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Modulation of hepatocyte nuclear factor-4 α function by the peroxisome-proliferator-activated receptor- γ co-activator-1 α in the acute-phase response

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Abstract

HNF-4 α (hepatocyte nuclear factor-4 α) is a key regulator of liver-specific gene expression. To understand the mechanisms governing the regulation of HNF-4 α function during the APR (acute-phase response), the effects of transcription co-activators, including p300, PGC-1 α (peroxisome-proliferator-activated receptor- γ co-activator-1 α) and SRC (steroid receptor co-activator)-1 α were investigated in an injury cell model. We have shown previously that the HNF-4 α -sensitive APR genes *ApoB* (apolipoprotein B), *TTR* (transthyretin) and *α 1-AT* (α 1-antitrypsin) were regulated at the DNA binding and transcriptional levels after cytokine stimulation. We now show that co-activators have a differential impact on the transactivation of HNF-4 α -sensitive genes via HNF-4 α -binding sites in *ApoB*, *TTR* or *α 1-AT* promoters. PGC-1 α strongly enhances the transactivation of *ApoB* and *α 1-AT* and, to a lesser extent, of *TTR*, whereas SRC-1 α and p300 only have a weak or no effect on these three genes. More importantly, it was found that PGC-1 α has a novel role in the modulation of the binding ability of HNF-4 α in response to cytokine treatment. Using *in vitro* and *in vivo* approaches, electrophoretic mobility-shift and chromatin immunoprecipitation assays, we demonstrate that the reduced HNF-4 α -DNA binding ability induced by cytokines is eliminated by overexpression of PGC-1 α . Cytokine treatment does not significantly alter the protein levels of HNF-4 α and PGC-1 α , but it does reduce the recruitment of PGC-1 α to HNF-4 α -binding sites and thereby decreases transcriptional activity. These results establish the importance of PGC-1 α for HNF-4 α function and describe a new HNF-4 α -dependent regulatory mechanism that is involved in the response to injury.

Keywords

acute-phase response (APR); cytokine; gene regulation; HNF-4 α (hepatocyte nuclear factor-4 α); transcriptional co-activator

INTRODUCTION

The APR (acute-phase response) is an orchestrated response to deviations in homeostasis, such as tissue injury, infection or inflammation. The systemic acute-phase reaction is initiated by proinflammatory cytokines such as IL (interleukin)-1 and TNF (tumour necrosis factor), as well as by acute-phase cytokines from the IL-6 family. The APR is accompanied by specific changes in the concentration of plasma proteins called the APPs (acute-phase proteins), which are largely attributable to alterations in their rate of synthesis in the liver

[1-3]. A better understanding of the regulation of APR gene expression is a crucial step to develop safe and effective ways to manipulate this important clinical response. Although the molecular mechanisms of APR regulation have not been totally defined, the expression of APR genes induced by binding of inflammatory mediators to their respective receptors on hepatocytes are primarily modulated at the transcriptional level through the manipulation of transcription factors [4,5].

HNF-4 α (hepatocyte nuclear factor-4 α) is one of the most important liver-enriched transcription factors for hepatocyte differentiation and phenotype. HNF-4 α plays important roles not only in the specification of the hepatic phenotype during liver development, but also in the transcriptional regulation of genes involved in glucose, cholesterol, fatty acids and xenobiotic metabolism, and in the synthesis of blood coagulation factors [6-9]. Disruption of *HNF-4 α* leads to an early embryonic lethal phenotype associated with a failure of differentiation of visceral endoderm [10]. Genome-scale location analysis revealed surprising results for HNF-4 α in hepatocytes. The number of genes that exhibit binding of HNF-4 α to their regulatory regions was much larger than that observed with other typical liver-specific regulators [11]. Therefore HNF-4 α has been recognized to be a broad regulator of liver function.

The regulation of gene activity at the transcriptional level has been thought to occur in part via changes in the amounts or activities of transcription factors. However, previous results has shown that important physiological control of gene-regulatory systems are not solely the effect of transcription factors. Co-activator proteins also participate in gene regulation. Indeed, it has been shown that HNF-4 α , like other nuclear receptors, provokes gene activation in concert with transcription co-activators and co-repressors through its AF (activation function) domains. Studies have reported that HNF-4 α interacts with the p160 family co-activators SRC (steroid receptor co-activator)-1, -2 and -3 [12-14] and that HNF-4 α activity can be enhanced by the presence of CBP [CREB (cAMP-response-element-binding protein)-binding protein]/p300 [15,16]. In addition, HNF-4 α has been shown to be involved in hepatic nutrient metabolism through interactions with the co-activator PGC-1 α (peroxisome-proliferator-activated receptor- γ co-activator-1 α) [17,18].

In the present study, utilizing a cytokine-stimulated HepG2 cell model of injury, we explore the possible roles that the transcription co-activators p300, PGC-1 α , and SRC-1 α play in the regulation of the HNF-4 α -sensitive APR genes *ApoB* (apolipoprotein B), *TTR* (transthyretin) and *α 1-AT* (α 1-antitrypsin). All three genes have unique HNF-4 α -specific binding sites in their promoter regions [19] and differential biological responses to injury in their clinical presentation. We demonstrate that PGC-1 α not only strongly enhances the transactivation potentials of the *ApoB*, *TTR* and *α 1-AT* genes in a HNF-4 α -binding-site-specific manner, but also it restores the HNF-4 α -binding ability which was repressed by treatment with cytokines. The observations presented here reveal a novel regulating mechanism for the control of HNF-4 α -dependent APR gene expression.

EXPERIMENTAL

Cell culture

HepG2 cell (human hepatoblastoma cell) and HEK-293 cell (human embryonic kidney cell) lines were obtained from the A.T.C.C. (Manassas, VA, U.S.A.). The cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with penicillin (100 units/ml), streptomycin (100 μ g/ml) and 10% (v/v) heat-inactivated fetal bovine serum (Mediatech, Herndon, VA, U.S.A.) at 37°C in a humidified atmosphere with 5% CO₂. At 6 h prior to the experiment, cells were placed into serum-free medium. The injury response in HepG2 cells was stimulated by incubation with a cytokine mixture consisting of 1 ng/ml of

recombinant human IL-1 β , 10 ng/ml of IL-6 and 10 ng/ml of TNF- α (PeproTech, Rocky Hill, NJ, U.S.A.) in serum-free medium for 18 h at 37°C.

Plasmids

The luciferase reporter plasmids, containing three tandem copies of the specific HNF-4 α -binding sites derived from *ApoB* (−63—81), *TTR* (−154—136) or *α 1-AT* (−106—124) promoter regions, and the HNF-4 α shRNA (short hairpin RNA) expression plasmids were constructed as described previously [20]. Rat HNF-4 α was a gift from Dr A. Kahn [21]; PGC-1 α (pcDNA3-HA-PGC-1 α) was a gift from Dr A. Kralli (Department of Chemical Physiology; The Scripps Research Institute, La Jolla, CA, U.S.A.); p300 was purchased from Upstate Biotechnology; and SRC-1 α (pCR3.1-SRC-1 α) was a gift from Dr B. W. O'Malley (Baylor College of Medicine, Houston, TX, U.S.A.).

RNA isolation and quantitative real-time PCR

Total RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions and treated with 27 units of RNase-free DNase I (Qiagen). Quantitative real-time PCR analysis was conducted using the ABI 7000 Sequence Detection System (Applied Biosystems) and relative mRNA expression was quantified using the comparative Ct ($\Delta\Delta$ Ct) method following the manufacturer's instructions. Amplification of β -microglobulin was used in each reaction as an internal reference gene. Assay-on-Demand PCR primers and TaqMan® MGB probe mix used are PGC-1 α (Hs00173304_m1) and β -microglobulin (Hs99999907 m1) (Applied Biosystems). Assays were performed in triplicate [20].

Transient-transfection assay

HepG2 cells were grown to approx. 80% confluence in 48-well tissue-culture plates. The luciferase reporter and expression plasmid were transfected into HepG2 cells as described previously [20]. Cells were treated with IL-1 β , TNF- α and IL-6 for 18 h as described above. Cells were harvested for assay of luciferase-reporter activity using the Dual-Luciferase assay system (Promega). Firefly luciferase activity values were divided by *Renilla* luciferase activity values to obtain normalized luciferase activity values. Each assay was performed in triplicate and results are means $\pm$.D. ($n = 3$).

EMSA (electrophoretic mobility-shift assay)

The preparation of nuclear extracts from HepG2 cells and EMSA were performed as described previously [20].

Immunoblot analysis

Cell lysates were isolated for immunoblot analysis using antibodies against PGC-1 α , HNF-4 α (Santa Cruz Biotechnology) or β -actin (Sigma) as described previously [20].

ChIP (chromatin immunoprecipitation) assay

HepG2 cells were grown in 100-mm diameter culture dishes to 80% confluence and transfected with the PGC-1 α expression plasmid using the FuGENE HD transfection reagent following the manufacturer's instructions (Roche) and 24 h post-transfection, the cells were grown in serum-free medium for 6 h and then incubated with or without cytokines for 18 h. ChIP assays were performed using an EZ ChIP kit (Upstate Biotechnology) following the manufacturer's protocol. An antibody against PGC-1 α (10 μ g; Santa Cruz Biotechnology) was used to immunoprecipitate DNA–protein complexes, and additional mock immunoprecipitations with normal rabbit Ig (2 μ g; Santa Cruz Biotechnology) were utilized to detect background DNA binding. Real-time PCR was used to analyse

immunoprecipitated DNA and input control DNA. Determinations were performed in triplicate on 3 μ l of bound chromatin, 3 μ l of normal rabbit control IgG immunoprecipitation, or 3 μ l of a 10-fold dilution of input chromatin in a 20 μ l reaction volume. *ApoB*, *TTR* and *α 1-AT* promoter-specific primers (Assays by Design, Applied Biosystems) were designed as follows: *ApoB*, forward primer 5'-GAAGCCAGTGTAGAAAAGCAAACAG-3', reverse primer 5'-GCGCCAGGATTGCAAAAGG-3' and TaqMan® FAM (6-carboxyfluorescein) dye-labelled probe 5'-TCCAAAGGGCGCCTCC-3'; *TTR*, forward primer 5'-CGAATGTTCCGATGCTCTAATCTCT-3', reverse primer 5'-CAAACCTGCTGATTCTGATTATTGACTT-3' and probe 5'-CCCATACAAATATGAACCTTG-3'; and *α 1-AT*, forward primer 5'-GGCGGGCGACTCAGATC-3', reverse primer 5'-CCAGTTATCGGAGGAGCAAACA-3' and probe 5'-CCAGTGGACTTAGCCCC-3'. Probes were designed to specifically probe the HNF-4 α -binding sites in the promoter region of *ApoB* (−63 to −81), *TTR* (−154 to −136), and *α 1-AT* (−106 to −124) [19]. Amplification of input chromatin was used as an internal reference gene in the same reactions. Relative quantification was determined by using the comparative Ct ($\Delta\Delta$ Ct) method.

Statistical analyses

Statistical analyses were performed using the Student's *t* test. $P < 0.05$ was considered to be statistically significant.

RESULTS

The effects of co-activators on HNF-4 α -mediated APR gene transactivation

To identify whether the co-activators p300, PGC-1 α and SRC-1 α regulate HNF-4 α -mediated APR gene transactivation potential, co-activator expression plasmids and luciferase reporters (there porter contains three copies of the HNF-4 α -specific binding site derived from *ApoB*, *TTR* or *α 1-AT* promoters) were transiently co-transfected into HepG2 cells. The results in Figure 1 show that transfection of co-activators, especially PGC-1 α , produced a dramatic increase in luciferase activity for the *ApoB*, *TTR* or *α 1-AT* genes via HNF-4 α -specific-binding sites (36-, 2.6- and 29-fold respectively), and SRC-1 α also led to a small but significant increase in activity above basal level ($P < 0.05$). In contrast, p300 did not substantially increase transactivation activity of any of the three promoters studied. Utilizing HepG2 cells in which RNA interference had been used to knockdown endogenous HNF-4 α , and the non-hepatic HEK-293 cells lacking endogenous HNF-4 α [22], we further tested whether the increase in luciferase activity elicited by PGC-1 α is dependent on HNF-4 α . It has been reported previously by our laboratory that the mRNA and protein expression levels of HNF-4 α can be successfully reduced by approx. 70–80% of the non-targeted control level by using RNA interference technology in HepG2 cells [20]. As shown in Figure 2, when the cells were transfected with shHNF-4 α or control shRNA in the presence or absence of the PGC-1 α expression plasmid, the reduction of HNF-4 α protein not only decreased the luciferase activity by approx. 60% of the control level, but also significantly attenuated the enhanced activity stimulated by overexpression of PGC-1 α for all three genes compared with non-targeted HNF-4 α controls ($P < 0.05$). In the HepG2 HNF-4 α knockdown cells transfected with the PGC-1 α expression plasmid, a higher level of luciferase activity was observed compared with the non-targeted control cells (Figure 2). This is likely to be the result of incomplete silencing of endogenous HNF-4 α by shRNA. To test this possibility, HEK-293 cells, lacking endogenous HNF-4 α , were used. The transfection of HEK-293 cells with the HNF-4 α expression plasmid alone caused a marked increase in ApoB-, TTR- or α 1-AT-luciferase activity (15-, 12- and 20-fold respectively) (Figure 3). The overexpression of both of PGC-1 α and HNF-4 α in HEK-293 cells exhibited

a synergistic effect on transactivation, where the co-transfection of both factors greatly increased all three HNF-4 α -dependent reporter gene activities (30-, 23- and 37-fold increase respectively). In contrast, transfection of PGC-1 α alone in HEK-293 cells revealed only a minimal (4-fold) increase in all three genes (Figure 3). This experimental evidence suggests that PGC-1 α can strongly enhance the transactivation potentials of the luciferase reporters containing HNF-4 α -binding sites from *ApoB*, *TTR* or *a1-AT* promoters. Furthermore, we demonstrate that the modulation effect of PGC-1 α is mediated through HNF-4 α . Similar experiments reveal that p300 and SRC-1 α have no or minimal impact on the transcriptional potential of these three genes.

PGC-1 α induces HNF-4 α -mediated APR gene transactivation in a dose-dependent manner

To further verify that the HNF-4 α -dependent APR gene transactivation potential could be regulated by PGC-1 α , the dose-response of PGC-1 α on the transactivation of ApoB-, TTR- and a1-AT-luciferase reporters was examined. As shown in Figure 4, an increasing level of PGC-1 α expression in HepG2 cells caused a dramatic increase in the transactivation of all three APR genes in a dose-dependent manner. Although overexpression of PGC-1 α was able to increase luciferase activity, different degrees of response to ectopic PGC-1 α was found among these three genes. *ApoB* was strongly activated by PGC-1 α and *a1-AT* was moderately changed, whereas *TTR* had the weakest response to PGC-1 α . The TTR-luciferase activity was more than 20-fold lower than the ApoB-luciferase activity with the same amount of PGC-1 α expression plasmid transfected. Increasing the amount of exogenous PGC-1 α further did not enhance the TTR-luciferase activity to that seen for ApoB or *a1-AT*. The impact of increasing PGC-1 α levels on HNF-4 α -dependent luciferase activity is, in general, positive, but the different degrees of co-activation of individual genes reflects the intrinsic and unique HNF-4 α -binding affinity for each individual binding site.

The protein levels of PGC-1 α and HNF-4 α are not significantly affected by cytokine treatment

It has been shown that cytokines can induce a classic APR in HepG2 cells [20]. To investigate whether PGC-1 α plays a role in the APR, the expression levels of PGC-1 α were determined in HepG2 cells treated with cytokines for 18 h or left untreated. The protein level of PGC-1 α detected by Western blot was barely visible after incubation in the presence or absence of cytokines (Figure 5B). Overexpression of PGC-1 α in HepG2 cells caused an approx. 8-fold increase in both mRNA (Figure 5A) and protein levels (Figure 5B) compared with untransfected cells. The protein level of HNF-4 α was not significantly affected by overexpression of PGC-1 α or treatment with cytokines alone or a combination of both compared with control (without any treatment) (Figure 5B). These experiments indicate that the protein expression levels of PGC-1 α and HNF-4 α do not change substantially in the cytokine-induced APR cell model.

Overexpression of PGC-1 α partially rescues the repressed HNF-4 α -DNA binding ability elicited by cytokine treatment

We have demonstrated previously that the binding activity of HNF-4 α decreases quickly after injury in our cell culture and mouse-burn-injury models [20,23,24]. In this study, we investigated whether co-activators could improve the binding activity of HNF-4 α in a cytokine-induced APR cell model. HepG2 cells were transfected with the co-activator expression plasmids expressing p300, PGC-1 α or SRC-1 α and then treated with cytokines for 18 h or left untreated. The binding ability of HNF-4 α was determined by EMSA. As shown in Figure 6, cytokine treatment caused a reduction in HNF-4 α binding to the *ApoB*, *TTR* and *a1-AT* genes in a binding-site-specific manner. The greatest suppression was observed for the *TTR* promoter HNF-4 α -binding site, where binding was reduced to approx. 35% of the untreated level, followed by *a1-AT* (59% of the control level), whereas *ApoB*

was reduced to 77% of the control level. The overexpression of PGC-1 α did not significantly increase the binding ability of HNF-4 α in cytokine-untreated cells. However, exogenous PGC-1 α partially reversed the binding inhibition of *ApoB*, *TTR* and *a1-AT* induced by cytokines, in which the increased PGC-1 α concentration eliminated the difference between cytokine-treated and -untreated cells ($P > 0.05$). The greatest improvement in HNF-4 α binding by overexpression of PGC-1 α in cytokine-treated cells was shown for *TTR*. The ability of HNF-4 α to bind to the *TTR* gene in PGC-1 α -transfected cells was significantly higher than in the non-transfected cells following cytokine treatment ($P < 0.001$). These findings indicate that PGC-1 α could partially overcome the inhibiting effect of cytokines on HNF-4 α binding, and this effect depends in part on the binding affinity of HNF-4 α for a specific binding site. We did not detect a significant effect of the transcription co-activators p300 or SRC-1 α on HNF-4 α -DNA binding to the *ApoB*, *TTR* and *a1-AT* genes in cytokine-treated cells (results not shown).

Cytokines inhibit HNF-4 α -binding ability by reducing recruitment of PGC-1 α to HNF-4 α

We further investigated within intact cells whether the interaction of PGC-1 α and HNF-4 α occurs, and what role cytokines would play in this process. ChIP assays were designed and performed to determine the effect of PGC-1 α on HNF-4 α binding to HNF-4 α -specific binding sites in the promoter regions of the *ApoB*, *TTR* and *a1-AT* genes *in vivo*. Fragmented chromatin from formaldehyde-cross-linked HepG2 cells was subjected to immunoprecipitation with a anti-PGC-1 α antibody or with normal rabbit Ig as a negative control. The immunoprecipitates were then analysed by real-time PCR using specific probes that flank HNF-4 α -binding sites in the promoters of the *ApoB*, *TTR* and *a1-AT* genes. As can be seen from Figure 7, PGC-1 α immunoprecipitates yielded an approx. 10-fold higher level of HNF-4 α -bound DNA than the non-specific control IgG immunoprecipitates, indicating that a specific interaction between PGC-1 α and HNF-4 α occurred *in vivo* for all three genes. Cytokine treatment decreased the amount of PGC-1 α immunoprecipitates bound to all three HNF-4 α -binding sites, a pattern similar to that observed with EMSA (Figure 6). These results demonstrate that PGC-1 α forms a complex with HNF-4 α inside intact cells, and that cytokines probably reduce the association of PGC-1 α with HNF-4 α and thus interfere with HNF-4 α -DNA binding ability. To further test these findings, PGC-1 α was transfected into HepG2 cells to determine if PGC-1 α affects HNF-4 α binding to its chromatin *in vivo*. The ectopic expression of PGC-1 α resulted in a significant increase in HNF-4 α binding to the *a1-AT* gene ($P < 0.01$) and a marginal enhancement in *ApoB* binding compared with untreated cells. Most importantly, exogenous PGC-1 α abolished the inhibitory effect of cytokines on HNF-4 α binding in all three genes (Figure 7). Taken together, these results suggest that cytokine-induced inhibition of HNF-4 α -DNA binding could be caused in part by interfering with the recruitment of PGC-1 α to HNF-4 α or by reducing the affinity of the interaction between PGC-1 α and HNF-4 α , and subsequently results in a reduction in the PGC-1 α -HNF-4 α complex available for efficient HNF-4 α binding and transactivation.

Overexpression of HNF-4 α enhances the basal and cytokine-repressed HNF-4 α -binding ability

To test the hypothesis that PGC-1 α facilitates HNF-4 α binding via the formation of a complex with HNF-4 α , HepG2 cells were transfected with exogenous HNF-4 α to determine whether the overexpression of HNF-4 α could mimic the effect of PGC-1 α . EMSA (Figure 8) shows that increased HNF-4 α binding to the *ApoB*, *TTR* or *a1-AT* gene was identified in both non-treated and cytokine-treated cells transfected with exogenous HNF-4 α , a pattern similar to that seen by ChIP (Figure 7). In contrast, knockdown of endogenous HNF-4 α abolished protein-DNA binding, indicating that a certain amount of HNF-4 α is required for

efficient binding. Figures 7 and 8 illustrate that overexpression of PGC-1 α or HNF-4 α has a comparable effect on HNF-4 α binding.

DISCUSSION

The role of transcription factors in the control of gene expression is well documented. However, the role played by transcription co-activators in this process needs to be better defined, and the effect of co-activators on HNF-4 α targeting APR genes in response to injury is little known. To gain insights into the function of co-activators in the regulation of HNF-4 α -sensitive APR gene expression, we analysed the impact of the transcription co-activators p300, PGC-1 α and SRC-1 α on a cytokine-induced injury cell model. We hypothesized that transcription co-activators exert their effects by docking to the transcription factor HNF-4 α , and recruiting the complex to HNF-4 α -specific recognition sequences on the promoter regions of HNF-4 α -sensitive APR genes, thereby mediating their transcriptional function during APR.

To test our hypotheses, we investigated the functional significance of co-activators on HNF-4 α -dependent reporter assays using a luciferase reporter plasmid containing the HNF-4 α binding site of the APR genes *ApoB*, *TTR* and *a1-AT*. We observed that overexpression of PGC-1 α significantly up-regulates the reporter activity for these three genes in HepG2 cells (Figures 1, 2 and 4), demonstrating that PGC-1 α enhances HNF-4 α -dependent reporter activity. To further determine if the action of PGC-1 α on transactivation is HNF-4 α -mediated, HepG2 cells knockdown endogenous HNF-4 α (by RNA interference) and non-hepatic HEK-293 cells lacking endogenous HNF-4 α were used. In both cases, the activity of luciferase reporter stimulated by overexpression of PGC-1 α significantly decreased when endogenous HNF-4 α was absent (HEK-293 cells, Figure 3) or artificially reduced by shRNA (HepG2 cells, Figure 2). These results suggest that PGC-1 α functionally contributes to HNF-4 α -dependent APR gene expression.

Although *ApoB*, *TTR* and *a1-AT* have been previously identified as HNF-4 α targets [19], and p300, PGC-1 α and SRC-1 α have been documented as HNF-4 α co-activators [14,15,17,18], the HNF-4 α -dependent transcriptional potential of *ApoB*, *TTR* and *a1-AT* genes is induced robustly only with overexpression of PGC-1 α , indicating a dominant and direct role of PGC-1 α . In contrast, p300 and SRC-1 α appeared to not act directly on the HNF-4 α -binding site in these three genes. It is also noteworthy that the reporter activities of *ApoB*, *TTR* and *a1-AT* are differentially increased by PGC-1 α . The activity induced by PGC-1 α in *TTR* is much lower than in *ApoB* or *a1-AT* (Figures 1, 2 and 4). This diversity among different genes probably reflects the effect of PGC-1 α being dependent on HNF-4 α and the specific HNF-4 α -DNA binding sequence found in each gene. This is consistent with the findings seen in HNF-4 α -binding assays (Figure 6). It has recently been shown that small differences in response elements in promoters may determine whether a productive interaction occurs between a bound transcription factor and a co-activator [25]. DNA-sequence variation in NF- κ B (nuclear factor κ B) sites dictates which co-activator will interact with bound NF- κ B [26]. Another possible explanation is that *TTR* has a more complex promoter which may require the presence of other transcription factors for its activation [27]. Alternatively, PGC-1 α may confer a subtle conformational change upon its partner transcription factor that affects its ability to effectively bind certain DNA sequences. This implies that the sequence alterations in binding sites may affect the conformation of a transcription factor, thereby determining how the co-activator could effectively act.

To further verify the dominant effect of PGC-1 α on HNF-4 α function, a luciferase reporter containing the proximal promoter of *TTR* (−191→+5) was constructed and tested. This reporter revealed a similar co-activation influence on activity of the reporter construct with

three copies of the isolated HNF-4 α -binding site (–154—136) from the *TTR* promoter on overexpression of PGC-1 α in HepG2 cells. In contrast, PGC-1 α failed to enhance the transcriptional activity when the HNF-4 α -binding site was mutated in the *TTR* proximal promoter (–191—+5) ([28] and results not shown). These results reinforce the role of PGC-1 α in the function of the HNF-4 α -dependent *TTR* gene.

In light of the results that PGC-1 α regulates the transactivation of several key APR genes, we asked whether co-activators are involved in the modulation of HNF-4 α -DNA binding during the APR. In previous studies, it has been demonstrated that HNF-4 α -DNA binding quickly decreased after injury [20,23]. The molecular mechanisms underlying this response are not understood. The results from ChIP (Figure 7) show that the complex of PGC-1 α and HNF-4 α is successfully immunoprecipitated with an anti-PGC-1 α antibody, suggesting that PGC-1 α and HNF-4 α can interact with each other in intact cells. Treatment of cells with cytokines, followed by immunoprecipitation with an anti-PGC-1 α antibody exhibited decreased HNF-4 α -DNA binding for all three genes, and the pattern was similar to a ChIP assays with an anti-HNF-4 α antibody reported previously [20] and to the *in vitro* HNF-4 α -binding assay (Figure 6). These results indicate that cytokine treatment results in less PGC-1 α being recruited to the HNF-4 α -binding site than in untreated cells. More importantly, the inhibitory effect of cytokines on HNF-4 α binding could be abolished by overexpression of PGC-1 α (Figure 7). In agreement with these findings, the effect of overexpression of PGC-1 α also could be imitated by overexpression of HNF-4 α (Figure 8). From these results, a plausible mechanism is proposed. The reduced HNF-4 α -binding ability in the presence of cytokines is probably not the result of a simple alteration in the protein levels of PGC-1 α or HNF-4 α (Figure 5); instead, cytokines lessen the recruitment of PGC-1 α to HNF-4 α or decrease the interaction of PGC-1 α and HNF-4 α , in turn reducing the availability of the HNF-4 α -PGC-1 α complex bound to HNF-4 α -binding sites. Thus, when ectopic PGC-1 α is transfected into cytokine-treated cells, the increased abundance of PGC-1 α provides more PGC-1 α -HNF-4 α complexes for binding to HNF-4 α -binding sites, thereby overcoming the cytokine-induced inhibitory effect.

When comparing the results from EMSA, ChIP and reporter assays, the response to overexpression of PGC-1 α is varied. Using an *in vitro* EMSA (Figure 6), the presence of ectopic PGC-1 α did not show an apparent increase in HNF-4 α -binding ability for the genes tested in cytokine-untreated cells. However, utilizing an *in vivo* ChIP assay, the presence of PGC-1 α caused a significant increase in HNF-4 α bound to *a1-AT* (Figure 7). A more potent effect of PGC-1 α for all three genes was found in the luciferase-reporter assay (Figures 1-4). This may be the result of a difference in the sensitivity for detecting changes among these assays. This discrepancy may also imply that, in intact cells, additional cofactors, protein modifications (e.g. phosphorylation) or some other aspect of the cellular environment may play a role in either DNA binding or transcriptional activity.

In summary, our results establish the importance of the co-activator PGC-1 α for HNF-4 α -mediated expression of APR genes in hepatic cells. Moreover, our results provide new insights into the regulatory mechanisms of the HNF-4 α -dependent *ApoB*, *TTR* and *a1-AT* gene response to cytokine stimulation. The physical interaction between PGC-1 α and HNF-4 α has been shown *in vitro* previously [18]. The interaction between PGC-1 α and HNF-4 α occurs between the LXXLL motif in the N-terminus of PGC-1 α and the AF-2 domain of HNF-4 α [18]. In the present study, we have found that PGC-1 α interacts with HNF-4 α *in vivo* by ChIP, and this co-activator interaction seems to be a key component in determining the activation of transcription by HNF-4 α . Since co-activator concentration is limited *in vivo*, it is logical to assume that their availability in cells plays a major role in determining the state of activation of a given transcription factor, such as HNF-4 α . The availability of co-activators, the interaction affinity between co-activators and transcription

factors, and protein–DNA binding ability that relies on specific binding-site sequences give further layers of complexity for controlling the transcriptional response to injury. A better understanding of these regulatory mechanisms *in vivo* is a mandatory prerequisite before any successful manipulation of the APR at the molecular level can be undertaken for therapeutic intervention after injury.

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Abbreviations used:

AF	activation function
ApoB	apolipoprotein B
APR	acute-phase response
α1-AT	α 1-antitrypsin
ChIP	chromatin immunoprecipitation
EMSA	electrophoretic mobility-shift assay
HEK-293 cell	human embryonic kidney cell
HNF-4α	hepatocyte nuclear factor-4 α
IL	interleukin
NF-κB	nuclear factor κ B
PGC-1α	peroxisome-proliferator-activated receptor- γ co-activator-1 α
shRNA	short hairpin RNA
SRC	steroid receptor co-activator
TNF	tumour necrosis factor
TTR	transthyretin

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Figure 1. PGC-1 α is an important co-activator for HNF-4 α -dependent APR reporter gene activity in HepG2 cells

The luciferase reporter plasmid of pGL3-promoter (75 ng) containing three in-tandem HNF-4 α -binding sites derived from (A) ApoB (-63—81), (B) TTR (-154—136) or (C) α 1-AT (-106—124) promoter regions (see right-hand panels for details) was transfected alone (control; Basal) into HepG2 cells or co-transfected with a co-activator expression plasmid (p300, PGC-1 α or SRC-1 α , 225 ng). All cells were also co-transfected with pRL-CMV (12 ng) to normalize for transfection efficiency. Cells in 48-well plates were harvested 48 h after transfection, and luciferase activities were assayed. Values represent firefly luciferase/*Renilla* luciferase enzymatic activity ratios. The normalized luciferase activity in the control sample was set to 1. Results are means $\pm$ S.D. (n = 4), with experiments performed in triplicate. * P < 0.05 and ** P < 0.01 compared with control. SV40, simian virus 40.

Figure 2. Knockdown of endogenous HNF-4 α impairs the co-activation effect of PGC-1 α on the luciferase activity of HNF-4 α target genes

HepG2 cells were targeted by shHNF-4 α or control shRNA and then transfected with reporter plasmids (A) ApoB-luciferase, (B) TTR-luciferase or (C) α 1-AT-luciferase in the absence or the presence of the PGC-1 α expression plasmid. The normalized luciferase activity in the cells transfected with control shRNA in the absence of PGC-1 α was set at 1. Results are means $\pm$ S.D. ($n = 3$) * $P < 0.05$ and ** $P < 0.01$ compared with non-shHNF-4 α -treated control cells-in the absence or the presence of PGC-1 α expression plasmid.

Figure 3. Effect of PGC-1 α on the transactivation of HNF-4 α -dependent APR genes is synergistically boosted by HNF-4 α in HEK-293 cells

Plasmids expressing PGC-1 α , HNF-4 α or a combination of PGC-1 α and HNF-4 α were co-transfected with the luciferase reporter into HEK-293 cells lacking endogenous HNF-4 α . Results are means $\pm$ S.D. for triplicate wells from three independent assays. * P < 0.05 and ** P < 0.01 indicate a significant difference compared with control cells (not transfected with HNF-4 α and PGC-1 α).

Figure 4. PGC-1 α dose-dependently increases luciferase-reporter activity of ApoB, TTR and α 1-AT

HepG2 cells were co-transfected with reporter plasmids ApoB-luciferase, TTR-luciferase or α 1-AT-luciferase and increasing quantities of the PGC-1 α expression plasmid as indicated. Results are means $\pm$ S.D. ($n=3$), with each experiment performed in triplicate.

Figure 5. Cytokine treatment does not significantly alter protein levels of PGC-1 α and HNF-4 α HepG2 cells were transfected with PGC-1 α expression plasmid or an empty vector (control; Basal) and 24 h after transfection, the cells were grown in serum-free medium for 6 h and then incubated with (white bars) or without (grey bars) cytokines (IL-1 β , 1 ng/ml; IL-6, 10 ng/ml; and TNF- α , 10 ng/ml) for 18 h. mRNA and whole-cell lysates were prepared. Real-time PCR (**A**) and Western blotting (**B**) were performed as described in the Experimental section. In (**A**), mRNA levels in each sample were normalized against the β -microglobulin mRNA level. The abundance of mRNA in the absence of PGC-1 α transfection (Basal) was set at 1. Results are means $\pm$ S.D. ($n = 3$) and no significant difference was found between cytokine-treated and untreated cells ($P > 0.05$). The result shown in (**B**) is a representative experiment, replicated three times with similar results. kD, kDa.

Figure 6. The impact of PGC-1α on HNF-4α binding to the ApoB, TTR, and α1-AT gene promoters

EMSA were performed to detect the effect of co-activators on HNF-4α–DNA binding ability. HepG2 cells were transfected with empty vector (control; Basal) or the PGC-1α expression plasmid and then treated with (white bars) or without (black bars) cytokines for 18 h. Nuclear proteins were extracted from cells. The ³²P-labelled oligonucleotide probe was based on the HNF-4α-specific binding site in the ApoB, TTR or α1-AT genes [20]. Anti-HNF-4α antibody was added for supershift (SS) assay of antibody–protein–DNA complexes. Histograms (right-hand panels) show densitometric analyses of HNF-4α–DNA complexes. Values represent mean ± SD of three separate experiments. The cytokine-untreated control cells were set at 1. **P* < 0.05 and ***P* < 0.01 compared with cytokine-untreated cells in the absence (Basal) or the presence of PGC-1α expression plasmid.

Figure 7. Overexpression of PGC-1α abolishes the repressing effect of cytokines on HNF-4α-binding ability

HepG2 cells were transfected with plasmids expressing PGC-1α or empty vector and then treated with or without cytokines for 18 h. Protein interaction of PGC-1α and HNF-4α was determined by ChIP with either antibodies against PGC-1α or rabbit IgG (IgG, control). Chromatin-immunoprecipitated DNA was analysed by real-time PCR with primers and probes specific for the HNF-4α-binding sites on the *ApoB*, *TTR* and *α1-AT* gene promoters. The control samples (PGC-1α-untransfected and cytokine-untreated cells) were set at 1. The results are means ± S.D. ($n = 3$) assayed in triplicate by real-time PCR. * $P < 0.05$ and ** $P < 0.01$ indicate that the value is significantly different from control. # $P < 0.05$ indicates that the value is significantly different from PGC-1α-untransfected and cytokine-treated cells.

Figure 8. Effect of ectopic PGC-1 α on HNF-4 α binding to the APR genes could be mimicked by exogenous HNF-4 α

HepG2 cells were transfected with a HNF-4 α expression plasmid [either 5 μ g (lanes 3 and 4) or 10 μ g (lanes 5 and 6)], and 5 μ g of shHNF-4 α (lanes 7 and 8). The cells were treated or untreated with cytokines for 18 h. EMSAs were performed using a 32 P-labelled oligonucleotide probe based on the HNF-4 α -specific binding site in *ApoB*, *TTR* and *α 1-AT* promoter regions. Histograms (right-hand panels) illustrate densitometric analyses of HNF-4 α -DNA complexes. Results are means $\pm$ S.D. ($n = 3$). The cytokine-untreated control cells (lane 1, left-hand panels) were set at 1. * $P < 0.05$ and ** $P < 0.01$ compared with cytokine-untreated control cells.