Hepatocyte nuclear factor 4 response to injury involves a rapid decrease in DNA binding and transactivation via a JAK2 signal transduction pathway

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The injury response is a complex set of events, which represents the reaction of a biological system to a perceived change in its environment in an attempt to maintain system integrity. Isolation of individual events or components of this response cannot describe the overall process, but may reflect general mechanisms that have evolved over time to solve the complex requirements of the injury response. The process, generally termed the acute phase response, is a series of organ-specific responses that begin shortly after a systemic injury. In the liver, this response involves both dramatic inductions and reductions in specific sets of genes, and an overall widespread global change in proteins produced. This can be thought of as a phenotypic change or ‘reprogramming’ of the liver. These changes in protein production are modulated and regulated at the level of transcription and involve significant manipulations of transcriptional regulatory mechanisms. Hepatocyte nuclear factor 4 (HNF-4) is a liver enriched transcription factor that regulates a large number

of liver-specific genes, which play important roles in the critical pathways modulated by the response to injury. HNF-4 also performs an essential role in overall development and is critical for the normal expression of multiple genes in the developed liver, as well as being upstream of HNF-1 in a transcriptional hierarchy that drives hepatocyte differentiation. The role of HNF-4 in regulating liver-specific transcriptional changes directed by injury remains to be defined. In our cell-culture and whole-animal models, we demonstrate that the binding activity of HNF-4 decreases quickly after injury due to post-translational modification by phosphorylation. The mechanisms by which HNF-4 is modified after injury involve the activation of Janus kinase 2 (JAK2) signal transduction pathways, but the direct or indirect interaction of JAK2 with HNF-4 remains to be defined.

Key words: acute phase, electrophoretic mobility-shift assay (EMSA), liver, phosphorylation, tyrosine kinase.

INTRODUCTION

The response to injury or trauma involves profound changes in the normal physiological processes of many tissues and activates unique cellular responses. These responses are achieved for the most part by changes in specific gene transcription. A pre-determined complex set of events, known as the acute phase response (APR), are executed to prevent further tissue damage and activate reparative functions. The liver plays a central role in the APR [1]. Within 24 h of the induction of the APR, the liver shifts its protein production, with some proteins increasing 2- to 1000-fold (positive APR proteins), while the production of others significantly decreases (negative APR proteins) [2]. The hallmark of this APR are the resultant changes in serum-protein concentrations. This wholesale change in steady-state protein production can be thought of as a reprogramming of the liver.

The liver responds to many external stimuli with modulation in protein production; however, not all of these responses require a change or ‘reprogramming’ in phenotype. The degree and extent of the response can vary greatly, and depends on the magnitude and type of the stress [3]. For instance, changes in serum glucose levels lead to a rapid modulation of both the gluconeogenic and glycogenolysis pathways, requiring transcriptional regulatory changes that do not necessitate an effect on other genes central to the hepatic phenotype [4]. Another such response by the liver is the induction of the cytochrome P450

system after exposure to phenobarbital [5]. On the other hand, regeneration in response to partial hepatectomy and the APR to injury do require reprogramming or a large-scale modification in gene transcription [6,7].

The maintenance and development of the hepatic phenotype appears to require the expression of a series of liver-enriched transcription factors. These nuclear factors, including hepatocyte nuclear factor (HNF)-1, HNF-3, HNF-4 and CAAAT/enhancer binding protein (C/EBP), are critical to the developmental regulation of the hepatic phenotype [8]. They are organized in a loose hierarchy of expression, with HNF-4 being most proximal [9]. HNF-4 is a homodimeric orphan nuclear hormone receptor, having neither known effectors nor regulating drugs [10]. This nuclear factor is critical to development and HNF-4 knockouts are lethal [11].

HNF-4 is known to regulate a number of liver-specific genes, which play central roles in fatty acid, glucose and amino acid metabolism, many of which are modulated during injury [10]. Additionally, HNF-4 performs a critical role in liver development and liver-specific gene expression and phenotype. Observation in developmental models shows that HNF-4 always precedes the development of the liver specific phenotype and chimaeric mice expressing HNF-4 deficient cells in the developing liver fail to express liver specific genes [10,12]. Ablation of the HNF-4 gene in newborn mice further produces a dramatic loss in differentiated liver function and morphology [13]. In cultured cell lines and

Abbreviations used: APR, acute phase response; ATF-2, activating transcription factor-2; CAT, chloramphenicol acetyltransferase; C/EBP, CAAAT/enhancer binding protein; CIP, calf intestinal alkaline phosphatase; CRE, cAMP responsive element; CREB, CRE binding protein; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assays; GH, growth hormone; HNF-4, hepatocyte nuclear factor 4; IL-1, interleukin-1; JAK2, janus kinase 2; L-PK, L-pyruvate kinase; MAP kinase, mitogen-activated protein kinase; MEM, minimal essential medium; NE, nuclear extract(s); NP40, Nonidet P40; PEPCK, phosphoenolpyruvate carboxykinase; PKA, protein kinase A; PKC, protein kinase C; TK, thymidine kinase; WT, wild-type.

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primary liver explants, the loss of HNF-4 expression is accompanied by the loss of the liver-specific phenotype, and over-expression of exogenous HNF-4 in these same cell lines can restore differentiated liver-specific functions [14,15]. Although the presence of HNF-4 is necessary for liver-specific protein production it is not always sufficient, as shown by cell lines that express HNF-4 but are unable to express liver-specific genes such as albumin. These uncoupled cell lines can generally be induced, through manipulation of conditions, to express a differentiated liver phenotype, indicating that the modification of HNF-4, or the presence or absence of cofactors, plays a role in HNF-4 maintenance of the liver phenotype [16,17].

Little is known, beyond the level of expression, about the possible mechanisms of regulation of HNF-4. Previous work by this group demonstrated significant changes in binding activities of several transcription factors involved in liver gene expression secondary to injury, including HNF-1, HNF-4 and C/EBP [18–20]. These binding activity changes are rapid, occur well before changes in acute phase gene mRNAs, and are not accompanied by decreases in the amount of the corresponding proteins. The most dramatic effects of injury were observed in HNF-4 [18–20]. In the present study, we further characterize the decrease in HNF-4 binding activity in response to injury. We show that HNF-4 binding *in vivo* is rapidly modulated by phosphorylation after injury. This phosphorylation event results in a decrease in binding activity and increase in K_d . Utilizing a cell culture model of APR, we further show that this decrease in HNF-4 binding activity affects its ability to transactivate target genes and that the phosphorylation observed occurs through a JAK2(Janus kinase 2)-dependent pathway.

MATERIALS AND METHODS

Injury model

An injury model utilizing a 15% body surface area/full thickness burn was utilized as described [18]. Briefly, female Balb/C mice (Taconic Farms, Germantown, NY, U.S.A.) were anaesthetized and exposed to water at a temperature of 90 °C for 8 s, creating a 15% body surface area/full thickness burn injury. Control animals were anaesthetized and exposed to water at 20 °C. Mice were then killed by cervical dislocation at 1.5 and 3 h after injury, and their livers were immediately harvested.

Cell culture injury model

HepG2 cells were maintained in minimal essential medium (MEM) containing penicillin (100 units/ml) and streptomycin sulphate (100 µg/ml), with 10% (v/v) foetal bovine serum, at 37 °C in a 5% CO₂ incubator. When 40–50% confluence was reached, HepG2 cells were changed to serum-free MEM for 5 h before stimulation. Cells were treated with 1 µg/µl forskolin (Sigma, St. Louis, MO, U.S.A.), 1 µg/µl PMA (Sigma), or a cytokine mixture consisting of 1 ng/ml recombinant human interleukin (IL)-1β (PeproTech, Rocky Hill, NJ, U.S.A.), 10 ng/ml IL-6 (PeproTech), 10 ng/ml tumour necrosis factor-α (Sigma) and 1 µM dexamethasone, in serum-free MEM for variable times before harvesting and immediate preparation of nuclear extracts (NE).

NE preparation

The preparation of mouse liver NE was carried out as described previously [19]. NE from HepG2 cells were prepared from 10 cm dishes which were washed once with HDB buffer [25 mM Hepes (pH 7.4), 0.7 mM Na₂HPO₄, 137 mM NaCl, 5 mM KCl], then

scraped with a plastic lifter and transferred into 1.5 ml tubes. Cells were pelleted and resuspended in 400 µl of cold buffer A [50 mM KCl, 20 mM Hepes, 1 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM PMSF, 5 µM leupeptin, 10 mM β-glycerophosphate and 1 mM sodium orthovanadate], stored on ice and vortexed every 5 min for 15 min. A 30 µl aliquot of 10% (v/v) Nonidet P40 (NP40) was added to the lysate and centrifuged at 4 °C, 10000 g for 1 min using a benchtop centrifuge. The nuclear pellets were resuspended in 50 µl of cold buffer B (as in buffer A but 400 mM NaCl instead of 50 mM KCl), stored on ice and vortexed every 5 min for 30 min, followed by centrifugation at 10000 g for 5 min. The supernatant containing NE proteins was transferred to new tubes and stored at –80 °C.

Total RNA isolation

The total RNA was collected from HepG2 cells grown in 6 cm plates and lysed in 0.4 ml of Trizol®LS reagent (Invitrogen, Carlsbad, CA, U.S.A.) after washing them once with HDB buffer and incubating for 5 min at 20 °C. Chloroform (0.1 ml) was added to the lysate, which was mixed and incubated at 20 °C for 15 min. The RNA in the aqueous phase was separated by centrifugation at 12000 g for 10 min at 4 °C, and transferred to new tubes. RNA was precipitated by adding 0.25 ml of isopropyl alcohol, incubating at 20 °C for 10 min, and then centrifuging at 12000 g for 10 min at 4 °C. RNA pellets were mixed with 0.5 ml of 75% ethanol, mixed, centrifuged at 7500 g for 5 min at 4 °C and air-dried. The total RNA was dissolved in 30 µl of diethyl pyrocarbonate water and stored at –20 °C.

Northern-blot analysis

Fibrinogen, L-pyruvate kinase (L-PK), phosphoenolpyruvate carboxykinase (PEPCK) and tubulin cDNA fragment were produced by enzymic digestion and isolated by agarose gel electrophoresis (a 1.2 kb *Pst*I fragment of rat fibrinogen β-chain, a 1.8 kb *Pst*I fragment of L-PK, and a 1.6 kb *Pst*I fragment of PEPCK) [18,21,22]. Fragments (25 ng of cDNA) were labelled by random priming with [γ -³²P]CTP with a DNA labelling system (Amersham Biosciences, Piscataway, NJ, U.S.A.). The unincorporated labelled nucleotide was removed through a G25 Quick Spin column (Roche Applied Science, Indianapolis, IN, U.S.A.) and labelled probes were stored at –20 °C. The membrane was prehybridized for 4 h at 42 °C in 50% (v/v) formamide, 5 × SSPE (1 × SSPE = 0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA), 0.5% SDS, 5 × Denhardt's solution, 200 µg/ml of herring-sperm DNA. Hybridization was performed at 42 °C in fresh prehybridization buffer containing (2–5) × 10⁶ c.p.m./ml of probe (denatured by boiling) for 24 h. Membranes were sequentially washed four times at 42 °C for 15 min in each of the following: 2 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate), 1% SDS and 1 mM EDTA, 1 × SSC and twice in 0.1 × SSC. The membranes were exposed to Kodak BioMax films at –70 °C overnight.

Electrophoretic mobility-shift assays (EMSAs)

HNF-4 oligonucleotide corresponding to the HNF-4 binding site of the mouse transthyretin gene (5'-CCCTAGGCAAGGTTT-ATATGGCC-3') was synthesized and labelled as described previously [19]. The transthyretin binding site, a physiological binding site, was chosen based on its use in the initial description of HNF-4 [8] and its classification as an acute phase protein with significant changes in concentration after injury. The gel mobility reaction was performed in a 15 µl reaction volume containing

3 μg of NE protein, 2 μg of poly(dI-dC), 100 $\mu\text{g}/\text{ml}$ BSA, 1 mM DTT, 25 mM Tris (pH 7.5), 50 mM KCl, 0.1 mM EDTA, 2 mM MgCl_2 , 10% (v/v) glycerol, 0.05% NP40, and 20000 c.p.m. (approx. 0.4–0.6 ng) of labelled probe. After incubation for 30 min at 20 °C, protein–DNA complexes were separated from free probe on a 4.5% polyacrylamide gel and visualized by autoradiography. To assess the specificity of protein–DNA interactions, competition experiments were performed by adding 100–400 ng of unlabelled specific (HNF-4) and double-stranded oligonucleotide nonspecific competitors to each individual binding reaction. Also, HNF-4 antibody was added to perform a supershift reaction. Quantification of changes in binding activity was performed using densitometric scanning as described previously [19].

Western-blot analysis

Proteins separated by SDS/PAGE (10% gel) were transferred to nitrocellulose membranes in Bio-Rad apparatus according to the manufacturer's instructions. After blocking of non-specific protein binding sites for 1 h at 20 °C in TBST [50 mM Tris/HCl (pH 8), 150 mM NaCl, 0.05% Tween] containing 5% non-fat dry milk, the blots were incubated with antibodies against cAMP response element binding protein (CREB) (244), phosphoCREB (5322), given by Dr M. Montminy (Peptide Biology Laboratories, Salk Institute for Biological Studies, La Jolla, CA, U.S.A.), or HNF-4 (Santa Cruz Biotech, Santa Cruz, CA, U.S.A.) for 1 h at 20 °C. Following three washes of 10 min each in TBST, the secondary antibody [goat anti-(rabbit IgG) or anti-(goat IgG)] was added and left at 20 °C for 45 min. The blots were washed again as described above, and peroxidase activity was detected by autoradiography with the enhanced chemiluminescence system (Amersham).

Dephosphorylation of NE

The dephosphorylation of liver NE was performed as described previously [23]. Briefly, 5–10 μg of liver NE were incubated with calf intestinal alkaline phosphatase (CIP; 2.5 units/10 μg of nuclear protein; Boehringer Mannheim, Indianapolis, IN, U.S.A.) at 20 °C for 30 min. In selected experiments, CIP extracts were incubated with several phosphatase inhibitors (50 mM NaF, 1 mM sodium vanadate, and 5 μM okadaic acid) before being used in EMSAs.

Transient transfection and chloramphenicol acetyltransferase (CAT) assays

HepG2 cells were cultured in 6 cm dishes at 37 °C in MEM until 40% confluence. Cells were transfected using the Lipofectin reagent (Invitrogen, Carlsbad, CA, U.S.A.) with 2 μg of each expression plasmid and incubated for 30 min in Opti-MEM (Gibco, Grand Island, NY, U.S.A.). The expression vector for wild-type (WT) HNF-4 under the control of a strong enhancer/thymidine kinase (TK) promoter (WT HNF-4) and a CAT reporter construct (HNF-4 CAT), consisting of four tandemly repeated HNF-4 binding sites [(L3)₄-54PK/CAT] from another negative APR protein gene, the L-PK gene [24], were provided by Dr A. Kahn (Unité de Recherches en Physiologie et Pathologie Génétiques et Moléculaires, Institut Cochin de Génétique Moléculaire, Inserm Unité 129, 24 Rue du Faubourg Saint Jacques, 75014, Paris, France). The protein kinase A (PKA) constructs, protein kinase C (PKC) constructs, Lac Z (under the control of a strong enhancer/TK promoter), wild-type constitutively-active JAK2 (JAK2 Act), and constitutively-non-active JAK2 (JAK2 Neg), PEPCAT and cAMP responsive element (CRE) CAT

expression constructs were provided by Dr M. Montminy (Salk Institute). Cells were harvested 30–48 h after transfection or, where indicated, were treated for 18 h with cytokines or forskolin before harvesting. Cellular lysate was collected by freezing and thawing three times in dry ice/ethanol and 37 °C water. After normalizing for transfection efficiency and TK promoter expression for each sample utilizing β -galactosidase activity, CAT assays were performed following standard procedures [25,26]. CAT activities were calculated as the percentage of the acetylated form of chloramphenicol versus the non-acetylated substrate and each experiment was repeated a minimum of three times.

RESULTS

In vivo injury model

To study the effects of injury on HNF-4 functions *in vivo*, we utilized a murine burn-injury model. NE from non-injured mouse liver yielded one band on EMSA that was specific for HNF-4. The specificity of this band was confirmed by (i) its absence in competition with low concentrations of a specific HNF-4 competitor DNA, but not with a 100-fold increased concentration of a non-specific DNA competitor and (ii) the fact that this band was supershifted in the presence of HNF-4-specific antibodies (Figure 1A) [20].

In vivo injury leads to a rapid decrease in HNF-4 binding activity, which dropped to 25% of control levels (anaesthetized sham-injured animals) 3 h after injury. Anaesthetized sham-injured animals showed no significant changes in HNF-4 binding at the time points studied (Figures 1B and 1C). The large and rapid changes in HNF-4 appear unique, in that the ubiquitously expressed transcription factor, NF-1 (nuclear factor-1), showed no significant changes after injury, whereas HNF-1, another liver-enriched transcription factor, and C/EBP showed decreased binding after injury. These decreases in HNF-1 and C/EBP, however, were not as dramatic in either degree or rapidity [19]. Western-blot analysis of NE before and after injury showed no significant changes in HNF-4 protein concentrations (Figure 1B). The equilibrium dissociation constant (K_d), a measure of the binding affinity, was determined with a saturating binding assay in which a constant amount of NE (3 μg) was titrated with increasing amounts of radiolabelled HNF-4 oligonucleotide probe [19]. Calculation of the K_d for HNF-4 before and after injury utilizing EMSA and Scatchard analysis showed that the K_d for HNF-4 increased 2-fold from 50 nM to 143 nM, 1.5 h after injury (results not shown). These data indicate that *in vivo* injury leads to a rapid and significant decrease in HNF-4 binding activity. This change is not due to a decrease in HNF-4 concentration, but to a post-translational modification of HNF-4 or its binding complex induced by the response to a systemic injury. Liver NE made from animals 3 h after injury, but treated *in vitro* with CIP, showed restored HNF-4 DNA binding to control levels (Figure 1D), indicating that the post-translational changes may in part be due to an injury-induced phosphorylation of HNF-4.

In vitro injury model

Previously, we have shown that a 15% body surface area burn injury induces a classical APR in the liver, defined at a molecular level as significant changes in acute phase protein mRNA levels [19]. Specifically, after burn injury, increases in fibrinogen mRNA and decreases in albumin mRNA concentrations are seen. Utilizing a mixture of pro-inflammatory cytokines and dexamethasone we, and others, are able to mimic this *in vivo* mRNA response in cultured HepG2 cells [27,28]. Cytokine treatment

Figure 1 Consequences of burn injury on HNF-4 binding to DNA *in vivo*

(A) EMSA of HNF-4 DNA binding of mouse liver NE with specific (sp) and nonspecific (nsp) oligo competitors, or HNF-4 antibody (ss = supershift). (B) EMSA and Western blot of HNF-4 DNA binding of mouse liver NE 1.5 and 3 h after anaesthetized burn and sham-burn treatments. (C) Graph of HNF-4 DNA binding activity of mouse liver NE as a percentage of control (anaesthetized sham burn mice at time = 0) before and after burn treatment. (D) EMSA of mouse liver NE of HNF-4 DNA binding with CIP and specific CIP inhibitor mix (CIP INH = 50 mM sodium fluoride, 1 mM sodium vanadate, and 5 μ M okadaic acid).

of HepG2 cells leads to the induction of fibrinogen mRNA in a pattern similar to that seen with the *in vivo* model, as well as decreases in mRNA levels of negative acute phase genes such as PEPCK and L-PK (Figure 2). Both of these metabolically important genes are considered negative acute phase proteins and are partly regulated by HNF-4, with levels of mRNA and protein decreasing as seen in various other injury models [29,30].

HNF-4 binding in cytokine-treated HepG2 cells

NE from HepG2 cells show one specific HNF-4 band in EMSA. As with whole-liver NE, the HNF-4 specific band can be specifically competed off and supershifted in the presence of HNF-4 antibodies (Figure 3A, supershift results not shown). Cytokine treatment leads to a rapid decrease in HNF-4 binding, dropping to 30% of control levels at 5 and 15 min, and Western blots showed no significant change in HNF-4 protein concentrations at the time points studied (Figure 3C). As was seen *in vivo*, this change in HNF-4 binding is due to a post-translational change. This effect was specific to cytokine treatment, as treatment with PMA or forskolin produced no significant changes in HNF-4 binding. Western-blot analysis with antibodies specific for CREB, or phosphorylated CREB, in Hep G2 cells showed a minimal increase in CREB phosphorylation after cytokine treatment,

Figure 2 Differential effect of cytokines on acute phase protein mRNAs in HepG2 cells

(A) Northern blot of mRNA of fibrinogen, PEPCK, L-PK and tubulin (control) over 24 h. (B) Quantification of mRNA changes of acute phase proteins shown in (A) relative to time (time 0 = 1.0).

Figure 3 Effect of cytokine-induced injury on HNF-4 binding to DNA in HepG2 cells

(A) EMSA of HNF-4 DNA binding of HepG2 cell NE with specific (sp) and nonspecific (nsp) oligo competitors. (B) EMSA and Western blot of HNF-4 DNA binding of NE versus time after cytokine treatment. (C) EMSA and Western blot of HNF-4 DNA binding of NE after a 30 min treatment of HepG2 cells with cytokines, forskolin, or PMA. (D) Western blot of NE of CREB and phosphorylated CREB (P-CREB) after cytokine or forskolin treatment.

whereas forskolin treatment produced the expected dramatic increase in levels of phosphorylated CREB (Figure 3D).

Effects of cytokine treatment on HNF-4 transactivation

HepG2 cells were transiently transfected with several different CAT reporter constructs. Figure 4(A) demonstrates that cytokines had no effect on baseline CAT activity, but inhibited the increase in PEPCK CAT activity seen with over-expression of WT HNF-4. The over-expression of active PKA showed the expected increase in CRE CAT activity, which indicated the viability of the system (Figure 4B). The various constructs and treatments appeared to have no general effect on basal transcriptional mechanisms as indicated by the observation that the β -galactosidase values for each condition did not vary significantly.

To study the effects of cytokine-induced injury on HNF-4 transactivation, in the absence of regulation of HNF-4 protein levels, HepG2 cells were transiently transfected with HNF-4 CAT and WT HNF-4 expression vectors. This allowed the reliable detection of HNF-4 transactivation activity. Cytokine treatment (14 h) led to a 10-fold decrease in HNF-4 CAT activity compared to non-treated controls (Figure 5). Decreases in HNF-4 CAT activity were not seen after treatment with forskolin, although Western-blot analysis of forskolin treated HepG2 cells showed the expected increase in phosphorylated CREB (Figure 3D). Hep G2 cells transiently transfected with a CRE-driven CAT reporter construct showed a significant increase in

CAT activity after forskolin treatment [31]. PMA treatment of HNF-4-CAT/WT HNF-4-transfected Hep G2 cells led to a minimal decrease in CAT activity compared to cytokine treatment (results not shown).

Co-transfection of Hep G2 cells with HNF-4-CAT/WT HNF-4 and constitutively active PKA or PKC constructs resulted in no significant decrease in HNF-4-driven CAT activity (Figure 6). However, over-expression of a constitutively active JAK2 construct led to a decrease in HNF-4-driven CAT activity similar to that seen with cytokine treatment. This decrease was not seen when a kinase-negative form of JAK2 was utilized (Figure 7). To confirm the role of JAK2, the complementary experiment was performed. NE was prepared from HepG2 cells that were pretreated with specific inhibitors of JAK2 (100 μ M AG490; Calbiochem, San Diego, CA, U.S.A.) or JAK3 (270 μ M JAK3 Inhibitor I; Calbiochem) for 45 min before cytokine stimulation. The results show that the cytokine-mediated decrease in HNF-4 binding could be blocked with the specific JAK2 inhibitor, but not by a specific JAK3 inhibitor (Figure 8).

These data show that cytokine treatment of Hep G2 cells leads to a rapid and specific decrease in HNF-4 binding activity. Furthermore, cytokine treatment also decreases HNF-4 transactivation in HepG2 cells as measured with a transiently transfected HNF-4 reporter construct. Over-expression of active JAK2 led to a decrease in HNF-4 transactivation, while blocking JAK2 activity before cytokine treatment inhibited their effect on HNF-4 binding. Over-expression of constitutively active

Figure 4 PEPCK CAT and CRE CAT activity in HepG2 cells

(A) PEPCK CAT activity with cytokines and WT HNF-4 over-expression. (B) CRE CAT activity with over-expression of active (PKA Act) and non-active (PKA Neg) PKA constructs.

PKA or PKC had no effect. These data would suggest that the cytokine/injury effect on HNF-4 binding activity and transactivation involves post-translational changes in HNF-4. These changes appear to be related to a rapid phosphorylation of HNF-4, which affects its transactivation activity. Furthermore, this phosphorylation event appears to be related to the activities of a JAK2 signalling pathway, but not to the activities of PKA or PKC.

DISCUSSION

The liver's response to a systemic injury *in vivo* involves rapid changes in the functions of several liver-enriched transcription factors. Other investigators utilizing various models of injury have shown significant changes in a number of liver-enriched transcription factors including C/EBP α , C/EBP β , C/EBP γ [32], HNF-3 α [33], HNF-1 and HNF-4 [34]. In a rabbit model of the APR, binding and transactivation by C/EBP β and C/EBP γ were noted to be significantly modulated and these changes in binding and transactivation were related, in part, to a phosphorylation of these two factors [23]. Others have shown that transactivation by C/EBP β is partially dependent on its phosphorylation, and several protein kinases have been implicated including mitogen-activated protein (MAP) kinase [35], calmodulin-dependent protein kinase II [36], PKA [37], and PKC [38,39]. HNF-4 has been shown to be a phosphoprotein, being phosphorylated on serine, threonine, and tyrosine [40,41].

Tyrosine phosphorylation of HNF-4 has been shown to affect subnuclear localization, as well as binding activity and transactivation [40].

Several different models of injury have been shown to have various effects on HNF-4. Partial hepatectomy and sham hepatectomy in rats both demonstrated small decreases in HNF-4 mRNA 46 h after injury, with minimal changes in HNF-4 protein levels [34]. Stress induced by a protein-restricted diet in rats resulted in no significant changes in the HNF-4 mRNA or protein levels. However, HNF-4 DNA-binding activity, as measured by EMSA, was down by 40% [42]. While our animal model of injury and stress was quite different from those above, we observed similar effects on HNF-4. HNF-4 mRNA levels declined over time [18], but there was no acute change in HNF-4 protein concentrations, while binding activity was rapidly and significantly decreased. Furthermore, this decrease in HNF-4 binding activity after injury appeared to be partly due to a modification via phosphorylation [43].

HNF-4 phosphorylation by PKA has been shown to decrease its binding and affect HNF-4 dependent transactivation [24]. However, the ability of cAMP to decrease HNF-4-driven transcription was retained in HNF-4 constructs containing mutations in the PKA site, indicating that PKA dependent phosphorylation at these sites was not critical to cAMP down-regulation of HNF-4. PKA's effect on HNF-4 may be part of a cascade that leads to a modification of HNF-4 at other sites or modifies cofactors of HNF-4 transactivation [44]. In the HepG2 cell-culture model of injury, cytokine treatment led to a rapid

Figure 5 Cytokine treatment decreases HNF-4-directed transactivation in HepG2 cells

(A) HNF-4 CAT activity before and after cytokine or forskolin treatment (14 h). (B) Quantification by densitometry of HNF-4 activity relative to HNF-4 CAT alone.

Figure 6 HNF-4-directed transactivation independent of PKA or PKC in HepG2 cells

Quantification by densitometry of HNF-4 activity relative to cells transfected with HNF-4 CAT alone.

decrease in HNF-4 binding. However, stimulation of the PKA/cAMP pathway with forskolin did not affect HNF-4 binding, while increases in phosphorylated CREB were clearly seen. Over-expression in Hep G2 cells of the catalytic subunit of PKA, co-transfected with wild-type HNF-4 and an HNF-4 reporter

Figure 7 JAK2 over-expression leads to a decrease in HNF-4-directed transactivation in HepG2 cells

Quantification by densitometry of HNF-4 activity relative to cells transfected with HNF-4 CAT alone. (JAK2 Act, WT constitutively active JAK2; JAK2 Neg, WT constitutively non-active JAK2)

Figure 8 JAK2 inhibition blocks cytokine-mediated decrease in HNF-4 DNA binding in cytokine-stimulated HepG2 cells

EMSA and western of HNF-4 DNA binding of nuclear extract from HepG2 cells treated with cytokines in the presence of JAK2 or JAK3 specific inhibitors (JAK2 INH and JAK3 INH).

construct, did not show any changes in HNF-4-driven transactivation. The discrepancy between our results and those in the literature [24,44], with regard to the effects of PKA on HNF-4-dependent transactivation, are likely due to variations in the models used (COS cells versus HepG2 cells) and the timing and construction of transient transfection assays. A similar lack of effect on HNF-4 was seen with PMA stimulation and over-expression of active PKC constructs. However, the over-expression of WT JAK2, causing an auto-phosphorylation and a state of constitutively active JAK2, led to a significant decrease in HNF-4-dependent transactivation. This was similar to the effect seen on HNF-4 transactivation after cytokine treatment. This decrease in HNF-4 transactivation was not seen when a kinase-negative form of JAK2 was over-expressed. These results would indicate that JAK2 plays a role in modulating the transactivation activity of HNF-4. *In vitro*, gel-shift analysis with the specific JAK2 inhibitor AG490 was able to block the cytokine-induced decrease in HNF-4 binding. This effect was not seen when a JAK3 specific inhibitor, JAK3 Inhibitor I, was used. These results would indicate that the activation of JAK2 by cytokines leads to a decrease in HNF-4 binding and subsequent transactivation.

A number of cytokines including IL-6, as used in this study to mimic *in vivo* injury, are known to utilize the JAK pathway of

signal transduction [45,46]. A direct JAK2 interaction with HNF-4, similar to those involved in STAT activation, seems unlikely given the protein sequence of HNF-4. JAK2 activation has been shown to play a role in other signalling cascades, leading to changes in transactivation by several transcription factors. In studies of growth hormone (GH) signal transduction, treatment with GH leads to the phosphorylation and increased transactivation of activating transcription factor-2 (ATF-2). These changes are dependent on JAK-2 as evident from the specific inhibition of JAK-2 with AG490 or by over-expression of JAK2, which lead to a decrease or increase in transactivation respectively by ATF-2 [47]. Furthermore, it has been shown that the GH stimulation of ATF-2-mediated transactivation requires p38 MAP kinase, which is dependent on JAK2 activation. Additionally, the transactivation of another transcription factor, CHOP (a C/EBP family member), by GH is also dependent on p38 MAP kinase and JAK2 [48].

Changes in HNF-4 binding have been seen in several clinically relevant models. In starvation [24], protein restriction [43] and, demonstrated here with burn trauma or pro-inflammatory cytokine treatment, HNF-4 binding activity is decreased without significant change in HNF-4 protein concentration. However, in a model of liver regeneration, neither liver resection nor sham hepatectomy leads to changes in HNF-4 protein concentration. Unfortunately, the effects on HNF-4 binding activity in these models were not defined [33,49]. In our models of burn injury and pro-inflammatory cytokine treatment, we have shown that these post-transcriptional changes in HNF-4 binding involve a modification by phosphorylation and affect both binding affinity and transactivation. Furthermore, we have shown that these effects on HNF-4 transactivation are not seen with stimulation of PKA or PKC, but can be mimicked or blocked by the activation or inhibition of JAK2.

The injury response is complex and highly regulated. Induction of the APR by injury, involving large changes in protein production by the liver, utilizes significant changes in the transcriptional apparatus. This transformation of the liver can be viewed as a reprogramming or change in phenotype. One can speculate that the rapid change in HNF-4 binding and transactivation after injury are part of the mechanism required to change the phenotype of the liver to one better equipped to enhance the organism's survival. While HNF-4 alone is not critical to the liver's APR, its manipulation by systemic events reflects the general mechanism at work that leads to these profound changes.

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