Dual Role of Insulin in Transcriptional Regulation of the Acute Phase Reactant Ceruloplasmin*

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Insulin is a potent negative regulator of the response of hepatic cells to pro-inflammatory cytokines, particularly, interleukin (IL)-6. The action of insulin is target-selective because it inhibits transcription of most but not all acute phase genes. We here show that ceruloplasmin (Cp), an acute phase reactant with important functions in iron homeostasis, is subject to a unique dual regulation by insulin. IL-6 increased Cp mRNA expression in HepG2 cells by ~5-fold. Simultaneous treatment with insulin reduced this stimulation by half. Surprisingly, insulin by itself caused a 2-4-fold induction in Cp mRNA expression. The mechanism of induction by insulin was studied by transfecting into HepG2 cells chimeric constructs of the Cp 5′-flanking region driving luciferase. The activity of a 4800-bp segment of the Cp 5′-flanking region was increased 3-fold by insulin. Deletion and mutation analyses showed the requirement for a single hypoxia-responsive element in a 96-bp segment ~3600 bp upstream of the initiation site. The domains required for the two activities of insulin were distinct: The distal, hypoxia-responsive element-containing site was sufficient for Cp transactivation by insulin; in contrast, an 848-bp region adjacent to the initiation site was sufficient for IL-6 transactivation of Cp and for the inhibitory activity of insulin. The role of hypoxia-inducible factor-1 in the induction of Cp by insulin was shown by electrophoretic mobility shift assays and by the absence of insulin-stimulated Cp promoter activation in mouse Hepa c4 cells deficient in hypoxia-inducible factor-1 activity. Taken together these results show that insulin functions as a bidirectional, condition-dependent regulator of hepatic cell Cp expression. The unique regulation of Cp may reflect its dual roles in inflammation and iron homeostasis.

Vertebrates respond to tissue damage and other inflammatory stimuli by implementing a coordinated series of processes known as the acute phase reaction. A characteristic of this response, and the basis of common clinical tests, is the increase in the plasma concentration of a limited group of proteins, e.g., C-reactive protein. The major function of the acute phase response is almost certainly to protect the host against injury, trauma, infection, and other inflammatory events (1, 2). The response is initiated by surveying leukocytes that respond to these events by secretion of inflammatory mediators. These mediators have been broadly classified into two groups: (i) cytokines, such as interleukin (IL)-1, IL-1, interferon-γ, and tumor necrosis factor-α, which are the primary regulators of acute phase gene expression; and (ii) insulin (and other growth factors) and glucocorticoids, which function as modulators of cytokine action (1, 2). Among the pro-inflammatory mediators, IL-6 is considered to be the major physiological regulator of acute phase gene expression (2, 3). The liver is a principal target of inflammatory mediators, and the specific actions of these mediators with respect to the pattern of acute phase gene expression have been defined in detail in hepatocarcinoma cell lines (2). Insulin is a highly effective negative regulator of the cytokine-stimulated acute phase response in vitro (4–8) and in vivo (9, 10). Insulin inhibits IL-6-mediated induction of haptoglobin, thiostatin, complement C3, and C-reactive protein in hepatocytes and HepG2 cells (6, 11). The extent of the inhibition depends on the target protein, and insulin does not alter IL-6-induced expression of several acute phase proteins including α1-antichymotrypsin and α1-acid glycoprotein (6, 11).

Ceruloplasmin (Cp) is a 132-kDa, copper-containing acute phase protein of mainly hepatic origin. Its plasma concentration during inflammation in humans increases by ~50–100%, much less than the 100–1000-fold increases for C-reactive protein and serum amyloid A (12). The function of Cp during inflammation is unclear. Several groups have reported an antioxidant activity of Cp that protects lipids and DNA against free radical-mediated injury (13). In contrast, we and others have shown that Cp copper can cause oxidative modification of lipoproteins (14–16). In addition to its participation in free radical reactions, Cp is an important regulator of iron homeostasis. It is the major ferroxidase in plasma, catalyzing the conversion of Fe2+ to Fe3+ for binding by apo-transferrin (17). The key role of Cp in iron homeostasis is supported by recent reports of iron overload in patients with hereditary Cp deficiency (18), and in mice with targeted disruption of the Cp gene (19). These findings, together with early organ culture and animal studies (20, 21), suggest that Cp is required for efficient release of iron from cells and tissues. In contrast, Cp has been shown to mediate inward iron flux as well in some cell culture systems. Cp increases iron uptake by iron-deficient cells of hepatic and erythroid origin (22, 23), and by glioblastoma cells (24, 25); the Cp homologue fet3p has a similar role in high affinity iron uptake in Saccharomyces cerevisiae (26, 27).

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¶ Interleukin; ARNT, aryl hydrocarbon receptor nuclear translocator; C/EBP, CCAAT/enhancer-binding protein; Cp, ceruloplasmin; EMSA, electrophoretic mobility shift assay; HIF, hypoxia-inducible factor; HRE, hypoxia-responsive element; IDDM, insulin-dependent diabetes mellitus; Luc, luciferase; NIDDM, non-insulin-dependent diabetes mellitus; STAT, signal transducers and activators of transcription; VEGF, vascular endothelial growth factor.

1 The abbreviations used are: IL, interleukin; ARNT, aryl hydrocarbon receptor nuclear translocator; C/EBP, CCAAT/enhancer-binding protein; Cp, ceruloplasmin; EMSA, electrophoretic mobility shift assay; HIF, hypoxia-inducible factor; HRE, hypoxia-responsive element; IDDM, insulin-dependent diabetes mellitus; Luc, luciferase; NIDDM, non-insulin-dependent diabetes mellitus; STAT, signal transducers and activators of transcription; VEGF, vascular endothelial growth factor.
The spectrum of agents that increases plasma Cp concentration in vivo, and hepatic cell synthesis of Cp in vitro, is consistent with its involvement in both inflammation as well as iron homeostasis. Plasma levels of Cp increase in animal models of inflammation, including after injection of tumor necrosis factor-α, turpentine, IL-6, and endotoxin (28–30). Cp secretion is increased in human hepatoma cells exposed to IL-6 (31, 32), but the underlying molecular mechanism has not been investigated in detail; a recent report suggests that regulation may be post-transcriptional (33). We have shown recently that iron depletion increases hepatic cell transcription of Cp via activation of hypoxia-inducible factor (HIF)-1, which binds to a hypoxia-responsive element (HRE) in the 5′-flanking region of Cp (22, 34). HIF-1 is a heterodimer containing HIF-1α and HIF-1β/aryl hydrocarbon receptor nuclear translocator (ARNT) subunits, two basic helix-loop-helix/Per-ARNT-Sim proteins (35). Upon cell activation, HIF-1α is induced and it translocates to the nucleus where it forms dimers with HIF-1β; these dimers bind to HREs in multiple genes to induce transcription. HIF-1 is activated by iron deficiency, hypoxia, and CoCl₂, and recent evidence shows activation of HIF-1 by insulin and insulin-like growth factor (36, 37). Thus, HIF-1 activation may provide a common mechanism for induction of multiple genes by hypoxia and insulin, e.g., erythropoietin, vascular endothelial growth factor, and the glucose transporter Glut1 (36, 38).

The known transcriptional responses of Cp present a potential paradox regarding the effect of insulin on Cp gene expression. As a negative regulator of the acute phase response, insulin would be expected to diminish transcriptional activation of Cp, particularly by IL-6. In contrast, as a HIF-1-responsive protein, insulin may increase Cp expression. To investigate this paradox, we have studied the effect of insulin (and IL-6) on the transcriptional response of Cp in HepG2 cells. Like multiple other acute phase proteins, the IL-6-mediated induction of Cp is inhibited by insulin. However, our results indicate that Cp is unique among acute phase proteins in that transcriptional induction of Cp is induced by insulin, in the absence of IL-6, through HIF-1 activation. This unusual dual response of Cp to insulin may be the result of its unique metabolic positioning at the intersection of inflammation and iron metabolism.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Human Cp was purchased from Calbiochem and bovine insulin from Invitrogen. Rabbit polyclonal anti-human Cp IgG and rabbit polyclonal anti-β/aryl hydrocarbon receptor nuclear translocator (ARNT) IgG were obtained from American Type Culture Collection and National Diagnostics, Atlanta, GA) was set at either 1% O₂ for hypoxia or 20% O₂

**HepG2 Cells and Culture Conditions—**HepG2 cells were obtained from American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 mg/mL streptomycin, and 2 mM l-glutamine (Invitrogen). Mouse hepatoma cell lines Hepa-1c1c7 and Hepa c4 were kind gifts of Oliver Hankinson and were cultured in modified Eagle’s medium with the same supplements used for HepG2 cells. Cells at 50–60% confluence were used in all experiments. Cells were maintained in a humidified atmosphere containing 5% CO₂ at 37 °C. For experiments in which cells were exposed to hypoxia, oxygen tension in the chamber (Billups-Rothenberg, San Diego, CA) was set at either 1% O₂ for hypoxia or 20% O₂ for normoxia.

**Immunoblot Analysis of Cp—**Conditioned medium from serum-deprived HepG2 cells was subjected to 7% SDS-PAGE using Protogel (National Diagnostics, Atlanta, GA) and transferred by a semi-dry method to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was incubated with anti-human Cp 1:11,000 as primary antibody, and then with peroxidase-conjugated secondary antibody (1:5,000). Cp was detected by chemiluminescence using ECL (Amersham Biosciences). The blot was subsequently incubated with Coomassie Blue dye to verify uniform loading of all samples.

**RNA Blot Analysis of Cp and Other Transcripts—**RNA was isolated from HepG2 cells using TRIzol reagent (Invitrogen). Total RNA (20 μg) was denatured in formamide/formaldehyde, electrophoresed through a 1% agarose gel containing 6% formaldehyde, and then blotted onto nylon membranes (Schleicher & Schuell). After cross-linking by ultraviolet irradiation (Stratalinker, Stratagene), the filters were hybridized to 

**Construction of Vectors Containing Cp Promoter and Enhancer Sequences—**Cp promoter/enhancer constructs, engineered to contain SacI and Xhol restriction sites, were made by PCR amplification using Pfu polymerase (Stratagene), primers containing these restriction sites, and a 4,774-bp fragment of the 5′-flanking region of the human Cp gene as template (pGEM-Cp). pGEM-Cp was obtained by a PCR-based screen of a human genomic library in the bacterial artificial chromosome vector pBeloBAC11 (Research Genetics, Huntsville, AL) as described (34). A proximal construct was made from –848 to –1 (the nucleotide upstream of the translation-initiation site, which is here defined as +1) and ligated into SacI and XhoI restriction sites, were made by PCR amplification using Pfu polymerase and constructed by a semi-dry method (35). Upon cell activation, HIF-1α is induced and it translocates to the nucleus where it forms dimers with HIF-1β; these dimers bind to HREs in multiple genes to induce transcription. HIF-1 is activated by iron deficiency, hypoxia, and CoCl₂, and recent evidence shows activation of HIF-1 by insulin and insulin-like growth factor (36, 37). Thus, HIF-1 activation may provide a common mechanism for induction of multiple genes by hypoxia and insulin, e.g., erythropoietin, vascular endothelial growth factor, and the glucose transporter Glut1 (36, 38).

**Preparation of Nuclear Extracts—**Nuclear extracts were prepared from HepG2, Hepa-1c1c7, and Hepa c4 cells as described (40). Briefly, 1 ml of complete wash medium was applied to each dish, the cells were washed twice with cold phosphate-buffered saline, and then with a solution containing 10 mM Tris-HCl, pH 7.8, 1.5 mM MgCl₂, and 10 mM KCl, supplemented with a protease inhibitor mixture containing 0.5 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each of leupeptin, pepstatin, and aprotinin (Sigma). After incubation on ice for 10 min, the cells were lysed by 10 strokes with a Dounce homogenizer and the nuclei were pelleted. The pellet was resuspended in a solution containing 420 mM KCl, 20 mM Tris-HCl, pH 7.8, 1.5 mM MgCl₂, and 20% glycerol, supplemented with the protease mixture described above, and incubated at 4 °C with gentle agitation. The nuclear extract was centrifuged at 10,000 × g for 10 min, and the supernatant was dialyzed twice against a solution of 20 mM Tris-HCl, pH 7.8, 100 mM KCl, 0.2 mM EDTA, and 20% glycerol. Protein concentration was determined using the Bio-Rad reagent with bovine serum albumin as standard.

**Electrophoretic Mobility Shift Assay (EMSA)—**Sequences of the sense strands of the oligonucleotide probes used for EMSA were as follows: 5′-TCT GTA GTC GAG CAC ACT CAC CTC-3′ (Cp HRE), 5′-TCT GTA AAA GAC CAC ACT CAC CTC-3′ (mutated Cp HRE), and 5′-GCA CTA GTC CCT GTC TCA CAG AGC-3′ (erythropoietin HRE). The HRE-containing probes were annealed, gel-purified, and end-labeled with [γ-³²P]ATP (PerkinElmer Life Sciences) using T4-polynucleotide kinase (Promega). Unincorporated nucleotide was removed by gel filtration using G-25 Sephadex columns (Quick Spin™ TE, Roche Molecular Biochemicals). To measure DNA-protein interaction, 1 × 10⁵ cpm of oligonucleotide probe was incubated with 5 μg of nuclear extract and 0.5 μg of sonicated, denatured salmon sperm DNA (Invitrogen) in the experimental procedures.
the presence of insulin at concentrations up to 100 nM for 18 h. Cp mRNA expression was quantitated densitometrically, normalized by the 28 S ribosomal subunit, and expressed with respect to untreated control cells. Surprisingly, Cp mRNA expression was increased by IL-6 was substantially reduced by approximately half by insulin, to a level that was still 2-fold higher than untreated control cells. The induction of several acute phase and other genes was determined. HepG2 cells were treated with insulin (100 nM) for 18 h and total RNA isolated. The expression of Cp, C-reactive protein (CRP), albumin, transferrin, and VEGF mRNA was determined by Northern blot analysis using gene-specific radiolabeled cDNA probes. Ultraviolet visualization of the 28 S ribosomal RNA subunit served as a control.

**RESULTS**

*Induction of Cp Expression by Insulin*—We first determined whether insulin blocked the IL-6-mediated induction of Cp gene expression. Northern blot analysis of HepG2 cells showed that IL-6 increased the steady state level of both major Cp mRNA transcripts by more than 4-fold (Fig. 1A). The induction by IL-6 was substantially reduced by approximately half by insulin, to a level that was still 2-fold higher than untreated control cells. Surprisingly, Cp mRNA expression was increased by insulin itself. A dose-response experiment was done to determine whether the inhibitory activity was observed at physiological concentrations of insulin (between 0.1 and 5 nM). Marked inhibition of IL-6-mediated Cp expression was observed at 0.3 nM insulin and maximal inhibition at ~1 nM (Fig. 1B). The average serum insulin concentration in fasting adults is ~0.1 nM, but insulin concentrations from 0.3 to 0.9 nM are transiently reached after meals (in normal adults and obese adults with impaired glucose tolerance, respectively), and lev-
steady-state level of 

amounts of insulin showed that half-maximal stimulation of 

inflammatory conditions. 

dual activity with respect to Cp expression; it is a negative 

including bacteremia or meningitis (43). Thus, insulin has a 

concentration in patients with acute inflammatory syndromes 

used in this experiment, 2.5 ng/ml, is typical of the serum 

in patients with insulinomas (41, 42). The concentration of IL-6 

were determined as in Fig. 1A. B, aliquots of conditioned media were 

subjected to 7% SDS-PAGE and immunoblot analysis using polyclonal 

rabbit anti-human Cp IgG (top panel). The amount of Cp was quanti-

tated densitometrically and expressed as relative units (lower panel).

els of 1 nM and higher are seen after insulin administration and 

in patients with insulinomas (41, 42). The concentration of IL-6 

used in this experiment, 2.5 ng/ml, is typical of the serum 

concentration in patients with acute inflammatory syndromes 

including bacteremia or meningitis (43). Thus, insulin has a 

dual activity with respect to Cp expression; it is a negative 

regulator of Cp under inflammatory conditions characterized by 

elevated IL-6, but it is a positive regulator under non-

inflammatory conditions.

Northern blot analysis of HepG2 treated with increasing 

amounts of insulin showed that half-maximal stimulation of 

steady-state level of Cp mRNA occurred at ~1 nM insulin, and 

a maximal 4-fold increase was observed at 10 nM. (Fig. 2A). In 

multiple repetition of this experiment, the maximal stimula-

tion of Cp mRNA expression by insulin was between 2- and 

4-fold compared with untreated controls. Analysis of secreted 

Cp, by immunoblot analysis of conditioned medium, showed 

that the increase in synthesis and secretion of the protein 

nearly paralleled the induction of the transcript (Fig. 2B). 

Half-maximal induction was observed between 0.3 and 1 nM, and 

maximal stimulation was seen at 3–10 nM insulin. Cp was 

secreted entirely in the intact, 132-kDa form required for sev-

eral of its biological activities (44, 45). The specificity of the 

induction of Cp was shown by Northern analysis of other se-

creted hepatic proteins. Insulin did not alter HepG2 cell mRNA 

expression of C-reactive protein, a positive acute phase protein, 

or albumin and transferrin, two negative acute phase proteins 

(Fig. 2C). In a positive control experiment, we measured the 

induction of VEGF gene expression as described previously for 

insulin (36) and insulin-like growth factor-1 (37); comparable 

inductions of VEGF and Cp mRNA were observed (Fig. 2C). A 

time-course experiment showed only marginal stimulation of 

Cp mRNA expression after 8 h, and essentially maximal 

expression by 16 h (Fig. 3A). A time course of protein secretion, as 

expected, showed delayed stimulation of Cp by insulin (Fig. 

3B). Very little stimulation was seen after 16 h, but substantial 

stimulation by 24 h.

Fig. 3. Time-course Cp induction in response to insulin. A, 

HepG2 cells were incubated with 30 or 100 nM insulin for up to 24 h. 

RNA was isolated and subjected to Northern blot analysis. Cp mRNA 

expression (upper panel), 28 S ribosomal subunit as loading control 

(middle panel), and normalized Cp mRNA expression (lower panel) 

were determined as in Fig. 1A. B, aliquots of conditioned media were 

subjected to 7% SDS-PAGE and immunoblot analysis using polyclonal 

rabbit anti-human Cp IgG (top panel). The amount of Cp was quanti-

tated densitometrically and expressed as relative units (lower panel).

Northern blot analysis of HepG2 treated with increasing 

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Transcriptional Activation of Cp by Insulin: Requirement for 
a HRE in the Cp 5'-Flanking Region—We have previously 
reported that the Cp gene contains a functional HRE activated 
by both iron deficiency and hypoxia (34). This finding, coupled 
with recent reports that insulin induces multiple genes 
through activation of HIF-1 (36–38), led us to examine the 
possible role of HIF-1 in insulin-inducible Cp expression. We 
first compared the magnitude of the inductions in response to 
insulin and hypoxia. Exposure of cells to hypoxic conditions, i.e. 
1% O2, induced an ~10-fold increase in Cp mRNA compared 
with normoxic control cells (range was between 6- and 19-fold 
in multiple experiments), an induction substantially greater 
than that by a maximal dose of insulin (2.6-fold in this exper-
iment) (Fig. 4A). Exposure of cells to both hypoxia and insulin 
did not increase Cp expression beyond that induced by hypoxia 
alone, indicating the absence of a synergistic effect. Because 
the induction of HRE-containing genes by hypoxia requires 
new protein synthesis, we tested whether protein synthesis 
was also required for Cp induction by insulin. Cycloheximide 
completely blocked the induction of Cp, indicating a common 
dependence on new protein synthesis for both agonists 
(Fig. 4B).
FIG. 5. Determination of insulin-responsive element by deletion and mutation analysis of Cp gene 5′-flanking region. A, mapping of Cp gene insulin-responsive element by deletion analysis. Chimeric pGL3-basic vectors were constructed to contain the proximal 4774, 3639, or 3576 bp of the Cp gene 5′-flanking region (upstream of the translation initiation site) driving luciferase. Consensus HIF-1 binding sites in the linear maps are indicated by open rectangles, and labeled with the 5′-positions of the 5-bp G/ACGTG cores. The constructs were transiently transfected into subconfluent HepG2 cells (with a plasmid containing β-galactosidase to correct for transfection efficiency) using Lipofectin. After recovery, the transfected cells were incubated with insulin (100 nM, black bars) for 18 h, or were subjected to hypoxic condition of 1% O2 for 18 h (striped bars), or were left untreated (open bars). Luciferase activity in cell extracts was measured and normalized for β-galactosidase activity.

FIG. 6. Determination of domains of Cp gene 5′-flanking region responsible for the stimulatory and inhibitory actions of insulin.

A, subconfluent HepG2 cell cultures were transiently transfected with a chimeric construct containing the proximal 4774 bp of the Cp gene 5′-flanking driving luciferase in pGL3-basic vector (Cp 4774−1−Luc). The active HIF-1 binding site is indicated by an open rectangle and inactive sites indicated by X-marked rectangles. B, cells were transiently transfected a segment of the Cp 5′-flanking region containing the active HRE upstream of the SV40 promoter driving luciferase in pGL3-prom (Cp 3639−3544-SV40-Luc). A second construct contained the same segment but with the core of the HIF-1 binding site mutated from CGT to AAA. The HIF-1 site is indicated by an open rectangle; the HIF-1 mutation is indicated by an X and by an underline below the mutated nucleotides. The constructs were transiently transfected (with a β-galactosidase plasmid) into HepG2 cells as in A. After recovery the cells were incubated for 18 h with insulin (100 nM, black bars), or with 1% O2 (striped bars), or were left untreated (open bars). Luciferase activity in cell extracts was measured and normalized for β-galactosidase activity.
To investigate the molecular mechanism of insulin-stimulated Cp gene expression, we prepared chimeric constructs of the Cp gene 5′-flanking regulatory region ligated with luciferase as a heterologous reporter. The human Cp 5′-flanking region contains six consensus HIF-1 binding sites, i.e. (G/A)CGTGC (35), arranged in three adjacent pairs between positions −4535 and −2447 (Fig. 5A). A series of promoter/enhancer fragments was constructed with progressively larger 5′-deletions, and the fragments were ligated upstream of luciferase in the promoterless reporter gene pGL3-basic. Subconfluent HepG2 cells were transiently transfected with these constructs and then incubated for 18 h with insulin or under hypoxic conditions. Transactivation of the entire 4774-bp construct (Cp−4774,−1-Luc) was increased −3-fold by insulin (Fig. 5A). Hypoxia also transactivated this construct; the -fold stimulation was approximately twice that for insulin, consistent with their relative increases in Cp mRNA expression. Insulin and hypoxia similarly transactivated a chimeric construct (Cp−3639,−1-Luc) lacking the upstream-most pair of consensus HIF-1 binding sites. However, activation by insulin and hypoxia was completely abrogated in a promoter/enhancer construct lacking the three distal consensus HIF-1 binding sites (Cp−3576,−1-Luc). Basal activity was similar for all Cp promoter/enhancer constructs. These results suggest that a single HRE located between positions −3639 and −3577 is required for Cp transactivation by insulin and hypoxia.

To test whether this single HRE was sufficient for Cp transactivation by insulin, a mutagenesis strategy was used. A 96-bp segment containing this HRE was subcloned upstream of the SV40 promoter driving luciferase in the pGL3prom vector (Cp−3639,−3544SV40-Luc). After transient transfection into HepG2 cells, insulin stimulated reporter gene expression by almost 3-fold (Fig. 5B). As before, the stimulation by hypoxia was approximately twice that by insulin. To show that the HRE in this enhancer region was responsible for the observed activation, the core 5′-ACGTG-3′ sequence was mutated to 5′-AAAG-3′. This construct was completely inactive with respect to transactivation by insulin or by hypoxia (Fig. 5B). These results suggest that a single HRE present in the Cp enhancer is necessary and sufficient for transactivation by insulin.

**Relationship between Cp Promoter/Enhancer Sites Required for Stimulatory and Inhibitory Activities of Insulin**—We investigated the relationship between the cis-acting site at which insulin transactivates Cp and the site required for the inhibition of IL-6-mediated Cp transactivation. HepG2 cells were transfected with the full-length promoter/enhancer construct (Cp−4774,−1-Luc) and then were treated with IL-6, insulin, or both, and luciferase activity was measured. IL-6 induced reporter gene expression by almost 5-fold. Insulin reduced IL-6-mediated induction to the level seen with insulin alone, between 2- and 3-fold (Fig. 6A). Thus, both the positive and negative regulatory activities of insulin are reconstituted using the full-length Cp promoter/reporter construct. To determine whether the two activities of insulin utilize the same site, fragments of the 5′-flanking region were tested. The vector containing the 96-bp segment containing the active Cp HRE (Cp−3639,−3544SV40-Luc) was transfected into HepG2 cells and treated with insulin and IL-6. As expected, insulin induced luciferase expression by −2.5-fold; however, there was essentially no induction by IL-6 (Fig. 6B). The inability of insulin to induce the inhibition by IL-6 suggested that the inhibitory site was not in the region containing the HRE. Cells were then transfected with a construct containing the presumptive basal Cp promoter (Cp−648,−1-Luc), previously shown to drive constitutive, but not hypoxia-stimulated, reporter gene expression (34). As expected, insulin did not increase luciferase expression, but IL-6 increased expression by −5-fold (Fig. 6C). The stimulatory activity of IL-6 was completely abrogated by insulin, indicating the presence of the site of negative regulation in this proximal construct. Together these results show that distinct cis-acting sites in the Cp gene 5′-flanking region are required for the stimulatory and inhibitory activities of insulin.

**Role of Hypoxia-inducible Factor-1 in Transcriptional Activation of Cp by Insulin**—To identify the transcription factor(s) involved in insulin-stimulated induction of Cp, EMSAs were done using a radiolabeled 24-bp Cp HRE probe. HepG2 cells were treated with insulin or hypoxia, and nuclear extracts were incubated with the Cp HRE probe. Treatment with insulin led to the formation of a single radiolabeled complex (Fig. 7A). The mobility of the complex induced by insulin was the same as that induced by hypoxia, and the same as that bound to an erythropoietin HRE probe, suggesting that a similar HIF-1-containing complex may be induced by both hypoxia and insulin. Competition experiments were done to show specificity of binding of the complex to the Cp HRE probe. The binding of radiolabeled Cp HRE probe was partially competed by a 10-fold molar excess of unlabeled Cp HRE and almost completely competed by a 100-fold molar excess (Fig. 7B). Binding was similarly blocked by an excess of unlabeled erythropoietin HRE probe, whereas a mutated Cp HRE probe showed only partial competition even at 300-fold molar excess.

Gel supershift studies were done to confirm the presence of HIF-1 in the insulin-induced complex that binds the Cp HRE. Rabbit monoclonal anti-HIF-1α shifted the complex formed in insulin-treated cells (Fig. 8A). Furthermore, a polyclonal antibody against ARNT/HIF-1β (and a mixture of both antibodies) completely blocked the formation of the complex. To further confirm the requirement for HIF-1 in the formation of insulin-stimulated, Cp HRE-binding complexes, we took advantage of the Hepa c4 mouse hepatoma cell line, which is deficient in ARNT/HIF-1β (46). These cells, derived from the parental Hepa-1c1c7 cell line, have been used to show the requirement for HIF-1 in gene transactivation by hypoxia (40), and more recently by insulin (36). Both cell lines were transfected with the chimeric reporter gene Cp−3639,−3544SV40-Luc. Treatment of the wild-type Hepa-1c1c7 cells with insulin increased luciferase expression by almost 2-fold, whereas the stimulation by hypoxia was −3-fold (Fig. 8B). Neither treatment increased Cp enhancer activity in the HIF-1−β-deficient cell line. The mouse cell lines were also used to establish HIF-1 binding by electrophoretic mobility shift assay. Hepa-1c1c7 and c4 cells were treated with insulin or with CoCl2, and the binding of the HIF-1 complex to the Cp HRE probe was determined; we previously showed that CoCl2, like hypoxia, activated HIF-1 and Cp transcription in these cells (34). Inducible binding was observed in nuclear extracts of wild-type Hepa-1c1c7 cells treated with either agonist, whereas essentially no HIF-1 binding was seen in the ARNT/HIF-1β-deficient Hepa c4 cells (Fig. 8C). Together, these studies clearly demonstrate the requirement for HIF-1 in activation of Cp transcription by insulin.

**DISCUSSION**

We have found that the effect of insulin on hepatic cell transcription of Cp depends on the inflammatory state of the cell. In the absence of a pro-inflammatory stimulus, i.e. treatment with IL-6, insulin enhances Cp gene expression and protein production in HepG2 cells. This induction requires activation of HIF-1 and binding to an HRE in the distal 5′-flanking region of Cp. In contrast, in the presence of IL-6, insulin markedly represses Cp gene expression in these cells. Thus, insulin functions as a bidirectional, condition-dependent regulator of hepatic cell Cp expression.

**Negative Regulation of Cp Transcription by Insulin**—We
have confirmed previous reports of induction of Cp in hepatoma cells by IL-6 (31, 32). Our finding that IL-6 increases the steady-state level of Cp mRNA and activates a chimeric Cp promoter-Luc reporter shows that regulation is at the level of transcription. Although these results are consistent with the mechanism of IL-6-mediated induction of other acute phase genes, transcriptional regulation of Cp by IL-6 has not been previously shown. We cannot account for the difference between our results and an earlier report that Cp induction by IL-6 is primarily post-transcriptional, but a variance in culture conditions is a possible explanation (33). Elucidation of the mechanism of transcriptional activation of acute phase genes by IL-6 is an area of intense investigation. An important role for C/EBPβ in IL-6-stimulated transactivation of multiple acute phase genes has been shown by up-regulation of C/EBPβ expression, and by its binding to specific sequences in the 5′-flanking regions of these genes (47–49). More recently, the IL-6-activated “acute phase response factor” has been shown to be STAT3, which binds to palindromic, TT(N)AA motifs (termed acute phase response elements) in the promoters of multiple (but not all (Ref. 50)) acute phase genes (51–54). The essential role of STAT3 in the acute phase response in vivo has been shown in mice with an inducible STAT3 gene deletion (55). The molecular mechanism of Cp transactivation by IL-6 has not been investigated in detail. Binding of C/EBPβ to the rat Cp promoter has been shown, but agonist-induced responses have not been examined (56). Likewise, the role of STAT3 in basal or agonist-induced Cp transcription has not been reported; however, the presence of three near-consensus STAT3-binding sites in the proximal Cp promoter between nucleotide positions −848 and −1 merits further analysis.

Our results show that insulin suppresses the IL-6-mediated increase in Cp mRNA level to nearly the same level as that seen with insulin alone. The experiments with a chimeric reporter gene (Cp−848−1−Luc) confirm that IL-6 transcriptional activity is completely blocked by insulin. Thus, Cp joins a family of insulin-inhibited acute phase genes including haptoglobin, thiostatin, complement C3, and C-reactive protein (6, 11). The negative regulation by insulin may be mediated by at least three pathways. Insulin reduces IL-6-induced STAT-3 activity as measured by gene transcription, mRNA accumulation, protein concentration, and DNA binding activity (7). Insulin also reduces the expression of the IL-6 receptor α-subunit and IL-6 receptor binding (7). Finally, insulin suppresses transactivation by C/EBPβ (8). The presence of an active C/EBPβ-binding site in the rat Cp promoter (56) and the consensus STAT3-binding sites suggest that either or both of these pathways may be involved. Our results indicate that the direction and magnitude of the response of the Cp promoter to insulin is condition-dependent; under normal physiological conditions, insulin increases Cp transcription, whereas, during the systemic acute phase response or localized inflammation, insulin reduces Cp transcription. We are not aware of other acute phase reactants that are transcriptionally activated by insulin; thus, the response of Cp may be unique. The dual response of Cp to insulin may reflect its pleiotropic nature, with
possibly distinct functions in inflammation and iron homeostasis. Suppressive activities of insulin have been reported in other enzyme systems, e.g. it inhibits transcriptional activation of fructose-2,6-bisphosphatase by dexamethasone in rat hepatoma PTO-2B cells (57). Insulin by itself causes transcriptional activation of this enzyme in hepatic FAO-1 (58). Thus, the function of insulin may be bidirectional in this system as well; however, a direct demonstration using a single type of cell has not been demonstrated.

Activation of Cp Transcription by Insulin—We observed a 2–5-fold insulin-stimulated increase in Cp transcription, in Cp mRNA level, and in the rate of Cp secretion in HepG2 cells. We also found that the stimulation of Cp gene expression by a maximal concentration of insulin was much lower than that seen upon treatment with 1% O2. Our observations are comparable with a previous report of 2- and 4-fold induction of aldolase A transcription in HepG2 cells by insulin and hypoxia, respectively (36). The differential expression may reflect the 2-fold higher level of HIF-1α in cells treated with hypoxia compared with cells treated with insulin (59); however, these results were obtained in embryonic kidney 293 cells, and cell type-specific differences in HIF-1α activation by insulin have been observed (36). The mechanism(s) of HIF-1α induction by hypoxia and by insulin is not clearly understood. Hypoxia increases the amount of HIF-1α by stabilization of the protein (60). Under normoxic conditions, rapid, ubiquitin-dependent degradation of HIF-1α occurs, whereas hypoxic stress inhibits this process (61). There is evidence that insulin utilizes a similar mechanism for stabilization of HIF-1α (36). Recent reports suggest that HIF-1α activation by both hypoxia and insulin utilize the phosphatidylinositol 3-kinase pathway (62, 63).

**Role of Insulin in Regulating Cp Levels in Vivo**—Our results may help to clarify contradictory studies on Cp expression in diabetic patients and in animal models of diabetes. Elevated plasma Cp levels have been reported in non-insulin-dependent diabetes mellitus (NIDDM, type II diabetes) (64–66), but normal Cp levels have been reported as well (67, 68). The reports on insulin-dependent diabetes mellitus (IDDM, type I diabetes) are similarly inconclusive, with one report indicating decreased plasma Cp (69), whereas others indicate increased Cp (70). The lack of a consistent response of serum Cp to diabetes has been ascribed to variability in the acute phase response (64), diabetic vascular complications (71), oxidative stress (70), extent of hyperglycemia (66), serum nitric oxide (72), and to methodological differences (70).

Our in vitro results suggest that the circulating level of insulin may be a critical regulator of hepatic Cp synthesis, and consequently, plasma Cp level. In this case, part of the variation in Cp levels in NIDDM patients may be a result of the large range in plasma insulin values from normal to very high (e.g. in early phases of the disease and in diabetes related to obesity), and possibly of the oscillatory nature of insulin levels in NIDDM. IDDM patients require insulin for survival, and thus the method and extent of diabetic control may influence Cp status. Animal models of diabetes provide additional insights. In both alloxan- and streptozotocin-induced diabetic rats, the decrease in insulin level is accompanied by substantial reductions in plasma Cp (73–75), and treatment of diabetic rats with insulin restores plasma Cp to normal levels (75). Similarly, treatment of rats with streptozotocin decreases Cp synthesis by the isolated perfused liver, but is restored to normal after treatment of the rats with insulin (76). Taken together, these data suggest a possible regulatory activity of insulin on hepatic Cp synthesis and Cp plasma concentration in vivo. Our results suggest that the relationship between plasma Cp and diabetes is likely to be a complex function including dependences on both insulin levels and the extent of inflammation.

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