COMMENTARY

Role of HNF4α in the superinduction of the IL-1β-activated iNOS gene by oxidative stress

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IL-1β (interleukin-1β) treatment of hepatocytes results in an NF-κB (nuclear factor-κB)-mediated activation of the iNOS (induced nitric oxide synthase) gene, and this increase in gene expression is further augmented by oxidative stress. Oxidative stress alone has no influence on the iNOS promoter, therefore indicating that the promoter needs to be primed by NF-κB. In this issue of the *Biochemical Journal*, Guo et al. extend their earlier work, showing that HNF4α (hepatocyte nuclear factor 4α) mediates the superinduction of iNOS observed by co-treating cells with IL-1β plus H_2O_2. A specific phosphorylation by p38 kinase at Ser-158 of HNF4α results in increased binding of HNF4α to the iNOS promoter, leading to enhanced transcription. The study by Guo et al. is the first to show definitively that HNF4α can be modulated to differentially activate specific genes. However, issues remain to determine the functional significance *in vivo* of the elevated iNOS activity, and the mechanism that governs the specificity of HNF4α towards the iNOS promoter element as compared with many other HNF4α target genes in the hepatocyte.

Key words: direct repeat-1 element (DR1), hepatocyte, hepatocyte nuclear factor 4α (HNF4α), inducible nitric oxide synthase (iNOS), interleukin-1β (IL-1β), oxidative stress.

INTRODUCTION

iNOS (the inducible nitric oxide synthase) catalyses the oxidation of L-arginine to L-citrulline and nitric oxide (NO), the latter of which has a number of beneficial effects that promote cell survival. A variety of stress stimuli induce iNOS expression, and the iNOS gene is under complex regulation: at least 19 transcription factors affect expression at the transcriptional level [1]. The cell type and particular stimulus determines which mechanisms of iNOS gene activation will predominate. In most cell types, NF-κB (nuclear factor κB) and STAT-1α (signal transducer and activator of transcription-1α) seem to have major roles in activating the iNOS gene. IL-1β (interleukin 1β), TNFα (tumour necrosis factor-α) and other pro-inflammatory cytokines, and bacterial LPS (lipopolysaccharide) induce iNOS expression through NF-κB. INF-γ (interferon-γ) also induces the iNOS gene through STAT-1α. In the liver, there is a robust induction of iNOS by treatment with LPS and INF-γ, which produce a biological response that mimics acute sepsis in humans. Induction of iNOS can be achieved in primary hepatocyte cultures by treatment with the pro-inflammatory cytokine IL-1β. A further 5-fold induction is found when cells are treated with IL-1β along with BZT (1,2,3-benzenetriol), a source of superoxide [2]. This is in keeping with a possible function of NO in protection against oxidative stress. The superinduction of iNOS by IL-1β plus BZT led to the search for the mechanism of iNOS gene activation by oxidative stress, and an upstream binding site for the transcription factor HNF4α (NR2A1) was uncovered [3]. The role of HNF4α in regulation of the iNOS gene was firmly established by studies with liver-specific HNF4α-null mice; although the gene was fully induced by IL-1β, there was no further induction by BZT in hepatocytes lacking HNF4α [4].

HNF4α

HNF4α is a member of the nuclear receptor superfamily. It is unique among this class of transcription factors in that it is not ligand-activated by the classical nuclear receptor pathway, where ligand binding alters the conformation of the receptor leading to increased interaction with transcriptional co-activators and DNA. The domain of the protein that would be expected to bind ligand, on the basis of homology comparisons, has embedded lipids that are thought to enter the protein during translation, since they cannot easily be exchanged with lipids exogenously added to the folded HNF4α. Thus HNF4α is considered a constitutively active nuclear receptor. HNF4α activates gene expression though binding to a DR1 (direct repeat-1) element, a 6 bp direct repeat separated by a single nucleotide that is usually located upstream of target genes. DR1 sites are also recognized by other nuclear receptors, such as PPARs (peroxisome proliferator-activated receptors) and RARs (retinoic acid receptors). Furthermore, consensus sites within the DR1 elements determine the specificity of binding to different nuclear receptors. HNF4α is essential for liver development; in the absence of this factor in early embryos, the liver does not develop due to a disruption in extra-embryonic ectoderm formation, as revealed by gene knockout studies [5]. In addition to the liver, HNF4α is expressed in the gut, pancreas and kidney. A human genetic deficiency in HNF4α expression in pancreatic β-cells causes the disease MODY-1 (maturity onset diabetes of the young-1). In adult liver, HNF4α regulates transcription of a large number of genes involved in liver-specific functions, such as bile acid and apolipoprotein synthesis, coagulation, urea synthesis, drug metabolism and gluconeogenesis [6–11]. The absence of HNF4α expression in adult mice results in increased mortality [6], thus indicating that HNF4α controls constitutive expression of genes that are essential for liver function. While the possibility cannot be excluded that HNF4α is activated by fatty acids or their metabolites, it is more likely that it is not readily activated by exogenous or endogenous ligands by a mechanism more typical of the nuclear receptor superfamily. However, HNF4α does participate in differential gene activation during certain metabolic conditions, such as starvation by a mechanism that is mediated by differential expression of the co-activator PGC1α (PPARγ

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co-activator 1α), which stimulates enhanced transcription of a number of genes involved in fatty acid β-oxidation, ketogenesis and gluconeogenesis through enhancing the activity of several transcription factors, including HNF4α [12]. For example, the gene encoding PEPCK (phosphoenolpyruvate carboxykinase), involved in gluconeogenesis, is under complex regulation by a number of transcription factors in the liver. Under normal feeding conditions, HNF4α has an insignificant role in PEPCK expression; however, during fasting, PGC1α is induced, resulting in co-activation of HNF4α on the PEPCK gene promoter [13,14].

HNF4α phosphorylation

Another means by which HNF4α can be regulated is through differential phosphorylation. Serine/threonine and tyrosine phosphorylation of HNF4α have been demonstrated in cultured cell systems, and this has been shown to affect DNA binding and transactivation, as well as protein stability [15–18]. These phosphorylations could be catalysed by a number of protein kinases involved in various signal transduction pathways, including extracellular-signal-regulated kinases-1/2, c-Jun N-terminal kinase, MAPK (mitogen-activated protein kinase), PKA and PKC (protein kinases A and C), cyclin-dependent kinase and JAK2 (Janus kinase 2). Indeed, modification of steroid and nuclear receptors by phosphorylation has been well documented [19]. The possibility that the activity of this constitutive factor is altered by phosphorylation is intriguing. However, it should be noted that studies linking HNF4α modification to gene activation have, in most cases, been limited to cultured cell systems, where the factor can be modified and overexpressed, or where the cells can be manipulated by treatment with various inhibitors. It has never been established with any certainty whether regulated phosphorylation of HNF4α occurs in the intact liver and alters the activity or stability of the protein.

In this issue of the *Biochemical Journal*, Guo et al. [20] present data showing that phosphorylation of the residue Ser-158 of HNF4α by p38 kinase enhances binding of HNF4α to the iNOS gene promoter, and increases its capacity to transactivate reporter gene constructs containing the iNOS HNF4α-binding site. Direct evidence for HNF4α phosphorylation was provided using His_6-tagged recombinant protein introduced into HepG2 cells, followed by direct sequencing of isolated HNF4α protein. Constitutive phosphorylation sites (Ser-133/Ser-134) were found in the untreated, IL-1β-treated or H_2O_2-treated cells, and four additional sites were found in the presence of IL-1β plus H_2O_2. ChIP (chromatin immunoprecipitation) and EMSA (electrophoretic mobility shift assay) revealed that, in IL-1β plus H_2O_2-treated cells, there was an increased binding of HNF4α to the iNOS promoter site; binding was decreased by the p38 kinase inhibitor staurosporine. Mutation of these serine and threonine target residues to alanine, which cannot be phosphorylated, revealed that only S158A blocked the increase in HNF4α binding to DNA after IL-1β plus H_2O_2 treatment of cells. In addition, a phosphomimetic of Ser-158 (changed to an aspartate) revealed that this modified form of HNFα had enhanced DNA binding in the presence of IL-1β. Furthermore, p38 kinase phosphorylated HNF4α Ser-158 *in vitro*. The role of Ser-158 phosphorylation in iNOS gene activation revealed (by reporter gene experiments using the iNOS promoter and various mutant HNF4α constructs) that Ser-158 is important for the superinduction of the promoter in the presence of IL-1β plus H_2O_2. Taken together, these data indicate that HNF4α is specifically phosphorylated at Ser-158 by p38, a kinase that is activated by oxidative stress, and that this modification augments DNA binding and activation of the iNOS promoter. This study is especially intriguing, because it shows, for the first time, that HNF4α can be differentially activated by post-translational modification to bind to and augment transcription of a specific promoter. However, this work leads to a number of additional questions that remain to be addressed.

Is the superinduction of iNOS by oxidative stress of functional significance? This could be addressed by abolishing the HNF4α-mediated activation by a knock-in of the endogenous iNOS promoter in which the HNF4α binding site has been destroyed. These mice could then be tested for susceptibility to experimentally induced sepsis or other treatments involving elevated oxidative stress. Studies could also be carried out on whole animal models in which the S158A variant was knocked-in in order to determine whether the superinduction of iNOS occurs *in vivo*, and whether the block of this phosphorylation site affects other HNF4α target genes. Additional studies are required to determine the mechanism(s) by which HNF4α causes superinduction of the iNOS promoter. In the absence of stimulation by pro-inflammatory cytokines, iNOS is not induced by the oxidative stress stimulus alone, indicating that HNF4α is not sufficient to activate transcription of the iNOS promoter. Of interest, HNF4α positively regulates a large number of genes. Why doesn't it activate transcription of the iNOS gene through the DR1 binding site? Perhaps in the context of the iNOS promoter, the NF-κB activation is required to open or remodel the chromatin and allow access of HNF4α to the DR1 site. In the context of other HNF4α-binding-site-containing promoters, the question of specificity arises. Are there other genes besides iNOS that are activated by IL-1β plus H_2O_2 through HNF4α? Most interestingly, are the typical housekeeping HNF4α target genes that are involved in the control of many aspects of liver function unrelated to protection against stress, affected by phosphorylated HNF4α? Why would the modified factor only interact with certain DR1s and not others? Perhaps it is due to the context of the DR1 relative to other important elements within specific promoters. This is an issue that could readily be addressed. In this connection, Guo et al. [20] found that the transcriptional co-activator PC4 is essential for up-regulation of the iNOS promoter by HNF4α in IL-1β plus H_2O_2-treated cells, and that Ser-158 phosphorylation was probably required for this association. This is reminiscent of the PGC1α augmentation of the PEPCK promoter in starved hepatocytes, as discussed above. Perhaps HNF4α phosphorylation by other kinases augments PGC1α interaction during glucose deprivation of hepatocytes. This also leads to the possibility that other signal transduction pathways may feed into HNF4α through phosphorylation events, thus opening up new areas for investigation of the regulation of liver gene expression that may impact on a large number of metabolic disorders or responses to various pathological conditions or stimuli.

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Received 3 January 2006; accepted 5 January 2006
Published on the Internet 10 February 2006, doi:10.1042/BJ20060005