Insulin Is a Prominent Modulator of the Cytokine-Stimulated Expression of Acute-Phase Plasma Protein Genes

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Several endocrine hormones which influence liver metabolism are known to increase in activity during the acute phase of injury or inflammation. We determined whether these hormones have the potential to influence acute-phase protein production in human and rat hepatoma cells. Catecholamines, glucagon, growth hormone, triiodothyronine, and cyclic nucleotides individually or in combination did not modulate the basal or the interleukin-1 (IL-1)-, IL-6-, and dexamethasone-stimulated levels of acute-phase plasma proteins. Insulin, however, was found to be a rapid, nonspecific, and dose-dependent inhibitor of the cytokine and glucocorticoid stimulation of acute-phase protein gene expression and to exert its effect at the transcriptional level. The insulin inhibition applied to all cytokines tested but to various degrees, depending upon the particular acute-phase gene. Insulin resulted in an early and prominent increase in the transcription of genes encoding the AP-1 components of JunA, JunB, and c-Fos, as has been observed for other growth factors. However, the effect of insulin on C/EBP β was unexpected and paradoxical: while insulin completely inhibited the transcriptional activation of the C/EBP β gene in cytokine- and dexamethasone-treated cells, the level of cytoplasmic C/EBP β RNA was elevated. Quantitation of C/EBP β mRNA by Northern (RNA) blot analysis and of C/EBP β DNA binding activity by Southwestern (DNA-protein) blot analysis showed that insulin, when combined with cytokines and dexamethasone, stimulated both the mRNA and DNA binding activity by a factor of 1.6 compared with that of cells treated with cytokines and dexamethasone alone. Transient transfection of H-35 and HepG2 cells with a chloramphenicol acetyltransferase (CAT) gene expression vector containing the C/EBP β response element also resulted in a 1.5-fold increase of C/EBP β -mediated transcription in insulin-treated cells. Transfection of CAT gene constructs containing increasing lengths of haptoglobin gene 5' flanking sequences indicated that insulin inhibition of IL-6 stimulation required the presence of the region from -4100 to -1030. These results suggest that insulin has the potential to control the transcription of acute-phase genes by at least two separate mechanisms.

In different mammalian species, systemic tissue injury induces a coordinate increase in the hepatic production and circulating concentration of acute-phase plasma proteins that include fibrinogen, haptoglobin, hemopexin, α_1 -antitrypsin, α_1 -antichymotrypsin, complement C3, and α_1 -acid glycoprotein (31). The fact that tissue injury at a distant site leads to activation of liver metabolism suggests mediation by humoral factors (4, 9). It has been shown that a variety of cell types, including activated monocytes, tissue macrophages, keratinocytes, and fibroblasts, synthesize and release factors which fulfill the postulated role of acute-phase mediators (22, 30, 33, 37). By utilizing *in vivo* and tissue culture systems, tumor necrosis factor α , interleukin-1 (IL-1), IL-6, IL-11, and leukemia inhibitory factor have been demonstrated to act as the principal mediators of the hepatic acute-phase response. Maximal expression of several acute-phase plasma protein genes is, however, dependent on the presence of glucocorticoids (10). For a number of the acute-phase reactant genes, separable and distinct *cis*-acting regulatory elements responsive to IL-1, IL-6, and glucocorticoids have been described (6, 9, 36, 49).

Although inflammatory cytokines and glucocorticoids have attracted much attention of late, circulating levels of several endocrine hormones, including catecholamines, in-

sulin, glucagon, growth hormone, and triiodothyronine, rise dramatically during acute systemic injury and stress (47). These hormones have been proposed to function primarily in the control of metabolic processes in various organs (17, 20, 41, 48). The role of these endocrine hormones on the regulation of acute-phase plasma proteins during systemic injury has not been previously described. Recognizing that the hepatic acute-phase response is the end effect of several cooperatively interacting factors, the potential contribution *in vivo* of these "metabolic" hormones needs to be evaluated. In this study, we have determined to what extent several of the prominent, acute-phase-regulated endocrine hormones can influence expression of acute-phase plasma proteins in human and rat hepatoma cells.

We have found that catecholamines, glucagon, growth hormone, triiodothyronine, and cyclic nucleotides do not individually or in combination affect acute-phase proteins, nor do they modulate significantly the cell response to cytokines with and without dexamethasone. In contrast, insulin was found to cause a dose-dependent, nonspecific inhibition of the cytokine response. The effect of insulin was accompanied by specific changes in the expression of transcription factor genes which are distinct from those changes caused by cytokines. Our data suggest that insulin exercises a prominent attenuation of some but not all immediate responses of hepatic cells to inflammatory cytokines.

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MATERIALS AND METHODS

Cells and cell treatment. HepG2 cells (29) were grown to confluency in minimal essential medium containing 10% fetal calf serum, and H-35 cells (clone T-7-18) (43) were grown to confluency in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Hormone treatments were carried out in serum-free media. The concentrations of the additions were as follows: dexamethasone, 1 μ M; human recombinant IL-1 β (Immunex Corp.), 10 U/ml; human recombinant IL-6 (COS cell derived; Genetics Institute), 100 U/ml; porcine insulin (Sigma), 0 to 5 μ g/ml; human growth hormone (Genentech, Inc.), 2.5 to 250 ng/ml; porcine-bovine glucagon (Lilly), 5.0 to 500 ng/ml; triiodothyronine (Sigma), 1 μ g/ml; 8' bromo-cyclic AMP and 8' bromo-cyclic GMP, (Sigma), 10 to 1,000 μ M; epinephrine and norepinephrine (Sigma), 0 to 100 μ M. Unless indicated otherwise, the standard treatment period was 24 h.

Analysis of cell response by rocket immunoelectrophoresis. Aliquots (20 to 70 μ l) of culture media from HepG2 and H-35 cells were analyzed by rocket immunoelectrophoresis using monospecific antibodies for plasma proteins (46). The areas under the precipitin peaks were used to measure the amounts of secreted plasma proteins.

RNA analysis. Total cell RNA was prepared by using guanidine thiocyanate extraction and cesium chloride gradient centrifugation (15). Total RNA or polyadenylated fractions (10 μ g) were analyzed for specific mRNAs by Northern blot (RNA) hybridization under standard stringency by using 32 P-labeled full-length cDNAs encoding human α_1 -antichymotrypsin (27) and haptoglobin (11); rat α -fibrinogen (5), haptoglobin (36), and phosphoenolpyruvate carboxykinase (PEPCK) (generously provided by R. W. Hanson, Case Western Reserve University); and the transcription factors JunB (7) and C/EBP β (14) (generously provided by S. L. McKnight, Carnegie Institute).

Nuclear run-on reaction. H-35 cell nuclei were prepared according to the methods of Almendral et al. (1). The entire nuclear preparation was subjected to the run-on reaction as outlined by Lamers et al. (32). Denatured cDNAs encoding rat hemopexin, α -fibrinogen, α_1 -acid glycoprotein, and haptoglobin; mouse AGP, C/EBP β , C/EBP α , JunA, JunB, c-Fos, N-Myc, and L-Myc; and human triosephosphate isomerase and pBR322 were slot blotted onto nitrocellulose strips. Equal aliquots of 32 P-labeled transcripts (5×10^7 cpm) were hybridized to the nitrocellulose strips for 2 days. After being washed, the strips were mounted and exposed simultaneously for autoradiography. Cytoplasmic RNA was isolated from the postnuclear supernatants of the cell lysate, serially diluted twofold, and analyzed by dot hybridization. The radioactivity bound to the membranes (Northern [RNA] blots, dot blots, run-on strips, and Southwestern [DNA-protein] blots) was quantitated (volume integration and background subtraction) by using a PhosphorImager (Molecular Dynamics).

DNA binding. Nuclear proteins were prepared from H-35 cells (39) and analyzed by Southwestern blotting as described previously (50). The DNA binding activity of C/EBP β was assessed by using 32 P-end-labeled restriction fragment that harbored the IL-1 response element (RE) of the rat α_1 -acid glycoprotein gene (positions 1 to 46 of the distal cytokine RE) (49) and the cytokine RE of the rat haptoglobin gene (positions -165 to -56) (36).

Cell transfection. H-35 and HepG2 cells were transfected with plasmid DNA as a DEAE-dextran complex (34) and a calcium phosphate precipitate (24), respectively. The DNA mixture (20 μ g/ml) was composed of 2 μ g of pIE-MUP (internal marker for transfection efficiency [42]) and 18 μ g of

FIG. 1. Influence of insulin on IL-1, IL-6, and dexamethasone stimulation of plasma protein secretion. HepG2 cells were treated for 24 h with the indicated factors (insulin, 500 ng/ml; dexamethasone [Dex], 1 μ M; IL-1 β , 10 U/ml; IL-6, 100 U/ml). Aliquots (20 μ l) of the media were analyzed by rocket immunoelectrophoresis for the amount of haptoglobin and albumin.

pHp(4100)-OCT, pHp(1030)-OCT, pHp(400)-OCT, or pHp(190)-OCT (each of which contains the indicated length of rat haptoglobin promoter and 5' flanking gene region in the promoterless and enhancerless pOCT [8]). After a 36-h recovery period, the cells were treated for 24 h. The chloramphenicol acetyltransferase (CAT) activity in cell homogenates and the amount of plasma proteins and major urinary protein (MUP) in the culture media were subsequently determined and expressed relative to those of the untreated control (23).

RESULTS

Insulin reduces the cytokine-mediated stimulation of acute-phase plasma proteins. Treatment of H-35 cells and HepG2 cells with combinations of IL-1, IL-6, and dexamethasone resulted in the predictable pattern of acute-phase plasma protein stimulation (4). The addition of growth hormone, glucagon, triiodothyronine, catecholamines, 8' bromo-cyclic AMP, and 8' bromo-cyclic GMP, either alone or in combination, did not alter the pattern of plasma protein production, nor did it modulate the cell response to IL-1, IL-6, and dexamethasone (data not shown). However, in the presence of insulin, the cells showed a general decrease in the IL-1-, IL-6-, or dexamethasone-stimulated secretion of several acute-phase proteins (representative example for HepG2 cells shown in Fig. 1). The dose response of HepG2 cells indicated that insulin concentrations of 5 ng/ml and greater consistently and significantly attenuated the stimulatory effect of IL-1, IL-6, and dexamethasone (Fig. 2). Inhibition to subbasal levels was seen only at an insulin concentration of 5,000 ng/ml. This inhibition probably reflected a cytotoxic

FIG. 2. Insulin dose-dependent response of acute-phase plasma protein secretion by HepG2 cells. HepG2 cells were treated for 24 h with media containing a combination of factors ($\times$, no addition; Δ , IL-1 β ; $\blacktriangle$, IL-1 β plus dexamethasone [Dex]; $\square$, IL-6; $\blacksquare$, IL-6 plus Dex; $\circ$, IL-1 plus IL-6; $\bullet$, IL-1, IL-6, and Dex). Serial dilutions of insulin were added to the media as indicated. The amounts of secreted plasma proteins (haptoglobin [HP], α_1 -acid glycoprotein [AGP], and albumin [ALB]) were determined by rocket immunoelectrophoresis (see Fig. 1). Data are expressed as percentages of the amounts secreted by untreated control cells. The values shown represent the mean values of three separate experiments. Virtually identical results were obtained in four additional experimental series.

effect since albumin secretion, which was not influenced by insulin at lower concentrations, was also inhibited at this concentration. Insulin concentrations below 5 ng/ml were not inhibitory; in fact, in some instances, a slight stimulatory effect was discernible. The insulin-mediated inhibition was not specific for any given treatment or for a specific plasma protein. The percent reduction in the secretion of a given plasma protein was comparable for all the treatments used.

The modulatory effect of insulin was not restricted to HepG2 cells, since a qualitatively similar effect was noted for H-35 cells (Fig. 3). Since there is no appreciable basal secretion of plasma proteins by H-35 cells, the inhibitory effect of insulin was expressed relative to levels produced by cells treated with cytokines and/or dexamethasone but not with insulin. As in HepG2 cells, the inhibition was dose dependent at concentrations above 5 ng/ml and seemed not to be cytokine specific.

The secretion of all major acute-phase proteins by HepG2 and H-35 cells (Fig. 1 to 3 and data from four additional experiments [not shown]) was reduced by insulin regardless of whether these were type I or type II acute-phase proteins. A notable exception was α_1 -antichymotrypsin in HepG2 cells, the secretion of which was not significantly affected by insulin treatment (Fig. 4B).

The insulin effect is demonstrable at the mRNA level. The amount of acute-phase plasma proteins secreted into the media paralleled the changes seen in the concentration of the mRNAs for these proteins. The 60% reduction in the level of

FIG. 3. Insulin dose-dependent response of acute-phase plasma protein secretion by H-35 cells. H-35 cells were treated for 24 h with media containing cytokines with or without dexamethasone (Dex) and serial dilutions of insulin as indicated. The amounts of complement C3 (C3), α_1 -acid glycoprotein (AGP), and thiostatin (TST) secreted into the media were determined by rocket immunoelectrophoresis. Since there is no detectable basal secretion of these plasma proteins by H-35 cells, insulin effect was expressed as a percentage of secretion by cells treated with cytokines with or without dexamethasone but not treated with insulin. The results are representative of four additional experiments.

cytokine-stimulated haptoglobin secretion by the HepG2 cells when treated with 500 ng of insulin per ml correlated with a similar reduction in the level of cytokine-stimulated haptoglobin mRNA. The same analysis also indicated the exceptional feature of α_1 -antichymotrypsin, whose cytokine-stimulated secretion and mRNA concentration were unaffected by insulin (Fig. 4). In response to insulin, the extent of the decrease in mRNA encoded by the various genes was not uniform; e.g., the level of haptoglobin mRNA was reduced more than either the level of α_1 -acid glycoprotein in HepG2 cells (see Fig. 7) or the level of α -fibrinogen in H-35 cells (see Fig. 5).

As previously demonstrated, the treatment of H-35 cells with IL-1, IL-6, and dexamethasone led to an increase in the level of other mRNAs coding for acute-phase proteins (examples in Fig. 5; see also references 6 and 9). The addition of insulin to all treatment combinations uniformly decreased

FIG. 4. Differential effect of insulin on acute-phase plasma protein in HepG2 cells. (A) HepG2 cells were treated for 24 h with minimal essential medium alone or with minimal essential medium containing the indicated hormones (insulin, 500 ng/ml). Total cellular RNAs were extracted, and 15- μ g aliquots were analyzed to determine relative amounts of antichymotrypsin (ACH) and haptoglobin (HP) mRNA. Ethidium bromide staining of the 18S rRNA is also shown. (B) For comparison, culture media from HepG2 cells treated with IL-1, IL-6, and dexamethasone (Dex) and serial dilutions of insulin were analyzed by rocket immunoelectrophoresis to determine amounts of secreted haptoglobin and α_1 -antichymotrypsin. Results are expressed as percentages of secretion by non-insulin-treated cells.

the concentration of these mRNAs (Fig. 5). To verify that insulin alone also exerted its known effect on these H-35 cells, the Northern blot shown in Fig. 5 was subsequently probed with cDNA encoding rat PEPCK. In agreement with published reports (25, 51), insulin reduced not only the basal level but also the dexamethasone-stimulated level of PEPCK mRNA (data not shown).

Insulin modulates transcription rates of genes coding for acute-phase proteins and transcription factors. The close quantitative correlation between the level of mRNA and the level of secretion of the encoded acute-phase protein indicates that production of these gene products is primarily regulated at the pretranslational level. To evaluate the insulin effect on transcription, transcription rates in H-35 cells were determined by nuclear run-on reactions (Fig. 6A and Table 1). There was an immediate activation of acute-phase protein genes by cytokines and dexamethasone. This activation was on average reduced twofold after only a 30-min

FIG. 5. Hormone-specific effect on mRNAs coding for acute-phase proteins and transcription factor. H-35 cells were treated for 4 h with the indicated combination of hormones (insulin, 500 ng/ml). Total cell RNA was extracted, and 15- μ g aliquots were analyzed by Northern blot hybridization to determine relative amounts of α -fibrinogen (α -FB), haptoglobin (HP), and C/EBP β .

exposure to insulin. The inhibitory effect of insulin persisted and resulted in a proportional reduction in the accumulation of cytoplasmic mRNA at 4 h (Fig. 6B). Insulin, like other growth factors, produced an early and prominent (three- to sixfold), but transient, increase in the transcription of the genes encoding JunA, JunB, and c-Fos (Fig. 6A and Table 1) and Egr-1 (not shown). For JunA and c-Fos, but not JunB, the increased gene expression seen after 30 min of insulin treatment exceeded the levels seen in response to IL-1, IL-6, and dexamethasone. There was no additive action on the transcription rate seen with the combined addition of insulin, cytokines, and dexamethasone. However, the RNA analysis indicated at least an additive effect on the accumulation of JunB mRNA (Fig. 6B).

A drastically different pattern of regulation was found for C/EBP gene members. Insulin and cytokines had no immediate effect on C/EBP α gene transcription, but insulin produced a fourfold increase in the transcription rate after 4 h of treatment (Fig. 6A). In contrast, transcription of the C/EBP β gene was enhanced 3-fold at 30 min and 10-fold at 4 h of cytokine and dexamethasone treatment. This transcriptional activation was completely inhibited by insulin. More surprising, however, was that the analysis of cytoplasmic RNA from cells treated with IL-1, IL-6, and dexamethasone alone or in combination with insulin showed equally elevated levels of C/EBP β mRNA (Fig. 6B). This suggested that insulin promotes enhanced nuclear processing or cytoplasmic stabilization of C/EBP β mRNA to compensate for the inhibited transcription. Since insulin alone increased C/EBP β mRNA only 1.6-fold at 4 h, the 9.3-fold increase found in cells treated with cytokines, dexamethasone, and insulin must be the product of the additive/synergistic action of all hormones on C/EBP β mRNA stability. The striking differential effect of insulin on acute-phase protein and C/EBP β mRNAs is manifested in H-35 cells treated with various hormone combinations (Fig. 5).

The function of C/EBP β is not inhibited by insulin. C/EBP β has been proposed to be the critical component in IL-6 signalling to acute-phase protein genes, such as the gene for haptoglobin (36). A paradox arose in that we have shown that insulin attenuates cytokine-stimulated expression of acute-phase proteins, including haptoglobin, but enhances C/EBP β mRNA concentration (Fig. 7). Therefore, we had to determine whether C/EBP β mRNA was translated and whether the C/EBP β protein exhibited the appropriate DNA binding properties.

Recently, we established (7a) that C/EBP β is identical to the IL-1 RE binding activity, NF-AB, that is recognized in

FIG. 6. Correlation of gene transcription rate and cytoplasmic RNA. (A) H-35 cells were treated for 30 min or 4 h with the indicated hormones (insulin, 500 ng/ml). Nuclei were prepared and used for run-on reactions. Labeled RNAs (5×10^7 cpm each) were hybridized to slot blotted cDNA encoding rat hemopexin (HPX), α -fibrinogen (α -FB), haptoglobin (HP), C/EBP β , C/EBP α , JunA, JunB, c-Fos, N-Myc, L-Myc, triosephosphate isomerase (TPI), and pBR322 (pBR). The autoradiogram after a 5-day exposure is shown. Membrane strips were subsequently quantitated in the PhosphorImager following a 3-h exposure. The net pixel values for selected slots from the control and 30-min treatments are presented in Table 1. (B) Serially diluted cytoplasmic RNAs isolated from postnuclear supernatants of the cell lysate were analyzed by Northern blot hybridization to determine amounts of the indicated mRNAs.

Southwestern blots as a 35-kDa nuclear protein. Indeed, NF-AB binding activity with the same characteristics as those shown for C/EBP β in Fig. 7 has been induced in H-35 cells by cytokines and dexamethasone (50). The effect of insulin on the DNA binding activity of C/EBP β (or NF-AB) was assessed by Southwestern blot analysis using the IL-1 RE and the cytokine RE of rat α_1 -acid glycoprotein and haptoglobin genes, respectively (Fig. 7). Quantitation of C/EBP β mRNA and C/EBP β DNA binding activity from cells treated for 17 h with IL-1, IL-6, dexamethasone, and insulin showed that both parameters were stimulated by a factor of 1.6 compared with what was seen with cells treated with IL-1, IL-6, and dexamethasone alone. Under the same conditions, insulin reduced protein secretion and mRNA levels for acute-phase reactants (the reduction in mRNA and protein was somewhat greater than observed in Fig. 3).

Since insulin did not lower but rather enhanced C/EBP β DNA binding activity, we wanted to determine whether cells treated with insulin would exhibit elevated transcription of reporter genes with C/EBP β REs. Therefore, HepG2 and H-35 cells were transiently transfected with pHp(190)-OCT, a plasmid in which the CAT gene is linked to the rat haptoglobin gene promoter region that spans positions -165 to +21 (36). This rat haptoglobin promoter has sequences that are recognized by C/EBP β (Fig. 7), and these sequences correspond to the functionally defined A and C regions of the cytokine RE (7a, 40). In agreement with the DNA binding activity of C/EBP β , insulin increased the transiently expressed CAT activity by a factor of 1.5 ± 0.1 ($n = 4$) above that induced by IL-1, IL-6, and dexamethasone (Fig. 8). As expected, endogenous haptoglobin gene expression was markedly suppressed. To determine whether insulin action was influenced by limited amounts of C/EBP β present in untreated hepatoma cells, HepG2 cells that overexpressed C/EBP β were prepared by transient transfection with a 1:1 molar ratio of pHp(190)-OCT and pCD-C/EBP β (a plasmid in which C/EBP β cDNA is driven by the simian virus 40 promoter). Although a 50-fold C/EBP β -mediated transactivation was achieved in untreated cells, treatment of these cells with insulin alone did not appreciably increase the extent of transactivation. However, insulin in combination with IL-1, IL-6, and dexamethasone stimulated reporter gene expression severalfold (data not shown). These results suggest (i) that insulin inhibits endogenous acute-phase protein genes by mechanisms independent of C/EBP β response elements and (ii) that insulin does not directly inhibit the function of the C/EBP β protein.

Insulin inhibition requires upstream promoter sequences.

The results shown in Fig. 8 clearly indicated that the 190-bp haptoglobin promoter region could not reproduce the characteristic regulation of the endogenous haptoglobin gene. To test whether the addition of 5' flanking regions would promote insulin inhibition, CAT gene constructs with increasing lengths of 5' flanking regions were tested (Fig. 9). Only the plasmid carrying a 4,100-bp flanking region displayed an insulin-sensitive suppression of IL-6 stimulation. The IL-6 stimulation was on average reduced by 40%. Although the region from -4100 to -1030 was clearly necessary for achieving this reduction, the more proximal region from -400 to -190 seemed also to interfere with IL-6 stimulation. Removal of that sequence from the 400-bp construct was accompanied by a twofold increase of the IL-6 response and enhanced expression in the presence of insulin.

TABLE 1. Effect of insulin on gene transcription rates

cDNA	Bound radioactivity ^a			
	Control	Insulin	IL-1 + IL-6 + Dex ^b	IL-1 + IL-6 + Dex + Insulin
Hemopexin	26 (1.0)	22 (0.7 ± 0.2)	1,071 (38.2 ± 1.8)	487 (15.8 ± 7.6)
α-Fibrinogen	6 (1.0)	12 (1.2 ± 0.4)	235 (42.4 ± 6.4)	144 (19.4 ± 10.8)
Haptoglobin	14 (1.0)	13 (1.0 ± 0.2)	177 (10.0 ± 2.0)	75 (5.2 ± 0.9)
C/EBPβ	20 (1.0)	24 (1.0 ± 0.3)	98 (2.7 ± 1.5)	18 (0.9 ± 0.1)
JunA	16 (1.0)	109 (6.3 ± 0.5)	70 (2.6 ± 1.3)	86 (6.7 ± 1.4)
JunB	84 (1.0)	362 (3.1 ± 0.9)	720 (6.3 ± 2.0)	672 (7.4 ± 1.4)
c-Fos	57 (1.0)	384 (5.2 ± 1.5)	169 (2.1 ± 0.6)	232 (3.4 ± 0.5)
TPI ^c	44 (1.0)	38 (1.2 ± 0.2)	34 (1.1 ± 0.2)	32 (1.1 ± 0.2)

^a The amounts of ³²P-labeled nuclear run-on reaction products from H-35 cells treated for 30 min and bound to the immobilized cDNAs in Fig. 6A were quantitated by a PhosphorImager. Each value represents the net pixel number (10⁴) integrated for the indicated slot. The values of five independent experiments were normalized to the respective untreated controls; means ± standard deviations are shown in parentheses.

^b Dex, dexamethasone.

^c TPI, triosephosphate isomerase.

DISCUSSION

In an investigation of the possible role of endocrine hormones on hepatic expression of the acute-phase reactants, insulin emerged as a nonspecific, dose-dependent inhibitor of the cytokine- and glucocorticoid-mediated stimulation of acute-phase plasma proteins. The results presented here show that insulin regulates the expression of acute-phase proteins at the transcriptional level.

Although insulin influences the rate of synthesis of many proteins, the molecular details involved in its control of gene transcription are still unknown. Messina (38) has detected significant induction of *c-fos* gene transcription within 5 min

of adding physiologic concentrations of insulin. We also demonstrated that insulin causes a rapid induction of *c-fos* along with the transcription factors JunA and JunB. AP-1 components have not been associated with the stimulation of acute-phase proteins. The only effect of AP-1 components that has been described is an inhibition of the C/EBP-mediated transactivation of the expression of several acute-phase protein genes by JunA and JunB in H-35 cells (7). Therefore, it seemed conceivable that the observed insulin inhibition of acute-phase protein gene expression was achieved at least in part by elevating the activity of AP-1 and thus reducing the ability of C/EBP and related factors to

FIG. 7. Insulin stimulation of C/EBPβ. H-35 cells were treated for 16 h with IL-1, IL-6, and dexamethasone with and without insulin (500 ng/ml). Secretion of haptoglobin (HP) and α₁-acid glycoprotein (AGP) was measured by rocket immunoelectrophoresis. Cell homogenates were fractionated into nuclei and cytoplasm. Proteins were extracted from the nuclei, and duplicate aliquots of 100 μg were subjected to Southwestern blot analysis using either the IL-1 RE of the rat α₁-acid glycoprotein gene or the cytokine RE of the rat haptoglobin gene as a probe. The characteristic pattern of C/EBPβ (or NF-AB), with molecular weights of 35,000 and 20,000, is shown. RNAs were isolated from the cytoplasmic fraction and analyzed by dot blot hybridization to determine amounts of the indicated mRNAs.

FIG. 8. Regulation of transfected plasmids is not affected by insulin. Plasmid pHp(190)-OCT was transfected into H-35 and HepG2 cells. Following the recovery period, subcultures of the transfected cells were treated for 24 h as indicated (insulin, 500 ng/ml). The media were used for rocket immunoelectrophoresis to determine the amount of secreted haptoglobin. Equal amounts of cell extract were assayed for CAT activity. Results shown are representative of four different experiments.

FIG. 9. Insulin sensitivity of haptoglobin gene promoter sequences. HepG2 cells were transfected with a mixture of pIE-MUP and pOCT containing the haptoglobin gene promoter of the indicated length. The cells were treated for 24 h with serum-free medium containing dexamethasone alone (control) or with the addition of insulin or IL-6 or both. The specific CAT activity was determined (percent conversion of substrate to product per hour and picogram of MUP) and expressed relative to the activity of control cells. The means $\pm$ standard deviations of four independent transfection experiments using two separate CAT plasmid preparations are shown.

transactivate acute-phase protein genes. However, the insulin inhibition of acute-phase protein gene expression appeared to be independent of C/EBP β and its corresponding response elements (Fig. 8 and 9). The casual involvement of insulin-stimulated transcription factors in the inhibition of acute-phase proteins still remains to be defined.

A most interesting aspect of future investigation will be the identification of the ultimate target or targets of the insulin signal pathway at the level of the acute-phase protein genes. Our current working model (Fig. 10) is that IL-1, IL-6, and dexamethasone stimulate transcription of "early" acute-phase protein genes by activating preexisting components through a yet to be defined reaction (7a). To sustain elevated transcription of acute-phase protein genes, transcription factor genes, including the C/EBP β gene, are also

activated by IL-1, IL-6, and dexamethasone. The C/EBP β mRNA and its translation product and transactivating function are proportionally enhanced. Insulin inhibits the immediate transcriptional activation of the acute-phase protein genes as well as of the C/EBP β gene by cytokines and dexamethasone. The biochemical nature of this inhibition is unknown. However, insulin elicits, probably through mRNA stabilization, an accumulation of C/EBP β mRNA. The resulting increase in the amount of C/EBP β allows the activation of transfected gene constructs which contain appropriate C/EBP β recognition sequences but are devoid of those sequences sensitive to insulin inhibition. The perfect correlation between C/EBP β expression (Fig. 7) and the activation of transiently introduced cytokine-responsive plasmids (Fig. 8) seems to rule out a direct or indirect modification of

FIG. 10. Schematic model of the interaction of insulin and cytokines on the regulation of transfected and endogenous haptoglobin gene elements. Dex, dexamethasone.

C/EBP β function by insulin. Therefore, we propose that insulin inhibits the transiently transfected pHp(4100)-OCT (Fig. 9) and the chromosomal haptoglobin gene, as well as other acute-phase plasma protein genes, through specific sequences separate from the C/EBP β recognition sites.

Insulin has not primarily been considered important for controlling plasma protein genes during inflammatory reaction. This stems from the fact that insulin has been tested in culture systems in the absence of recognized, plasma protein gene-regulating cytokines and has been unable to induce any changes. Our present experiments confirm that insulin is indeed unable to modulate basal-level expression of plasma proteins (Fig. 1 and 2). Of practical importance in tissue culture experimental systems is that commercially available serum-free tissue culture media for hepatocytes usually contain 5 μ g of insulin per ml (28), which we have found to be detrimental to our cells (Fig. 2). This observation highlights the need to redetermine the optimal concentration of insulin for tissue culture media.

The biological significance of the insulin effect on acute-phase proteins seen in these tissue culture systems must be viewed in the context of in vivo conditions. In vivo, the acute-phase liver is exposed to elevated insulin concentrations in the presence of IL-1, tumor necrosis factor alpha, IL-6, and glucocorticoids (13, 26, 44, 45, 47). Measurements of circulating concentrations of insulin following sepsis and injury range from 0.5 to 5 ng/ml (13, 16, 18, 21, 44). These concentrations are comparable to those found to be active in our tissue culture systems (Fig. 2 and 3). From that, we infer that the expression level of acute-phase plasma proteins during inflammatory responses is attenuated proportionally to the level of insulin. Moreover, the broad spectrum of insulin inhibition renders to this hormone a powerful homeostatic role in counteracting cytokines and restricting the hepatic acute-phase reaction.

The clinical significance of our finding needs to be addressed. Theoretical considerations are diverse. During acute injury, as in endotoxemia or severe burn, a wide variety of hemodynamic and metabolic alterations occur (13, 18, 44). Management of "diabetes of injury" warrants special consideration of our findings, especially since further lowering of acute-phase protein responses by exogenous insulin treatment may be counterproductive for the patient. Lowered or impaired acute-phase protein response in sepsis has been described as having an increased association with a fatal outcome (19).

Patients with insulin-dependent diabetes mellitus are regarded as being at high risk for infection. Defective granulocyte chemotaxis, adherence, and phagocytosis have been implicated to explain these patients' propensity to infection. These functional impairments appear to be correlated to poor diabetic control, particularly chronic hyperglycemia and recurrent ketoacidosis, rather than to an inherent immunologic abnormality (2, 3, 35). Restoration of a eumetabolic state after ketoacidosis generally requires large doses of insulin. Boluses of insulin used in response to marked hyperglycemia in a noncompliant diabetic acutely increase serum insulin concentrations (12). This may result in the suppression of acute-phase protein secretion, which, along with the reversible immunological derangements, may exaggerate the impaired capacity to resist infection in these patients.

Our findings attribute a new homeostatic role to insulin. Although the precise effect of insulin on hepatic acute-phase plasma protein response still needs to be defined, the influence of insulin appears to be sufficient to warrant further

characterization at the tissue, cellular, and molecular levels. Surely, the antagonistic involvement of a tyrosine kinase-dependent signalling system adds a new level of complexity to the already intricate network of regulatory pathways controlling acute-phase protein genes.

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