Phosphorylation of Ser¹⁵⁸ regulates inflammatory redox-dependent hepatocyte nuclear factor-4 α transcriptional activity

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In IL-1 β (interleukin 1 β)-stimulated rat hepatocytes exposed to superoxide, we have previously identified an IRX (inflammatory redox)-sensitive DR1 [direct repeat of RG(G/T)TCA with one base spacing] *cis*-acting activator element (nt –1327 to –1315) in the iNOS (inducible nitric oxide synthase) promoter: AGGTCAGGGGACA. The corresponding transcription factor was identified to be HNF4 α (hepatocyte nuclear factor-4 α). HNF4 α DNA binding activity and transactivation potential are tightly regulated by its state of phosphorylation. However, the functional consequences of IRX-mediated post-translational phosphorylation of HNF4 α have not been well characterized. In the setting of IL-1 β + H₂O₂, HNF4 α functional activity is associated with a unique serine/threonine phosphorylation pattern. This indicates that an IRX-sensitive serine/threonine kinase pathway targets HNF4 α to augment hepatocyte iNOS transcription. In the present study, following identification of phosphorylated residues in HNF4 α , serial mutations were performed to render the

target residues phosphorylation-resistant. Electrophoretic mobility-shift assays and transient transfection studies utilizing the iNOS promoter showed that the S158A mutation ablates IRX-mediated HNF4 α DNA binding and transactivation. Gain-of-function mutation with the S158D phosphomimetic HNF4 α vector supports a critical role for Ser¹⁵⁸ phosphorylation. *In vitro* phosphorylation and kinase inhibitor studies implicate p38 kinase activity. Our results indicate that p38 kinase-mediated Ser¹⁵⁸ phosphorylation is essential for augmentation of the DNA binding and transactivation potential of HNF4 α in the presence of IL-1 β + H₂O₂. This pathway results in enhanced iNOS expression in hepatocytes exposed to pro-inflammatory cytokines and oxidative stress.

Key words: hepatocyte nuclear factor-4 α , inducible nitric oxide synthase (iNOS), inflammatory redox, oxidative stress, phosphorylation, p38 mitogen-activated protein kinase (MAPK).

INTRODUCTION

In the presence of oxidative stress, the hepatocellular IRX (inflammatory redox) state up-regulates iNOS (inducible nitric oxide synthase) expression as an antioxidant function. In IL-1 β (interleukin 1 β)-treated rat hepatocytes, we have demonstrated that iNOS gene transcription and promoter activity are increased by oxidant stress mediated by peroxide, superoxide or acetaminophen [1–3]. Subsequently, in IL-1 β -stimulated rat hepatocytes exposed to superoxide, we identified an IRX-sensitive DR1 [direct repeat of RG(G/T)TCA with one base spacing] *cis*-acting activator element (nt –1327 to –1315) in the iNOS promoter: AGGTCAGGGGACA. The term IRX applies to the combination of pro-inflammatory cytokines and oxidant stress that serve as a biochemical correlate to conditions such as hepatocyte ischaemia–reperfusion injury. The corresponding transcription factor was isolated by DNA affinity chromatography, sequenced and identified to be HNF4 α (hepatocyte nuclear factor-4 α) [1,2].

HNF4 α is a member of the nuclear receptor superfamily of transcription factors and was originally identified in the regulation of liver-specific genes [4]. Subsequently, more than 55 distinct target genes involved in lipid, amino acid and glucose metabolism, liver differentiation, cell structure, and immune function have been identified for HNF4 α . HNF4 α is highly conserved; amino acid identity between rat and human varies from 89.7 to 100% among the various functional domains of HNF4 α . It binds DNA exclusively as a homodimer, is localized primarily in the

nucleus, and binds DR1 response elements with the consensus sequence: AGGTCAGGGG(T/A)CA. It also binds several different co-activators in the absence of exogenously added ligand [4–7]. HNF4 α DNA binding activity and transactivation potential are tightly regulated by its state of phosphorylation.

IRX-mediated post-translational phosphorylation of HNF4 α has not been well characterized. We have previously demonstrated that HNF4 α increases IRX-associated hepatocyte iNOS expression at the level of protein, mRNA and promoter activation [8]. In this context, HNF4 α functional activity is associated with a unique serine/threonine phosphorylation pattern that is NO (nitric oxide)-independent. These results indicate that an IRX-sensitive serine/threonine kinase pathway targets HNF4 α to augment hepatocyte iNOS transcription. In the present study, we extend our previous findings and demonstrate that HNF4 α Ser¹⁵⁸ is specifically phosphorylated in the presence of oxidative stress and IL-1 β to up-regulate HNF4 α DNA binding and transactivation potential.

MATERIALS AND METHODS

Expression vector construction and site-directed mutagenesis

Total RNA and mRNA were isolated from human HepG2 cells (A.T.C.C., Manassas, VA, U.S.A.) using the Qiagen mRNA purification kit. cDNA was synthesized using the purified mRNA and oligo(dT) as a primer using Thermoscript RT (reverse transcriptase)–PCR system (Invitrogen, Carlsbad, CA, U.S.A.). The

Abbreviations used: ARE, antioxidant response element; CAT, chloramphenicol acetyltransferase; ChIP, chromatin immunoprecipitation; DBD, DNA-binding domain; DR1, direct repeat of RG(G/T)TCA with one base spacing; EMSA, electrophoretic mobility-shift assay; ERK1/2, extracellular-signal-regulated kinase 1/2; HNF4 α , hepatocyte nuclear factor-4 α ; IL-1 β , interleukin 1 β ; iNOS, inducible nitric oxide synthase; IRX, inflammatory redox; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEM, minimal essential medium; MS/MS, tandem MS; PBST, PBS/0.025% Tween 20; PC4, positive co-activator 4; PKA, protein kinase A; PKC, protein kinase C; SAPK, stress-activated protein kinase; SV40, simian virus 40; WT, wild-type.

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product was used to amplify full-length HNF4 α cDNA. The amplified HNF4 α cDNA PCR product was inserted into the AT sites (adenine/thymine-rich sites) of pGEM-T vector (Promega, Madison, WI, U.S.A.); positive clones were screened by DNA Miniprep kit (Qiagen, Chatsworth, CA, U.S.A.) and fully analysed by automated DNA sequencing. The vector was then cut by the restriction enzyme EcoRI and run in 1 % agarose gel. The HNF4 α cDNA fragment was recovered and inserted into the EcoRI restriction enzyme site of pCMV-FLAG expression vector (Sigma, St. Louis, MO, U.S.A.); the resultant vector, WT-HNF4 α (where WT is wild-type), allows production of a protein with a FLAG peptide (MDTKDDDDKH) fused at its N-terminus. S5Y134CAA-HNF4 α , S158A-HNF4 α , S181A-HNF4 α , S304A-HNF4 α , T450A-HNF4 α and S158D-HNF4 α designate mutant FLAG-HNF4 α expression vectors, in which the designated amino acids were mutated to an alanine or aspartic residue. All mutant vectors were generated by using QuikChange II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) and primers as described below.

Ser¹³³-Ser¹³⁴-Tyr¹³⁵ to Cys-Ala-Ala:

S134A sense: 5'-aaggtgtgcagcagaggacagcagcctgccctcc-3'

S134A antisense: 5'-cctctgtcgcacaccttcgagtgctgatccggctc-3'

Ser¹⁵⁸ to Ala:

S158A sense: 5'-gatcgagagcccgtctccggatcaacggc-3'

S158A antisense: 5'-cgggctctgcgatctgtcgggacaggacct-3'

Ser¹⁸¹ to Ala:

S181A sense: 5'-gtgtgaggcaatgaaggagcagctgctggtt-3'

S181A antisense: 5'-ttcattgctcacacatctgcgatgct-3'

Ser³⁰⁴ to Ala:

S304A sense: 5'-ctcgctgcacaggtgcaggtgagcttggag-3'

S304A antisense: 5'-acctgtgcacgcagccgcttgatcttccc-3'

Thr⁴⁵⁰ to Ala:

T450A sense: 5'-accatcgcaaagcaggaagtattctagcaa-3'

T450A antisense: 5'-tgctttgcgatggtcggctgggggatggc-3'

Ser¹⁵⁸ to Asp:

S158D sense: 5'-gatcaccgaccccgctctccgggatcaacggc-3'

S158D antisense: 5'-cggggtcgggtgatctgtcgggacaggacct-3'

Cell culture

Human hepatoblastoma HepG2 and monkey kidney COS-1 cells were purchased from the A.T.C.C. and grown in MEM (minimal essential medium) or Dulbecco's modified Eagle's medium (as appropriate), supplemented with 10 % (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin at 37 °C under 5 % CO₂. Cells (1 $\times$ 10⁶) were transfected with 10 μ g of WT-HNF4 α expression vector, and/or mutant HNF4 α vectors using Lipofectin[®] (Invitrogen) according to the manufacturer's recommendation. After 24 h, the cells were treated with IL-1 β (1000 units/ml), H₂O₂ (50 μ M) or IL-1 β + H₂O₂ for 8 h. In selected experiments, cells were also treated with protein kinase inhibitors prior to stimulation with IL-1 β , H₂O₂, or IL-1 β + H₂O₂. The cells were then washed twice with ice-cold PBS and harvested for further assays.

Purification of recombinant complexes

Cells were transfected with the human FLAG-HNF4 α expression vector, WT-HNF4 α or mutants, as described above. At 24 h post-transfection, cells were treated as detailed above. Cells were harvested, and the cell pellet was suspended in a buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA and 1 % Triton X-100) and kept on ice for 30 min. It was then homogenized and centrifuged at 12 000 *g* for 10 min. The supernatant was incubated with anti-FLAG M2-agarose affinity resin for 2 h to allow WT-

HNF4 α protein to bind to the resin. The resin was washed three times with 20 vol. of ice-cold buffer (50 mM Tris/HCl, pH 7.4, and 150 mM NaCl), and fusion protein was eluted with a buffer containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl and 0.15 μ g of 3 $\times$ FLAG peptide. Purified protein was further concentrated by ultrafiltration in an Amicon concentrator (PM-30 membrane) and then dialysed against 50 mM Tris (pH 7.8) containing 10 % (v/v) glycerol. Aliquots of the purified protein were maintained at -70 °C until further use.

Transient transfection analysis of iNOS promoter activity

Cells were transfected using the Lipofectamine[™] technique [9]. After cells were washed twice with MEM, 10 μ g of plasmid DNA containing the iNOS promoter construct (1845 bp; GenBank[®] accession no. X95629) coupled with a CAT (chloramphenicol acetyltransferase) reporter gene were added per 10⁷ cells in 1 ml of medium without serum. In selected instances, an empty vector or an HNF4 α expression vector (10 μ g) encoding FLAG-tagged WT or mutant variants was co-transfected with the iNOS promoter plasmid construct. Following cell treatment, the supernatant was assayed for CAT activity using a CAT ELISA technique (Roche, Indianapolis, IN, U.S.A.). Transfection efficiency was normalized by co-transfection of a β -galactosidase reporter gene with a constitutively active early SV40 (simian virus 40) promoter. All values are expressed as pg of CAT/mg of protein.

Western blot of recombinant human HNF4 α

Protein (30 μ g) was resolved on SDS/4–20 % polyacrylamide gels and transferred on to PVDF membranes. Membrane was blocked in 5 % (w/v) dried milk in PBST (PBS/0.025 % Tween 20). Anti-FLAG M2 antibody (Sigma) and anti-HNF4 α antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) were diluted 1:800 in 5 % dried milk in PBST and incubated with the membrane for 60 min at room temperature (24 °C). Two 10 min washes with PBST followed antibody incubation. Horseradish peroxidase-conjugated donkey anti-goat or goat anti-mouse IgG (Santa Cruz Biotechnology) was diluted 1:2000 in 5 % dried milk in PBST. Reactivity was detected using chemiluminescent reagents (Pierce, Rockford, IL, U.S.A.).

Phosphopeptide mapping of HNF4 α

WT-HNF4 α was isolated from cell lysates (as above) and separated by PAGE. The resolving gel was stained; the WT-HNF4 α band was excised and stored at -70 °C until use. The gel slices were subjected to in-gel tryptic and chymotryptic digestions after reduction and carboxyamidomethylation. The resultant digests were pooled just prior to liquid chromatography MS/MS (tandem MS) injection. Phosphorylated peptide sequences were determined using a 75 μ m reverse-phase microcolumn (New Objective, Woburn, MA, U.S.A.) terminating in a custom nanoelectrospray source directly coupled with an LCQ DECA XP Plus quadrupole ion-trap mass spectrometer (Thermo Electron, San Jose, CA, U.S.A.). Flow was nominally 200 nl/min. The ion trap repetitively surveyed the range *m/z* 395–1600, executing data-dependent MS/MS for peptide sequence information on the four most abundant ions in each survey scan. MS/MS spectra were acquired with a relative collision energy of 30 % and an isolation width of 2.5 Da, and recurring ions were dynamically excluded. After database correlation with the algorithm SEQUEST, phosphorylated peptides were confirmed by manual *de novo* interpretation of the MS/MS spectra using the FuzzyIons program [10,11]. When needed, a second targeted ion MS/MS experiment was conducted to increase detection sensitivity and spectrum

quality. In this run, the predicted precursor mass-to-charge ratio (m/z) of the phosphopeptide was subjected to MS/MS for the entire chromatographic run, capturing the peptide of interest at the moment of its chromatographic elution.

EMSAs (electrophoretic mobility-shift assays)

EMSAs were performed as previously described [12]. Sense and antisense single-stranded DNAs were annealed, and labelled probe was obtained using T4 polynucleotide kinase with [γ - 32 P]ATP. A 10 μ g sample of purified FLAG-tagged WT or mutant HNF4 α , as indicated, 0.035 pmol of labelled probe, 4 % glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris/HCl (pH 7.5) and 0.5 μ g of poly(dI-dC)·(dI-dC) were mixed in a total volume of 10 μ l. Unlabelled target oligonucleotide was added at 200 M excess in specific or non-specific competition assays. For supershift experiments, anti-FLAG antibody (Sigma) was included in the binding reaction. The mixtures were incubated for 10 min at room temperature and then analysed by electrophoresis on a 4 % polyacrylamide gel by using 0.5 $\times$ TBE (1 $\times$ TBE = 45 mM Tris/borate/1 mM EDTA) as running buffer. The gels were subsequently dried and autoradiographed. The following oligonucleotides were used: sense, 5'-ctcagctgtacttgggtacaacta-3', and antisense, 5'-tagttgtaccaaagtacaagctgag-3'.

In vitro phosphorylation assay

In vitro phosphorylation was carried out by incubating 0.5 μ g of purified FLAG-tagged HNF4 α protein with 1 μ l of protein kinase: p38 (0.3 μ M), JNK (c-Jun N-terminal kinase; 0.25 μ M), or ERK1/2 (extracellular-signal-regulated kinase 1/2; 0.3 μ M; Calbiochem, San Diego, CA, U.S.A.) in kinase buffer (25 mM Hepes, pH 7.5, 10 mM MgCl₂ and 50 μ M ATP) containing 5 μ Ci of [γ - 32 P]ATP for 30 min at 37 °C. The reaction was quenched by adding 1 vol. of 2 $\times$ SDS/PAGE sample buffer [125 mM Tris/HCl, pH 6.8, 4 % (v/v) SDS, 10 % (w/v) 2-mercaptoethanol and 20 % glycerol]. The reaction products were immunoprecipitated with anti-FLAG M2-agarose affinity gel (Sigma). The gel was washed four times with wash buffer (50 mM Tris/HCl, pH 7.4, and 150 mM NaCl), followed by separation on SDS/4–20 % polyacrylamide gels; the gel was then dried and analysed by autoradiography.

ChIP (chromatin immunoprecipitation) assay

HepG2 cells were grown in 10 cm dishes, and DNA–protein cross-linked by addition of formaldehyde at room temperature for 10 min. Assays were performed using the ChIP assay kit (Upstate Biotechnology, Waltham, MA, U.S.A.) following the manufacturer's instructions. Goat anti-human polyclonal HNF4 α antibody (5 μ g; Santa Cruz Biotechnology) was used for each immunoprecipitation. The DNA was recovered and subjected to analysis by PCR. The primers had the following sequence: 5'-tgaccattgactgtgtgtg-3' (sense strand) and 5'-gctggcgtgggagatggctga-3' (antisense strand) to yield a PCR product of 280 bp. The PCR program was 94 °C for 4 min, followed by 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s for a total of 28 cycles, and then 72 °C for 7 min. The amplified DNA was visualized by electrophoresis on 1 % agarose gel in 1 $\times$ TAE (Tris/acetate/EDTA) buffer after staining with ethidium bromide.

Co-immunoprecipitation of HNF4 α and PC4 (positive co-activator 4)

HepG2 cells were transfected with WT-HNF4 α or S158A-HNF4 α by Lipofectin® (Invitrogen), incubated at 37 °C for 36 h and then

Table 1 HNF4 α serine/threonine phosphorylation sites

Phosphopeptide analysis of putative serine and threonine phosphorylation sites in HNF4 α under various treatment conditions. The bold underlined amino acids represent identified phosphorylation targets.

	Peptide	Ser/Thr position
Controls	RSSY EDSSLPSIN	133/134
IL-1 β	RSSY EDSSLPSIN	133/134
H ₂ O ₂	RSSY EDSSLPSIN	133/134
IL-1 β + H ₂ O ₂	RSSY EDSSLPSIN	133/134
	QITSPVSGINGDIR	158
	IASIADVCE SMK	181
	SQVQV SLDYINDR	304
	IVKPLSAIPQPT TK	450

treated with IL-1 β + H₂O₂ for 8 h. The cells were rinsed twice with PBS and a lysis buffer containing protease and phosphatase inhibitors was added [50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.8 μ M aprotinin, 50 μ M bestatin, 20 μ M leupeptin, 10 μ M pepstatin A, 25 μ M *p*-bromotetramisole oxalate, 5 μ M cantharidin and 5 nM microcystin-LR (microcystin-leucine arginine)]. After a 30 min incubation, the cells were scraped, collected and homogenized. The lysate was precleared with normal mouse IgG; immunoprecipitation was performed with anti-FLAG M2-agarose affinity gel overnight at 4 °C. The gel was washed four times with wash buffer (50 mM Tris/HCl, pH 7.4, and 150 mM NaCl), followed by SDS/PAGE and transfer on to PVDF membranes. The membranes were blocked with 5 % (w/v) non-fat dried milk and probed overnight at 4 °C with polyclonal anti-PC4 antibody (Santa Cruz Biotechnology). After multiple washes with PBST, the membranes were exposed to secondary antibody which was coupled with horseradish peroxidase in 5 % non-fat dried milk in PBST for 1 h at room temperature. After extensive washing, the complexes were visualized using the West Pico chemiluminescent kit (Pierce).

Statistical analysis

Results are expressed as means $\pm$ S.E.M. Statistical analysis was performed using Student's *t* test; *P* < 0.05 was considered significant.

RESULTS

Redox-dependent phosphorylation of HNF4 α

In the context of hepatocyte iNOS transcription, we have previously demonstrated that IRX-dependent HNF4 α functional DNA binding activity is associated with a unique serine/threonine phosphorylation pattern [8]. In the present study, phosphopeptide analysis was performed using WT-HNF4 α isolated from HepG2 cells that had been exposed to IL-1 β , H₂O₂ or IL-1 β + H₂O₂. Untreated cells served as controls. The cDNA encoding WT HNF4 α was inserted into the pCMV-FLAG vector containing a FLAG epitope and a CMV (cytomegalovirus) promoter region. The predominant constituent of the eluate was a 60 kDa protein. Western blot using anti-human HNF4 α antibody and MS confirmed the 60 kDa protein to be human HNF4 α (results not shown). We performed phosphopeptide analysis of HNF4 α by microcapillary reverse-phase HPLC nanoelectrospray MS/MS. When expressed with the FLAG epitope tag in HepG2 cells, WT-HNF4 α under control conditions is constitutively phosphorylated at Ser¹³³/Ser¹³⁴ (Table 1). In the presence of IL-1 β alone or H₂O₂ alone, no

additional phosphorylation sites were discovered and HNF4 α is phosphorylated solely at Ser¹³³/Ser¹³⁴. In contrast, when exposed to IL-1 β + H₂O₂, additional phosphorylation sites emerge at Ser¹⁵⁸, Ser¹⁸¹, Ser³⁰⁴ and Thr⁴⁵⁰ in addition to those previously noted at Ser¹³³/Ser¹³⁴. In conjunction with our previously published results, these results indicate that oxidative stress in combination with IL-1 β induces a serine phosphorylation pattern in HNF4 α that is unique from that found in the presence of IL-1 β or peroxide alone. To determine the potential functional relevance of these phosphoserine sites, each was individually mutated to a non-phosphorylatable amino acid (see the Materials and methods section), expressed, isolated and tested in subsequent studies.

DNA binding of HNF4 α protein

ChIP assays were performed in HepG2 cells to confirm the enhanced DNA binding activity of HNF4 α to the iNOS promoter in the context of IL-1 β + H₂O₂ (Figure 1). As previously demonstrated, there is constitutive HNF4 α binding under unstimulated conditions [8]. The extent of DNA binding is not different among the unstimulated control, IL-1 β - or H₂O₂-treated cells. However, in IL-1 β + H₂O₂-stimulated cells, HNF4 α DNA binding to the hepatocyte iNOS promoter is increased over 8-fold ($P < 0.01$ versus control, IL-1 β - or H₂O₂-treated cells). When staurosporine (0.5 μ M) is added as an inhibitor of serine/threonine kinase activity, HNF4 α binding is reduced to levels similar to that of unstimulated controls. In the presence of IL-1 β + H₂O₂, our findings indicate that up-regulated HNF4 α DNA binding activity requires serine/threonine phosphorylation.

To assess the role of IRX-mediated phosphorylation in HNF4 α DNA binding, EMSAs were performed using a target oligonucleotide bearing a consensus HNF4 α DNA-binding site and various FLAG-HNF4 α variants, as expressed in COS-1 cells. As HepG2 cells express HNF4 α constitutively, COS-1 cells, which normally do not express HNF4 α , were utilized (results not shown). EMSAs were initially performed using WT-HNF4 α isolated from COS-1 cells that were exposed to IL-1 β , H₂O₂ or IL-1 β + H₂O₂. Untreated cells served as controls (Figure 1). Although constitutive WT-HNF4 α DNA binding is present under all conditions, specific WT-HNF4 α DNA binding is significantly increased in the presence of IL-1 β + H₂O₂ stimulation more than 8-fold ($P < 0.01$, IL-1 β + H₂O₂ versus control, IL-1 β or H₂O₂). The mutated FLAG-HNF4 α expression vectors were then individually transfected into COS-1 cells subjected to the previously described treatment conditions. These mutant HNF4 α proteins were then isolated and tested for *in vitro* DNA binding to the target oligo. In unstimulated control, IL-1 β - and H₂O₂-treated COS-1 cells, there was detectable binding noted for all of the HNF4 α mutant variants. This level of DNA binding was not significantly different between the treatment groups. However, in the presence of IL-1 β + H₂O₂, the previously demonstrated 5–8-fold increase in DNA binding was found with all of the mutants except for S158A-HNF4 α in which Ser¹⁵⁸ was mutated to alanine (S158A). In this IL-1 β + H₂O₂ treatment group, S158A-HNF4 α DNA binding was not different from that of unstimulated controls. We then used SSY134CAA-HNF4 α as a negative control as these residues are typically phosphorylated in WT HNF4 α from unstimulated controls. In this instance, no HNF4 α DNA binding is exhibited under any treatment conditions. The specificity of our results was confirmed using supershift assays with anti-FLAG antibody and competition experiments with excess unlabelled specific and/or non-specific oligonucleotide competitors. These results indicate that phosphorylation of Ser¹⁵⁸ in HNF4 α is critical for *in vitro* DNA binding in the setting of IL-1 β + H₂O₂ stimulation.

Figure 1 HNF4 α DNA binding

(A) ChIP assay of iNOS promoter HNF4 α binding. Chromatin from hepatocytes was fixed and immunoprecipitated using the ChIP assay kit as recommended by the manufacturer. Goat anti-human polyclonal HNF4 α antibody (5 μ g, Santa Cruz Biotechnology) was used for each immunoprecipitation; 5 μ l of rabbit IgG served as a control. The DNA was recovered and subjected to analysis by PCR. The primers had the following sequence: 5'-tgaccaat-tgactggtatgtgtg-3' (sense strand) and 5'-gctgggctggggagatggctga-3' (antisense strand) to yield a PCR product of 280 bp. The input fraction corresponded to 0.1% of the chromatin solution before immunoprecipitation. After DNA purification, the presence of the selected DNA sequence was assessed by PCR. The blot is representative of four experiments. H₂O₂ (50 μ M); STA, staurosporine (0.5 μ M). (B) EMSA analysis of *in vitro* WT and mutant HNF4 α binding. EMSAs were performed using 10 μ g of FLAG-tagged WT or mutant HNF4 peptide. Unlabelled target oligonucleotide was added at 200 M excess in specific or non-specific competition assays (Spec Comp or Nonspec Comp respectively). For supershift experiments, FLAG antibody was included in the binding reaction. Probe was prepared by end-labelling the WT 28 bp double-stranded ARE (antioxidant response element) with [γ -³²P]ATP (2500 Ci/mmol) using T4 polynucleotide kinase, followed by gel purification on 15% polyacrylamide. The gel is representative of five experiments. Ab, antibody.

FLAG-HNF4 α and iNOS promoter activation

To determine the effect of Ser¹⁵⁸ phosphorylation on HNF4 α trans-activation potential, co-transfection studies were performed in COS-1 cells using WT and mutant (SSY134CAA-, S181A-, S304A-, T450A- and S158A-HNF4 α) FLAG-HNF4 α expression vectors and a CAT reporter plasmid containing the human iNOS promoter (Figure 2). The four conditions were unstimulated controls, IL-1 β -, H₂O₂- and IL-1 β + H₂O₂-treatment. In selected instances, an empty FLAG expression vector was used as an additional negative control. In studies using empty FLAG vector or the non-functional SSY134CAA-HNF4 α vector, iNOS promoter activity, as normalized for transfection efficiency, was increased over 10-fold in the presence of IL-1 β , in contrast with that

Figure 2 Transient transfection analysis of iNOS promoter activity in COS-1 cells

Cells were transfected using the Lipofectamine™ technique. After cells were washed twice with MEM, 10 μ g of plasmid DNA containing the iNOS promoter construct (1845 bp; GenBank® accession no. X95629) coupled with a CAT reporter gene were added. In selected instances, an empty vector or an HNF4 α expression vector (10 μ g) encoding FLAG-tagged WT or mutant variants was co-transfected with the iNOS promoter plasmid construct. After 24 h, cells were stimulated with IL-1 β (1000 units/ml) in the presence and absence of H₂O₂ (50 μ M). Unstimulated cells served as controls. After 6 h, the supernatant was assayed for CAT activity using a CAT ELISA technique. Transfection efficiency was normalized by co-transfection of a β -galactosidase reporter gene with a constitutively active early SV40 promoter. All values are expressed as pg of CAT/mg of protein. Results are expressed as means $\pm$ S.E.M. for three experiments (* P < 0.01 versus control, IL-1 β - or H₂O₂-treated cells).

noted in unstimulated controls, H₂O₂ or IL-1 β + H₂O₂ (P < 0.01, IL-1 β versus IL-1 β + H₂O₂, controls or H₂O₂). Interestingly, the absence of HNF4 α (as COS-1 cells do not express HNF4 α) or the presence of non-functional HNF4 α ablated the typical increase in iNOS promoter activity seen in IL-1 β + H₂O₂ cells in comparison with IL-1 β .

When the WT HNF4 α expression vector was utilized, iNOS promoter activity was increased over 10-fold in the presence of IL-1 β , in contrast with that noted in unstimulated controls or H₂O₂ (P < 0.01 in IL-1 β versus controls or H₂O₂). In this instance, iNOS promoter activity increased an additional 5-fold in IL-1 β + H₂O₂ cells over that noted in IL-1 β cells (P < 0.01 in IL-1 β + H₂O₂ versus IL-1 β). Finally, with S158A-HNF4 α in which there was a serine to alanine mutation, the expected increase in iNOS promoter activity was again noted in IL-1 β cells. However, in contrast with that seen with WT HNF4 α co-transfection, the additional incremental increase in CAT activity was not seen with IL-1 β + H₂O₂ treatment in S158A-HNF4 α cells. The S181A, S304A and T450A mutations did not behave differently from WT-HNF4 α under any of the four treatment conditions. These results suggest that phosphorylation of Ser¹⁵⁸ is critical for IRX-dependent up-regulation of HNF4 α trans-activation of the iNOS promoter.

Phosphorylation of FLAG-HNF4 α and kinase inhibitors

To determine a specific kinase pathway responsible for HNF4 α Ser¹⁵⁸ phosphorylation in the presence of IL-1 β + H₂O₂ treatment, we first submitted the sequence for database analysis using motif scan software (<http://scansite.mit.edu>). This database indicated that HNF4 α Ser¹⁵⁸ may serve as a potential substrate for PKC (protein kinase C), PKA (protein kinase A) and/or p38 kinase activities. Specific kinase inhibitors were identified for PKC, PKA and/or p38 kinase activities: 4-cyano-3-methylisoquinoline, GF 109203X and p38 MAPK (mitogen-activated protein kinase)

Figure 3 Kinase inhibitors and *in vitro* DNA binding of WT and mutant HNF4 α peptides

COS-1 cells were transfected with empty vector or FLAG-tagged WT or mutant (S158A or S304A) HNF4 α expression vectors. Cells were treated with IL-1 β + H₂O₂ in the presence or absence of increasing concentrations of p38, PKA or PKC inhibitors. Unstimulated cells served as a control. EMSAs were performed using 10 μ g of isolated HNF4 peptide. For the supershift experiments, anti-FLAG antibody (Kodak) was included in the binding reaction. Probe was prepared by end-labelling WT 28 bp double-stranded ARE with [γ -³²P]ATP (2500 Ci/mmol) using T4 polynucleotide kinase, followed by gel purification on 15% polyacrylamide. The gel is representative of five experiments. NP, not performed; Ab, antibody.

inhibitor III respectively (Calbiochem). COS-1 cells were then transfected with the FLAG-HNF4 α expression vectors (empty FLAG vector, WT-HNF4 α , SSY134CAA-HNF4 α , S304A-HNF4 α and S158A-HNF4 α) and subjected to IL-1 β + H₂O₂ treatment. For 2 h prior to initiation of treatment, the cells were cultured with increasing concentrations of the kinase inhibitors (5, 10 and 20 μ M). The cells were then harvested, nuclear protein extracted and EMSA performed using a target oligonucleotide bearing a consensus HNF4 α DNA-binding site (Figure 3). In WT-HNF4 α and S304A-HNF4 α vector transfection, DNA binding was significantly increased in IL-1 β + H₂O₂ in the absence of inhibitors and in the presence of inhibitors for PKC and PKA. However, in the presence of the p38 kinase inhibitor, this augmented DNA binding was decreased in a dose-dependent fashion for both WT-HNF4 α and S304A-HNF4 α . In contrast, with S158A-HNF4 α cells, minimal DNA binding was noted under both unstimulated control and IL-1 β + H₂O₂ conditions, and this was not altered by the presence of the various kinase inhibitors. In empty FLAG vector and SSY134CAA-HNF4 α cells, no DNA binding was found. These results indicate that p38 kinase is required for up-regulation of HNF4 α DNA binding in the presence of IL-1 β + H₂O₂ treatment and that WT-HNF4 α Ser¹⁵⁸ may serve as the target substrate for p38.

We then made a phosphomimetic mutant of HNF4 α in which Ser¹⁵⁸ was mutated to an aspartic residue, S158D-HNF4 α . This vector was then transfected into COS-1 cells subjected to the four treatment conditions: control, IL-1 β , H₂O₂ and IL-1 β + H₂O₂; EMSAs were subsequently performed (Figure 4). In this setting, equivalent DNA binding is again present in unstimulated control and H₂O₂ cells. However, in the presence of this phosphomimetic mutant HNF4 α , DNA binding is enhanced in IL-1 β -treated cells to the same extent as that noted in IL-1 β + H₂O₂ cells; indeed, the extent of binding is equivalent to that found with transfection of

Figure 4 *In vitro* DNA binding of an S158D phosphomimetic mutant of HNF4 α

COS-1 cells were transfected with empty vector or FLAG-tagged WT or mutant (S158A or S158D) HNF4 α expression vectors. Cells were treated as indicated. Unstimulated cells served as a control. EMSAs were performed using 10 μ g of isolated HNF4 peptide. Probe was prepared by end-labelling the WT 28 bp double-stranded ARE with [γ - 32 P]ATP (2500 Ci/mmol) using T4 polynucleotide kinase, followed by gel purification on 15% polyacrylamide. The gel is representative of three experiments.

WT-HNF4 α in IL-1 β + H $_2$ O $_2$ cells. The DNA binding of the phosphomimetic HNF4 α is not altered in the presence of the p38 kinase inhibition. These results indicate that phosphorylation of Ser 158 is required for enhanced DNA binding noted in IL-1 β + H $_2$ O $_2$ cells.

p38 kinase phosphorylates HNF4 α Ser 158 *in vitro*

In vitro phosphorylation studies were performed using full-length purified p38, JNK or ERK1/2 and WT or mutant FLAG-HNF4 α (Figure 5A). The HNF4 α constructs were immunoprecipitated

using anti-FLAG antibody (Sigma). The results show that full-length WT HNF4 α can be efficiently phosphorylated by p38, JNK and ERK1/2 kinases. Additional studies were then performed using S158A-HNF4 α in which there was an S158A mutation. In this instance, when *in vitro* phosphorylation studies were repeated, the band corresponding to p38 kinase-mediated phosphorylation was now absent. These results indicate that p38 kinase can phosphorylate Ser 158 in HNF4 α *in vitro*.

To determine the functional correlate of this observation, the p38 kinase inhibitor was used in co-transfection experiments with the human iNOS promoter reporter construct and the various FLAG-HNF4 α expression vectors, as above (Figure 5B). In IL-1 β + H $_2$ O $_2$ -treated COS-1 cells, transfection of S158A-HNF4 α was associated with significantly decreased iNOS promoter activation compared with WT-HNF4 α or S158D-HNF4 α . When p38 MAPK inhibitor III (Calbiochem) was added with WT HNF4 α , the extent of iNOS promoter activation in the IL-1 β + H $_2$ O $_2$ -treated COS-1 cells was not statistically different from that found with S158A-HNF4 α . In contrast, the activity associated with S158D-HNF4 α , the phosphomimetic mutation of HNF4 α , was not altered. These results suggest that p38 kinase phosphorylation of HNF4 α Ser 158 is required for the IRX-dependent HNF4 α -mediated increase in iNOS transcription.

HNF4 α and PC4 interaction

We have previously demonstrated that the transcriptional co-activator, PC4, is essential for up-regulation of HNF4 α activity in the presence of IL-1 β + H $_2$ O $_2$ treatment. In this regard, previous co-immunoprecipitation studies demonstrate that PC4 and HNF4 α associate under IL-1 β + H $_2$ O $_2$ exposure [13]. To determine the potential role of Ser 158 in PC4 binding, HepG2 cells were co-transfected with WT-HNF4 α or S158A-HNF4 α . Following exposure to IL-1 β + H $_2$ O $_2$, co-immunoprecipitation studies were performed (Figure 6). PC4 was found to associate with WT-HNF4 α , but not with S158A-HNF4 α . This result would suggest

Figure 5 HNF4 α phosphorylation and transactivation

(A) *In vitro* phosphorylation of HNF4 α . Purified FLAG-tagged WT or mutant HNF4 α protein was incubated with 1 μ l of protein kinase (p38, JNK, or ERK1/2). The reaction products were immunoprecipitated with anti-FLAG antibody at 0 and 20 min, followed by separation on SDS/4–20% polyacrylamide gels; the gel was then dried and analysed by autoradiography. The blot is representative of three experiments. (B) Transient transfection analysis of iNOS promoter activity in COS-1 cells. Cells were transfected using the LipofectamineTM technique. After cells were washed twice with medium, 10 μ g of plasmid DNA containing the iNOS promoter construct (1845 bp; GenBank[®] accession no. X95629) coupled with a CAT reporter gene were added. In selected instances, an empty vector or an HNF4 α expression vector (10 μ g) encoding FLAG-tagged WT or mutant variants was co-transfected with the iNOS promoter plasmid construct. In selected instances, the p38 kinase inhibitor was also added. Cells were stimulated with IL-1 β (1000 units/ml) in the presence and absence of H $_2$ O $_2$ (50 μ M). Unstimulated cells served as controls. After 6 h, the supernatant was assayed for CAT activity using a CAT ELISA technique. Transfection efficiency was normalized by co-transfection of a β -galactosidase reporter gene with a constitutively active early SV40 promoter. All values are expressed as pg of CAT/mg of protein. Results are expressed as means $\pm$ S.E.M. for three experiments (* P < 0.01 versus control or H $_2$ O $_2$ -treated cells; ** P < 0.01 versus control, IL-1 β - or H $_2$ O $_2$ -treated cells).

Figure 6 Co-immunoprecipitation of HNF4 α and PC4

HepG2 cells were transfected with FLAG-tagged WT-HNF4 α or S158A-HNF4 α by Lipofectin® (Invitrogen) and exposed to IL-1 β + H₂O₂. Immunoprecipitation (IP) was performed with anti-FLAG M2-agarose affinity gel (Sigma). Polyclonal anti-PC4 antibody (Santa Cruz Biotechnology) was used for immunoblot (IB) analysis. Membranes were then exposed to secondary antibody coupled with horseradish peroxidase and visualized. The blot is representative of three experiments.

that phosphorylation of Ser¹⁵⁸ is required for association of HNF4 α with PC4 in the presence of IL-1 β + H₂O₂. This may represent one mechanism by which HNF4 α up-regulates iNOS expression in the presence of pro-inflammatory cytokines and oxidative stress.

DISCUSSION

In sepsis and shock, the host response is characterized by production of pro-inflammatory cytokines and reactive oxygen species [14]. This combination of oxidative and inflammatory liver injury occurs in a number of clinical conditions, including haemorrhagic shock, liver transplantation, hepatic resection and thermal injury. The response is typically biphasic and initiated by production of free radicals by Kupffer cells followed by polymorphonuclear leucocyte infiltration and cytokine/chemokine production. Although the role of NO in this setting has been extensively studied, its relative hepatoprotective or hepatotoxic role remains controversial. Oxidative stress derived from xenobiotics such as hypochlorite and carbon tetrachloride is reduced by NO in hepatocytes [14]. Moreover, pre-exposure of hepatocytes to NO prevents subsequent peroxide-induced hepatic damage. The *in vivo* hepatoprotective effects of endogenous NO synthesis have also been demonstrated in a mouse model of BCG (Bacille Calmette–Guérin)/endotoxin-mediated immune injury, and rat models of tumour necrosis factor/D-galactosamine-induced apoptosis, endotoxin-induced hepatic injury, and hepatic reperfusion injury [15–17]. In these settings, NO functions as an antioxidant. In murine models of endotoxaemia, inhibition of NO synthesis exacerbates free radical-mediated hepatocyte injury [18,19]. In a rat model of hepatic ischaemia–reperfusion injury, inhibition of NO synthesis increases hepatocyte injury, systemic levels of lipid hydroperoxide and hepatic superoxide production, suggesting increased oxidative injury [20]. iNOS-deficient (–/–) knockout mice treated with endotoxin have significant elevations in serum transaminase values and exhibit pyogranulomata infiltration, multifocal cellular necrosis and endothelial injury in the liver [21]. However, studies also suggest that iNOS may play an hepatotoxic role in IRX injury. Lee et al. [14] reported that iNOS knockout mice exhibited less liver damage derived from ischaemia–reperfusion, indicating the deleterious function of iNOS-derived NO. Furthermore, they showed that iNOS is not expressed until 3 h following the initiation of reperfusion and

that an extra-hepatic source of iNOS appears to contribute to the efficiency of reperfusion. The toxicity of iNOS-derived NO in this setting was hypothesized to be related to the formation of ONOO[–]. There are clearly overlapping states characterized by oxidative stress and pro-inflammatory cytokines in which hepatocyte iNOS expression may be protective or toxic.

Our previous work suggests that the hepatocellular redox state up-regulates iNOS expression as an antioxidant function [1]. We found HNF4 α to be an activator of redox-associated hepatocyte iNOS expression at the levels of protein, mRNA and promoter activation and was associated with a unique serine/threonine kinase-mediated phosphorylation pattern. As this suggested that an IRX-sensitive kinase pathway targets HNF4 α to augment hepatocyte iNOS expression, we embarked upon the present set of experiments to identify the target amino acid residues in HNF4 α and the potential kinase pathway. Our results indicate that p38 kinase forms an essential part of the IRX-sensitive pathway that phosphorylates Ser¹⁵⁸ to up-regulate HNF4 α activity and iNOS expression.

The transcription factor HNF4 α (NR2A1), a member of the nuclear hormone receptor superfamily, is a key regulator of diverse metabolic pathways, through regulation of a large number of genes involved in glucose, cholesterol and fatty acid metabolism [22]. In mice, HNF4 α transcripts have been detected as early as day 4.5, whereas knockout of the gene impairs gastrulation and is embryonic lethal [23]. Moreover, production of a conditional gene knockout of HNF4 α has elucidated its central role in the maintenance of the hepatic phenotype in mature hepatocytes and has suggested a putative role for HNF4 α as a lipid sensor of the cell [24]. HNF4 α exhibits distinct functional domains typical of nuclear hormone receptors [22]. A highly conserved DBD (DNA-binding domain), composed of two zinc-co-ordinated modules, is responsible for specific binding to cognate response elements. Aggelidou et al. [22] recently determined the critical role of various residues in the ligand-binding domain of HNF4 α ; Ser¹⁵⁸ is not included among these.

In contrast with other members of the nuclear receptor superfamily, HNF4 α has been shown to be a constitutively active transcription factor. When HNF4 α was recently crystallized in the absence of added ligands, the ligand-binding pocket of both receptors contained a mixture of endogenous saturated and mono-unsaturated fatty acids in the crystals [6,7]. The fatty acids could not be displaced from the pockets even after prolonged dialysis or partial denaturation of the proteins, leading to the proposal that these endogenous molecules/ligands may be constitutively bound to the ligand-binding domain of HNF4, acquired soon after or even during the folding of the protein. This hypothesis offered an explanation for the constitutive transcriptional activity of HNF4, and it was proposed that additional modifications or co-factor recruitment could be the critical factors for modulating HNF4 activity in cells [6,7]. Certainly, in the present study, we find that post-translational modification via phosphorylation and co-activator PC4 activity are required for HNF4 α 's role in mediating the IRX-dependent increase in hepatocyte iNOS transcription [13].

Post-translational modification by phosphorylation is known to alter HNF4 α DNA binding activity, transactivation potential, and nuclear translocation and/or degradation [25,26]. HNF4 α DNA binding activity and transactivation potential are tightly regulated by its state of phosphorylation. HNF4 α potentially contains 21 serine, six threonine and seven tyrosine phosphorylation sites [25]. In COS-7 cells, serine/threonine phosphorylation of HNF4 α increases affinity and specificity of DNA binding by altering its tertiary structure [25]. Tyrosine phosphorylation of HNF4 α is required for DNA binding, transactivation and subnuclear

localization in primary cultures of rat hepatocytes [27]. In contrast, PKA-dependent phosphorylation within the DBD inhibits DNA binding in HepG2 and COS-1 cells [26]. Additional work by Jahan and Chiang [28] indicated that a JNK-dependent pathway inhibits HNF4 α expression and DNA binding in the presence of IL-1 β . Further work by Hong et al. [29] shows that AMP-activated protein kinase phosphorylates HNF4 α at Ser³⁰⁴ to inhibit homodimer formation and DNA binding. *In vivo* experiments support the functional importance of HNF4 α phosphorylation state. In a murine model of 15% burn injury, hepatocyte HNF4 α DNA binding is enhanced by serine phosphorylation [30,31]. Dietary protein restriction or overnight fasting decreases hepatic HNF4 α DNA binding activity as a result of decreased serine/threonine phosphorylation [4,26]. Although HNF4 α activity is certainly regulated by post-translational modification, IRX-mediated post-translational phosphorylation of HNF4 α has not been previously examined.

Cellular stressors such as reactive oxygen species and pro-inflammatory cytokines regulate intracellular signal transduction cascades and modulate transcription factor activity through calcium signalling, protein kinase and protein phosphatase pathways [32]. Oxygen free radicals may directly activate kinases by altering thiol-dependent protein-protein interactions, inhibiting phosphatase activity by oxidation of an active site cysteine residue, and/or stimulating proteolysis of kinase regulatory proteins. Ultimately, redox-regulated tyrosine- and serine/threonine-phosphorylation of transcription factors through tyrosine kinase and SAPK (stress-activated protein kinase) activities alter transcription factor subcellular localization, DNA binding properties and transactivation potential. In particular, the SAPKs [ERK1/2, BMK1 (big MAPK1), JNK and p38 isoforms] are often the ultimate (and best characterized) regulatory proteins in a series of sequential kinase reactions that target transcription factor modification in the setting of cellular stresses similar to those of our model [33–35]. In our setting, p38 kinase-dependent phosphorylation of HNF4 α is certainly in keeping with this existing paradigm of signal transduction of extracellular stress via the SAPK pathway [36]. The individual components of this pathway remain to be identified. Future experiments are required to characterize the molecular regulatory pathway by which HNF4 α integrates multiple extra- and intra-cellular signals mediated by kinase cascades to precisely regulate IRX-dependent hepatocyte iNOS gene expression. These considerations are crucial to our understanding of HNF4 α as a novel and, as yet, poorly described IRX-sensitive mechanism that regulates hepatocyte iNOS expression as an anti-apoptotic and antioxidant function.

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