the 3′-UTR of the human iNOS mRNA destabilized the mRNA of a luciferase reporter gene. A high affinity interaction of the embryonic lethal abnormal vision (ELAV) protein HuR with the human iNOS 3′-UTR could be demonstrated. HuR is known to increase the stability of several inducible mRNAs (Brennan and Steitz, 2001). HuR binds to the two distal AUUUA-elements of the 3′-UTR of the human iNOS mRNA (Rodriguez-Pascual et al., 2000). Stable overexpression of HuR in human DLD1 cells resulted in an upregulation of cytokine-induced iNOS expression. Consequently, a downregulation of HuR reduced cytokine-induced iNOS expression in DLD1 cells (Rodriguez-Pascual et al., 2000).

Whereas HuR has been described as an important stabilizer of AUUUA-containing mRNAs, the hnRNP D family of proteins (also named AU binding factor 1; AUF1) is believed to destabilize such mRNAs (Misquitta et al., 2001; Mitchell and Tollervey, 2000). p37AUF1 showed high affinity complex formation with the human iNOS 3′-UTR, binding to different AUUUA elements than HuR. Accordingly, stable overexpression of p37AUF1 in DLD1 cells downregulated human iNOS expression (Mangasser-Stephan et al., 2002).

In addition to HuR and AUF1, other RNA binding proteins seem to be involved in the regulation of iNOS mRNA stability. Tristetraprolin (TTP), the T-cell-restricted intracellular antigen-1-like protein (TIAR) and hnRNP A1 are known to regulate mRNA-stability or translation of different cytokine-induced genes like TNF-α or granulocyte-macrophage colony-stimulating factor (GM-CSF) (Carballo et al., 2000; Gueydan et al., 1999; Hamilton et al., 1997; Lai et al., 1999). Overexpression of these proteins in human DLD1 cells resulted in an enhanced cytokine-mediated iNOS mRNA expression and iNOS-mediated NO production (Fechir et al., 2003). Also, the RNA binding proteins KH-type splicing regulatory protein (KSRP), hnRNP E1 and the poly A tract binding protein (PABP) were found to interact with the 3′-UTR sequence of the human iNOS mRNA. Overexpression of KSRP in human DLD-1 cells markedly reduced human iNOS expression (Linker et al., 2004).

Thus, a complex array of different RNA binding proteins seems to be involved in the regulation of human iNOS mRNA stability.

6. Regulation of iNOS mRNA translation and protein stability

Human primary cardiomyocytes in cell culture express iNOS mRNA, but no iNOS protein. The inability of the cardiomyocytes to translate iNOS mRNA was correlated with the presence of the 5′- or 3′-UTR of the human iNOS mRNA (Luss et al., 1997). The inhibition of iNOS expression by different agents, such as TGF-β1 in primary murine macrophages (Vodovotz et al., 1993) or dexamethasone in rat mesangial cells (Kunz et al., 1996), has been described to result from iNOS mRNA and protein destabilization.

In human kidney HEK293 cells overexpressing iNOS, the proteasome pathway has been shown to contribute to the degradation of iNOS protein (Musial and Eissa, 2001). Also, in human epithelial RT4 cells or murine RAW 264.7 macrophages, the proteasome inhibitor lactacystin added 48 h after iNOS induction enhanced iNOS protein levels (Musial and Eissa, 2001).

Finally, in human intestinal carcinoma HT29 and DLD1 cells, overexpression of caveolin-1 (Cav-1) decreased cytokine-induced iNOS protein expression, whereas induction of iNOS mRNA expression was not changed. In the presence of proteasome inhibitors, the amount of iNOS protein was enhanced and a direct Cav-1-iNOS interaction has been shown. These data suggest that the interaction of Cav-1 with iNOS enhanced the proteosomal degradation of the enzyme (Felley-Bosco et al., 2000).

7. Alternative iNOS mRNA species

The expression of a small amount (~6%) of 5′-truncated iNOS mRNA was shown in cytokine-induced human macrophages and epithelial cells (Chu et al., 1995). These minor transcripts seem to result from transcription reactions initiated at different minor start sites. In addition, alternative splicing of the iNOS mRNA has been described resulting in different iNOS mRNA isoforms (Eissa et al., 1998). One of these mRNAs codes for an iNOS protein lacking exons 8

and 9, which are important for iNOS dimerization (Eissa et al., 1998).

Recently, new iNOS mRNA splicing variants have been described in normal B-lymphocytes and B-CLL cells in pro- and anti-apoptotic conditions (Tiscornia et al., 2004). A variant with a complete deletion of exon 14 (iNOS 13–16(14del)) was detected preferentially in normal B-lymphocytes. A second variant containing partial deletion of exons 13 to 16 (the flavodoxin region—iNOS 13–16(neg)) was correlated with a decreased B-CLL cell viability (Tiscornia et al., 2004).

However, at this time, it is unclear whether these iNOS mRNA variants are translated and whether any specific function can be attributed to them.

8. Conclusions

The inducible isoform of NOS is mainly regulated on the level of expression, with transcriptional, post-transcriptional and translational mechanisms involved. The stimuli and conditions that determine iNOS expression are cell- and species-specific. Thus, nature has invented a large array of regulatory mechanisms controlling the expression of this enzyme. Once expressed, iNOS does not seem to be subject to any major regulation of its enzymatic activity.

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