Insulin Modulates STAT3 Protein Activation and Gene Transcription in Hepatic Cells*

(Received for publication, August 25, 1995, and in revised form, July 3, 1996)

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Treatment of rat hepatoma cells with insulin attenuated the interleukin 6 (IL-6) stimulation of acute phase plasma protein genes. To identify the potential mechanism of this action, the influence of insulin on IL-6 signal transduction was determined. An insulin dose-dependent reduction in signal transducer and activator of transcription 3 (STAT3) gene transcription, mRNA accumulation, protein concentration, and IL-6-inducible DNA binding activity was detected. A reduction in the IL-6 activated sis-inducible element binding of STAT3 was observed within 4 h of insulin treatment, whereas a maximal 3–4-fold lower STAT protein concentration was measured after 8–24 h of insulin treatment. Insulin mediated a similar magnitude reduction in the amount of mRNA encoding the IL-6 receptor α subunit and IL-6 binding activity. These effects of insulin appear to contribute to the strongly suppressed transcriptional induction of the IL-6-responsive acute phase plasma protein genes.

Several pathways are involved in signaling by hematopoietic cytokine receptors; one such pathway involves ligand-induced receptor aggregation and concomitant activation of the receptor-associated cytoplasmic protein tyrosine kinases, particularly the Janus kinases. The activated Janus kinases phosphorylate themselves, the receptor, and members of a family of transcription factors termed signal transducers and activators of transcription (STATs)† (1, 2). Recent structure-function analysis of the IL-6 receptor has implicated STAT3, and to a lesser extent STAT5B, as being involved in the IL-6 induction of acute phase protein (APP) genes (3, 4). STAT3, also termed acute phase responsive factor (5, 6), is rapidly phosphorylated on tyrosine and serine residues in response to IL-6 and related cytokines (7) and coimmunoprecipitates with glycoprotein 130 and Janus kinase 1 (8). The phosphorylation of the receptor-recruited STAT3, as well as STAT1, leads to STAT protein dimerization and display of DNA binding activity that is detectable as high affinity binding to the sis-inducible element (SIE) of the c-fos gene, yielding the complexes termed SIF-A, -B, and -C. These SIF complexes represent SIE binding activity of STAT3 homodimers, STAT1 and STAT3 heterodimers, and STAT1 homodimers, respectively (9). The activated STAT3 proteins are translocated to the nucleus, where it is presumed to bind to specific gene sequences and in combination with other DNA-binding proteins to induce or modulate gene transcription (1–10).

We (11) and others (12, 13) have reported insulin to be a prominent inhibitor of the IL-6-mediated stimulation of APP genes in rat and human hepatoma cells. The molecular mechanism for this inhibitory effect of insulin has not yet been established. Although insulin mediated an increase of both CAAT-enhancer-binding protein mRNA and protein, this transcription factor could not explain the inhibitory action of insulin on IL-6 response, since the regulation of several IL-6-specific APP genes proved to be independent of CAAT-enhancer-binding protein (11). Hence, we proposed an alternative mechanism that suggests that signaling by the insulin receptor interferes with the IL-6 control of the STAT pathway. The current model of insulin action is that insulin binding to its receptor causes activation of its receptor tyrosine kinase activity, which leads to tyrosine phosphorylation of the receptor β subunit as well as several cellular proteins (14). Insulin receptor substrates 1 (IRS-1) (15) and 2 (IRS-2) (16) are the most prominent endogenous substrates of insulin receptor action that undergo tyrosine phosphorylation during insulin stimulation. The resulting phosphorytrosine residues on IRS-1 and IRS-2 serve as docking elements for a plethora of potential downstream regulatory proteins, including phosphatidylinositol 3-kinase, Grb2, Syp, Nck, GTPase-activating protein, and phosphotyrosine phosphatase, all of which contain Src homology 2 domains (17). Although STAT proteins contain in their carboxyl terminus an Src homology 2 domain (2), its binding to IRS-1 or IRS-2 has not been demonstrated. Moreover, no evidence exists that links signaling by insulin with the STAT pathway. We have therefore determined that IL-6- and insulin-responsive rat hepatoma H-35 cells whether the insulin receptor signal pathway has the potential to converge with the IL-6R signal pathway by influencing STAT3. Here we demonstrate that insulin inhibition of acute phase plasma protein gene expression correlates with insulin reduction of STAT3 and IL-6Rα expression.

EXPERIMENTAL PROCEDURES

Cell Lines—Rat hepatoma H-35 cells (18) (subclone of T-7–18 clone; Ref. 19) 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 alone or containing, in addition: human recombinant IL-6 (COS cell derived; Genetics Institute), 100 ng/ml; or porcine insulin (Sigma), 0.5–5000 ng/ml. To measure protein degradation, de novo protein synthesis was inhibited by incubating the cells with 10 μg/ml cycloheximide (Sigma).

RNA Analysis—Total cell RNA was prepared by guanidine thiocya-
Insulin and IL-6 Action

RESULTS AND DISCUSSION

Insulin Reduces STAT3 and IL-6 Response in H-35 Cells—The salient features of the insulin effect on IL-6 stimulation of APP expression in H-35 cells are summarized in Fig. 1A. H-35 cells were pretreated with insulin, challenged with IL-6 for 4 h, and then analyzed for the level of induced mRNA encoding the representative APPs, αFB, and HPX and for the expression and

nate extraction and cesium chloride gradient centrifugation (20). The polyadenylated fraction was recovered by chromatography on mini oligo(dT) cellulose spin columns. RNA was analyzed by Northern (RNA) blot hybridization using 32P-labeled cDNA encoding rat hemopexin (HPX), rat α-fibrinogen (αFB) (21), rat STAT3 (24), and rat IL-6R (22). Hybridization to the housekeeping gene transcript for mouse triose phosphate isomerase (TPP) (23) served as an endogenous marker for RNA loading. The hybridization signals were scanned with a PhosphorImager (Molecular Dynamics) and quantitated using the ImageQuant program.

Western Blot Analysis—Cells were lysed in 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM orthovanadate, 1 mM phenylmethylsulfonlfanyl fluoride, 10 mg/ml leupeptin, and 10 μg/ml aprotinin. Aliquots from the crude lysate containing 30 μg of protein were mixed with an equal volume of SDS buffer, boiled, and electrophoresed on a 6% SDS-polyacrylamide gel. The proteins were transferred to an Immobilon membrane (Millipore), incubated with anti-STAT3 monoclonal antibody (Transduction Laboratories), or anti-phosphotyrosine STAT3 (New England BioLabs, Inc.), and processed for enhanced chemiluminescent reaction according to the specifications of the supplier (Amersham Life Science, Inc.).

Immunoprecipitation was performed by incubating lysates from H-35 cells with either anti-IRS-1 antibody (3 μg/ml) or anti-IRS-2 antibody (3 μg/ml) (Upstate Biotechnology Inc.) for 2 h in the presence of 25 μl of protein G-Sepharose 4B (Pharmacia Biotech Inc.). The beads were washed five times with phosphate-buffered saline containing 1 mM orthovanadate, 5 mM NaF, and 1 mM phenylmethylsulfonlfanyl fluoride. Proteins were eluted by boiling with SDS sample buffer, separated on a 6% SDS-polyacrylamide gel, and transferred to Immobilon-P membranes. The membranes were reacted with antibody to either IRS-1, IRS-2, STAT3 (Transduction Laboratories), anti-phosphotyrosine (Upstate Biotechnology), or anti-phosphoinositide 3-kinase (Upstate Biotechnology) and processed for chemiluminescent reaction.

Electrophoretic Mobility Shift Assay (EMSA)—Whole cell extracts were prepared according to the procedure of Sadowski et al. (9). Double-stranded SIE m67 oligonucleotide served as a binding substrate for STAT proteins. Aliquots of whole cell extracts containing 5 μg of protein were preincubated in 20 μl of reaction buffer (50 mM HEPES, pH 7.9, 250 mM KCl, 5 mM EDTA, 25 mM MgCl2, 50% glycerol, 25 mM dithiothreitol, and 5 mg/ml bovine serum albumin) containing 5 μg of poly(dI- dC) for 15 min on ice, followed by the addition of labeled SIE probe (20,000 cpm), and the binding reaction continued for 15 min at room temperature. To identify the contribution of STAT1 and STAT3 to the SIF patterns, the binding reaction was carried out in the presence of anti-STAT1 (anti-interferon-stimulated gene factor-3; Transduction Laboratories), anti-STAT3 (C-20; Santa Cruz Biotechnology), or control antibodies (Cappel). Ten μl of the reaction mixture were loaded onto a 4% polyacrylamide gel in 0.5 × Tris/borate/EDTA buffer and electrophoresed at 4°C. The radioactive pattern was visualized by autoradiography and quantitated by PhosphorImager scanning.

IL-6 Receptor Binding—Binding of human recombinant IL-6, labeled with 125I by Bolton-Hunter reagent (DuPont NEN; 1600 Ci/mmol) were determined in a single point binding assay on confluent monolayers of H-35 cells in six-well cluster plates. Control cells and cells treated with insulin for 4, 8, and 24 h were washed four times with 2 ml of ice-cold binding medium (minimal essential medium containing 1% bovine serum albumin and 25 mM HEPES, pH 7.2). Replicate culture wells were incubated for 2 h at 4°C in 1 ml of binding medium containing 10−8 M IL-6 alone or with 1 × 10−7 M unlabelled human IL-6. The cells were washed four times with 2 ml of binding buffer, drained of all excess binding solution, solubilized in 0.1 N NaOH, transferred to scintillation vials, and neutralized with acetic acid, and cell-bound radioactivity was determined by scintillation counting. Parallel culture wells not included in the ligand binding assay were trypsinized, and trypan blue dye exclusion was used to determine the number of cells/well. The cell-associated radioactivity, corrected for nonspecific binding, was calculated as IL-6 binding sites/cell (expressed as mean ± S.D.).

Nuclear Run-on Reaction—Nuclei were isolated from H-35 cells and used for run-on as described (24). cDNA encoding STAT3, the acute phase plasma proteins, HPX, αFB, and haptoglobin, and TPI were slot blotted onto nitrocellulose strips. Plasmid pUC13 DNA was used as the control for background hybridization. Equal amounts of 32P-labeled transcripts (2 × 106 cpm) were hybridized to the nitrocellulose strips for 2 days. Strips were washed, digested with RNase A, and mounted together for autoradiography and PhosphorImager scanning.

FIG. 1. Influence of insulin on the expression of STAT3 and APPs. A, H-35 cells were pretreated for 24 h with serum-free medium alone (Control) or containing insulin (500 ng/ml) or IL-6, followed by a 4-h incubation with medium alone (−) or medium containing IL-6. Whole cell extracts were prepared from one-half of the cultures and analyzed by Western blot for STAT3 protein (top panel) or anti-STAT1, or anti-STAT3 and then subjected to EMSA.
DNA binding activity of STAT3. The expected IL-6 stimulation of αFB and HPX mRNA was detected in the control cell cultures. Insulin pretreatments substantially lowered the IL-6 regulation of both APP mRNAs. Moreover, in these cells, the basal expression was also reduced, as is apparent for HPX mRNA. IL-6 treatment for 4 h resulted in the stimulation of SIE binding activity of the STATs, which was primarily restricted to SIF-A complex (Fig. 1A, middle panel), previously determined to contain STAT3 homodimers (25) and verified by antibody supershift assay (Fig. 1B). Cells pretreated with insulin showed reduced SIE binding activity, which appeared to be in part due to reduced STAT3 proteins as detected by Western blot (Fig. 1A, top panel). These results suggested that insulin might affect IL-6 regulation of APP genes by modulating the
IL-6R-signaling potential.

**Insulin Dose-dependent Inhibition of STAT3 Proteins**—To define the insulin action on IL-6 signaling, we first determined the optimal condition for the insulin suppression. The STAT3 protein expression indicated an inverse relationship between the insulin dose and the amount of STAT3 protein detected by Western blotting (Fig. 2A, top panel). A close to maximal reduction was reached at an insulin dose of 500 ng/ml; this dose was therefore used in all subsequent experiments. With a decrease in STAT3 protein, we expected a correspondingly reduced activity of DNA binding with IL-6 treatment. A 15-min exposure to IL-6 revealed that the SIF-C complex (STAT1 homodimers) was most prominent. The pattern changed with a 4-h exposure to IL-6, at which time the SIF-A complex (STAT3 homodimers) became more prominent than SIF-C. This characteristic change in the profile of activated STAT proteins during the course of IL-6 treatment (Fig. 2A, middle panel) has been previously noted in hepatic cells, including HepG2 cells (9) and H-35 cells (3). An increasing dose of insulin resulted in a proportional reduction in both the SIF-C band after a 15-min IL-6 treatment and the SIF-A band after a 4-h IL-6 treatment. Although STAT1 (SIF-C band) is activated by the IL-6 receptor; it is not a trans-activating factor for APP genes, and it appears that the sole role of STAT1 is the regulation of a set of genes that collectively provide innate immunity (2). Our interest has therefore remained focused on STAT3. The insulin-mediated decrease in SIE binding activity of STAT3, measured after a 4-h treatment, correlated with a similar insulin dose-dependent reduction of IL-6-induced APP mRNA (Fig. 2A, bottom panel).

In addition to reducing the available amount of STAT3 proteins, insulin could conceivably influence the level of DNA binding activity of STAT3 by interfering with phosphorylation of preformed STAT3. Particularly critical is phosphorylation of tyrosine residue 705, which is crucial in dimerization and DNA binding of the STAT proteins (25). We determined the IL-6-induced tyrosine phosphorylation of STAT3 by Western blotting using phosphotyrosine-specific anti-STAT3 antibody (Fig. 2B). The level of inducible phosphorylated STAT3, detectable either after a 15-min or 4-h IL-6 treatment, was proportional to the amount of STAT3 present in the cells. Simultaneous IL-6 and insulin treatment did not appreciably change the immediate IL-6 action on STAT3 phosphorylation (Fig. 2B, top right panel). In response to different insulin doses, the values determined by PhosphorImager scanning of the EMSA bands representing SIF-A and SIF-B (Fig. 2A), and the values obtained by densitometric quantitation of the Western blot signals for phosphotyrosine STAT3 (Fig. 2B) showed identical changes, suggesting that the SIE binding activities of STAT3 are primarily a function of phosphorylated STAT3 proteins, which in turn depend on the total amount of STAT3.

**Delayed Kinetics of Insulin Action**—The time course of insulin effect on STAT3 protein expression indicated that insulin treatment alone resulted in a temporally delayed reduction in STAT3 protein level (Fig. 3A, top panel). Increasing duration of exposure to insulin followed by a 15-min IL-6 treatment produced a corresponding delayed reduction in phosphorylated STAT3 protein (Fig. 3A, bottom panel). Western blot analyses of six separate experiments showed that the inhibitory action of insulin became apparent after 4 h and reached maximal values (4-fold reduction) after 8–24 h. Compilation of several experimental data on cells treated for 24 h with insulin revealed a 3.4 ± 1.6-fold (mean ± S.D., n = 7) lower STAT3 signal than that seen in control cells.

Although the kinetics of insulin effect on STAT3 protein level...
did not temporally correlate with APP inhibition (Fig. 3B), the ability of insulin to reduce the STAT3 DNA binding activity (Fig. 3B, middle panel) in part correlated with the insulin inhibition of APP mRNA accumulation (Fig. 3B, bottom panel). The decline in APP mRNA appeared to be proportional to the decline in the amount of SIF-A complex recorded at the same time point. This suggests that the earlier effects of insulin are not due to a decrease in STAT3 protein but rather reflect either the ability of insulin to activate kinases and phosphatases that counteract the stimulatory effects of IL-6 (including those not detectable at the level of STAT3) or, as shown below, the ability of insulin to modulate IL-6R.

Insulin control of STAT3 expression occurred in part at the level of STAT3 mRNA accumulation (Fig. 4). The amount of STAT3 mRNA detected by Northern blot hybridization declined with the same delayed kinetics as STAT3 protein (Fig. 4A). STAT3 mRNA was reduced 2-fold after 8- and 24-h insulin treatment (Fig. 4B). Insulin Reduces mRNA for IL-6Ra—We used IL-6 treatment as an experimental tool to correlate the SIE binding activity with the amount of STAT3 protein that had been established in the insulin-treated cells (Figs. 1–3). This approach proved only applicable when the IL-6 treatment was limited to a few hours. Two additional cell regulatory processes needed to be considered in the interpretation of the results. One process was that the treatment with IL-6 alone enhanced STAT3 mRNA and protein 2–3-fold within 4 h (Fig. 4A). In the presence of insulin, this IL-6 response was reduced 50% on average (data not shown). The second process was that insulin also reduced the level of IL-6Ra mRNA (Fig. 5, top panel). This decrease in IL-6Ra mRNA would be expected to result in a corresponding reduction in IL-6Ra protein expression. We therefore determined IL-6 binding to H-35 cells during the course of insulin treatment and detected a maximal 2-fold decline of IL-6 binding sites/cell after a 24-h insulin treatment (Fig. 5, bottom panel). The decrease in IL-6 binding activity provides another mechanism by which insulin potentially interferes with the IL-6 action; with a reduction of IL-6Ra, the IL-6 signaling inducing the magnitude of STAT activation and APP gene induction is expected to be likewise diminished.

Aside from the down-regulation of STATs and IL-6Ra expression, additional pathways by which insulin conceivably contributes to the observed APP gene regulation need to be considered. Recent studies have proposed that kinases, such as growth factor receptor-activated mitogen-activated protein kinase, have the potential to modify serine residues on STAT3 (26) and that such modification can influence DNA binding (27) and transcriptional induction (28) by the STAT protein pathways. Although we could not assign a functional role for the mitogen-activated protein kinase phosphorylation site (serine 727) of STAT3 for the induction of gene expression in hepatic cells, the influence of other modifications still remains to be determined.

Insulin Effect is Manifested at the Level of APP Gene Transcription—The parallel reduction in STAT3 mRNA and protein level by insulin indicated that these gene products are regulated at the pretranslational level. To evaluate the effect of insulin on STAT3 gene transcription, the transcription rate was determined in H-35 cells treated with increasing doses of insulin alone. At a dose of 500 ng/ml insulin, there was a 2-fold reduction in STAT3 gene transcription compared with control (Fig. 6A). This inhibitory action of insulin on STAT3 gene transcription would also explain the lower STAT3 mRNA level detected in insulin-treated cells (Fig. 4B). To document that the insulin-mediated decrease of STAT3 protein, as seen in Fig. 2, correlated with a proportionally lower ability of the cells to induce immediate early APP genes, we determined the effect of insulin pretreatment on the IL-6-induced transcription of such genes (Fig. 6B). We restricted the IL-6 treatment to 30 min to circumvent complications arising from secondary changes in the expression of STAT3 and IL-6Ra. An insulin dose-depend-
ent impairment of IL-6 action was observed, resulting in a maximal 25-fold reduction in the transcriptional activity of HPX and αFB genes compared with that in cells treated with IL-6 alone. IL-6 enhanced 4-fold STAT3 gene transcription. However, this effect was attenuated 2-fold by insulin pretreatment.

**Effect of Insulin on STAT3 Turnover**—The data thus far indicated that insulin controls STAT3 expression by a 2-fold reduction of mRNA (Fig. 4B) likely by lowering STAT3 gene transcription (Fig. 6A). Since the relative decrease in STAT3 protein exceeded the decrease in STAT3 mRNA by a factor of 1–2, we suspected that insulin might also affect STAT3 protein turnover. To assess this possibility, we measured the STAT3 protein levels by Western blot analysis over 3 h in H-35 cells whose protein de novo synthesis was inhibited by cycloheximide (Fig. 7). This experimental approach is not optimal in that the inhibition of protein synthesis may indirectly influence the precise posttranslational processes of STAT3 turnover and/or the process controlled by insulin. Nevertheless, the results indicate that STAT3 protein, under the selected conditions, had a half-life of approximately 1 h and that this value was not significantly changed by either cotreatment or pretreatment of the cells with insulin.

**STAT3 Does Not Detectably Interact with IRS-1 or IRS-2**—Aside from reduced production and/or elevated turnover of STAT3, there are other potential mechanisms by which the insulin receptor signal could affect STAT3 function, ranging from modulation of the kinase signal pathway acting on STAT3 or associated factors (28) to sequestration of STAT3. Since IRS-1 and IRS-2 are major intermediaries in insulin signal transmission, their involvement in insulin regulation of STAT3 protein activation seemed conceivable and would provide a pathway linking the activated insulin tyrosine kinase receptor with the transcriptional process for APP genes. We hypothesized that IRS-1 or IRS-2, following phosphorylation, serves as an alternative binding site for STAT3 via its Src homology 2 domain, thus diverting it away from IL-6R signal or even leading to its degradation. The end effect would be that less
STAT3 becomes available for homomeric or heteromeric (with STAT1) complex formation, nuclear translocation, and DNA binding activity. To evaluate a possible link of IRS-1 or IRS-2 to STAT3, whole cell lysates from cells treated with or without insulin for 15 min were subjected to immunoprecipitation with antibodies against either IRS-1 or IRS-2. To determine whether STAT3 co-immunoprecipitates with IRS-1 or IRS-2, the immunoprecipitates were subsequently analyzed by Western blotting with anti-STAT3. The results revealed that insulin strongly promoted tyrosyl phosphorylation of IRS-1 (Fig. 8A), but that STAT3 did not significantly co-precipitate with IRS-1 (Fig. 8B) or IRS-2 (Fig. 8D). Anti-phosphoinositide 3-kinase was used as a positive control in the Western blot analysis to clearly demonstrate the expected insulin-induced association of phosphoinositide 3-kinase with IRS-1 (Fig. 8C) and IRS-2 (Fig. 8D).

In this study, we provide the first evidence linking insulin signaling with STAT3 and show that insulin interferes with STAT3 and IL-6Rα expression. We have demonstrated that the insulin effects on STAT3 correlate with and may in part account for insulin suppression of the IL-6 action on the APP gene. Recently, we have identified that STAT3 has a mediator role in IL-6R signaling by activating transcription through IL-6-responsive elements, as found in fibrinogen and hemoxygenin gene promoters (3, 10, 29). However, the regulation of the chromosomal genes in contrast to transient transfected reporter gene constructs proved to be dependent on a larger array of transcription-controlling factors (30). Recognizing the complexity of the signal transduction pathways existing in liver cells, which converge on individual acute phase plasma protein genes, the identification of the molecular and biochemical processes underlying the cross-talk among receptor signals, such as between insulin and IL-6, remains a future challenge.

Acknowledgments—We thank Juergen Ripperger for providing STAT3 cDNA, Francois Guillonneau for the data on IRS-2, and Marcia Held for secretarial assistance.

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