Prolonged insulin treatment inhibits GH signaling via STAT3 and STAT1

Jie Xu, Shaonin Ji, Derwei Y Venable, John L Franklin and Joseph L Messina

Department of Pathology, Division of Molecular and Cellular Pathology, University of Alabama at Birmingham, Birmingham, Alabama 35294-0019, USA

Requests for offprints should be addressed to J L Messina, Department of Pathology, Division of Molecular and Cellular Pathology, Volker Hall, G019, 1670 University Blvd, University of Alabama at Birmingham, Birmingham, AL 35294-0019, USA; Email: messina@path.uab.edu

Abstract

Growth hormone (GH) and insulin are important regulators of cellular and whole body metabolism as well as somatic growth and body composition. Studies have indicated complex feedback effects of GH on insulin action and of insulin on GH signaling pathways. Previous studies in our laboratory have shown that GH induction of signal transducers and activators of transcription (STAT)5B tyrosine phosphorylation is inhibited by prolonged insulin treatment, probably via downregulation of GHR. Here, we find that in rat H4IIE hepatoma cells GH-induced tyrosine phosphorylation of two other STATs (STAT3 and STAT1) was also greatly reduced following prolonged insulin pretreatment compared with that induced by GH alone. In the present work, total STAT5B and STAT1 protein levels were not altered by prolonged insulin treatment. However, prolonged insulin treatment (16 h; 10 or 100 nM) resulted in a 30–40% reduction of total STAT3 protein, with little change at 0·1 and 1·0 nM insulin. Thus, there is a selective reduction of total STAT3 protein levels by insulin, but only at high concentration of insulin. Basal tyrosine phosphorylated (PY)-STAT3 was also significantly reduced by prolonged insulin treatment, and to a greater extent than total STAT3 protein levels. The inhibitory effect of insulin on total STAT3 protein and basal PY-STAT3 levels was dependent on activation of the MEK-ERK pathway, rather than the PI3K pathway. In contrast, the MEK-ERK pathway did not play a major role in insulin’s inhibition of GH-induced PY-STAT3 and PY-STAT1. The present studies indicate that prolonged hyperinsulinemia, such as that found in some obese patients or patients with Type 2 diabetes mellitus, may have profound effects on GH signaling via STAT3 and STAT1.

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Introduction

Growth hormone (GH) is one of the prime regulators of body composition (Ho et al. 1996). Along with other hormones and growth factors it increases muscle mass and decreases subcutaneous and visceral fat (Bjorntorp 1997, Cuneo et al. 1998). Abdominal adiposity is prevalent in human diseases of impaired GH function, including Laron syndrome, a GH-resistant syndrome due to mutation of the GH receptor (GHR), and Prader-Will syndrome in which there is diminished circulating GH (Laron 1995). Abdominal obesity is also associated with human peripheral insulin resistance, hyperinsulinemia, and Type 2 diabetes mellitus (Bjorntorp 1997). Common to all of these conditions is an increase in the ratio of insulin to GH (Ho et al. 1996, Bjorntorp 1997).

The GHR belongs to the superfamily of cytokine receptors. Binding of GH to its receptor results in dimerization of the receptor followed by tyrosine phosphorylation of GHR itself and tyrosine phosphorylation and activation of Janus Activating Kinase 2 (JAK2) (Argetsinger et al. 1993, Carter–Su et al. 1996). Activation of JAK2 by GH leads to phosphorylation and activation of one or more signal transducers and activators of transcription (STAT) (Witthuhn et al. 1993, Dusant-Fourt et al. 1994, Carter-Su et al. 1996). STATs are latent cytoplasmic transcription factors that contain an SH2 domain, a tyrosine phosphorylation site, and DNA-binding and transactivation domains (Frank & Messina 2002). GH has been demonstrated to utilize STAT1, STAT3, STAT5A and STAT5B for the regulation of a variety of genes (Bergad et al. 1995, Waxman et al. 1995, Choi & Waxman 2000, Delesque-Touchard et al. 2000, Lahuna et al. 2000, Waxman 2000). Activation of STAT1 and STAT3 by GH has been reported in mouse 3T3F442A fibroblasts, human skin fibroblasts, cultured hepatocytes and rat liver (Campbell et al. 1995, Han et al. 1996, Ram et al. 1996, Freeth et al. 1998, Phornphutkul et al. 2000, Schaefer et al. 2001). Although STAT5 is the predominant STAT utilized by GH to regulate gene transcription including

In our previous studies, we found that insulin pretreatment for 8–24 h downregulates GHR levels and inhibits the acute effect of GH on STAT5B phosphorylation via the GHR/JAK2/STAT5B pathway (Ji et al. 1999). In the present study, we further investigated the effects of prolonged insulin on GH signaling via the other two STATs (STAT3 and STAT1) in rat H4IIE hepatoma cells. We found that prolonged insulin pretreatment inhibited not only GH-induced tyrosine phosphorylation (PY) of STAT3 and STAT1 but also basal tyrosine phosphorylation of STAT3. Furthermore, prolonged insulin treatment at higher concentrations (10 or 100 nM) specifically reduced total STAT3 protein levels without altering total STAT1 and STAT5B protein levels. The inhibition of insulin on total STAT3 protein and basal PY-STAT3 levels was dependent on the MEK-ERK pathway, rather than phosphatidylinositol-3-kinase (PI3K) pathway. Our work suggests that prolonged hyperinsulinemia may inhibit GH activation of all of the three STATs (STAT1, STAT3 and STAT5B), but the mechanism of STAT3 may be different from STAT1 and STAT5B.

Materials and Methods

Materials

Bovine GH (APF10325C) was kindly provided by Dr A F Parlow, Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center (Torrance, CA, USA) and the National Institutes of Health, National Institute of Diabetes & Kidney Diseases (NIDDK) National Hormone & Pituitary Program. Fetal bovine serum, calf serum, and horse serum were purchased from Invitrogen (Carlsbad, CA, USA). Enhanced chemiluminescent detection reagents were obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, England). Other materials were purchased from Sigma (St Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA) unless otherwise noted.

Antibodies

Anti-STAT1 (mouse) and anti-STAT3 (rabbit) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-STAT5B (mouse) antibody was purchased from Zymed Laboratories (San Francisco, CA, USA). Rabbit antibodies for PY701-STAT1, PY705-STAT3 and PY694-STAT5B antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The rabbit antibodies for ERK1/2, P-ERK1/2 and P-Akt were purchased from New England Biolabs (Beverly, MA, USA). Secondary antibodies including the peroxidase-linked sheep anti-mouse serum and the peroxidase-linked donkey anti-rabbit serum were obtained from Amersham Biosciences. Other secondary antibodies such as the donkey anti-goat IgG were obtained from Santa Cruz Biotechnology.

Cell culture and treatments

Rat H4IIE hepatoma cells were cultured in Swim’s medium supplemented with 10% serum (5% horse serum, 3% newborn calf serum, and 2% fetal calf serum) and 2 µg/ml gentamycin sulfate. At ~50% confluence, cells were removed from serum and maintained in serum-free (and bovine serum albumin–free) medium for 24 h prior to the start of experimental treatments (Ji et al. 1999).

Protein extraction

Hormone treatment (detailed in the text) was terminated by rinsing the cells once with 20 °C TBS (Tris-buffered saline; 10 mM Tris–HCl, pH 7–4, 150 mM NaCl), cells were collected in 20 °C SDS lysis buffer (1% SDS, 10 mM Tris–HCl, pH 7–4, 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, 0.5 mM Na3VO4) and boiled for 5 min, and the cell lysates were rigorously triturated with an Eppendorf pipet and a 100 µl tip. One volume of 4X Laemmli sample buffer (8% SDS, 250 mM Tris–HCl, pH 6–8; 40% glycerol, 4% 2-mercaptoethanol, 0.02% bromophenol blue) was added to three volumes of whole cell lysates, and the solution was boiled for an additional 5 min and stored at ~80 °C until subjected to polyacrylamide gel electrophoresis (Ji et al. 1999).

Electrophoresis and immunoblotting

The protein lysates in Laemmli sample buffer were then subjected to 9–12% gradient SDS-PAGE. Western transfer of proteins was performed as described previously (Ji et al. 1999), except for the use of a BA85 Protran membrane from Schleicher & Schuell (Keene, NH, USA). The membranes were blocked in 0.4–4% milk, 5% bovine serum albumin in TBS, 0.7% Tween 20, pH 8.0. Immunoblotting with the primary antibodies was performed at 4 °C overnight with the rabbit primary antisera, whereas incubation with mouse monoclonal antibodies was at room temperature for 1 h. Washing times after primary and secondary antibodies were at room temperature for 10 and 20 min respectively. All primary antibodies were used in 0.7% Tween 20 in TBS, pH 8.0, with 0.02% azide. Detection of bound antibodies by enhanced chemiluminescence and stripping and reprobing of blots were accomplished according to the manufacturer’s suggestions.
All blots measuring phosphorylated proteins were probed at least twice, the second probing using an antibody to detect total amounts (phosphorylated and non-phosphorylated) of the same protein. These repeat probings were to ensure even loading from lane to lane and an unchanging amount of the total protein following experimental treatments; several of the reprobed Westerns are included in the figures. The bands were analyzed by imaging densitometer as described (Ji et al. 1999), and the relative protein levels were normalized by comparison to the levels of total ERK1/2 (T-ERK).

**Densitometric and statistical analysis**

Enhanced chemiluminescent images of immunoblots were analyzed by scanning densitometry. Multiple exposures of each blot were used to obtain gray-scale images of each chemiluminescent band and were quantified with the Scion Image Analysis program (release beta 2) from Scion Corp. (Frederick, MD, USA). All data were analyzed by analysis of variance using the InStat statistical program (version 3) by GraphPad Software, Inc. (San Diego, CA, USA).

**Results**

GH induced PY-STAT3 and PY-STAT1 in rat hepatoma H4IIE cells

In H4IIE cells, GH significantly induced tyrosine phosphorylation of STAT3 by 10 and 20 min and returned to basal level by 60 min (Fig. 1A, row 1), with a maximum induction of PY-STAT3 by GH of 8–9 fold of control (Fig. 1B). Similarly, the peak of tyrosine phosphorylation of STAT1 by GH was at 10–20 min and returned towards basal levels by 60 min (Fig. 1C, row 1). Following GH exposure, PY-STAT1 induced by GH was about 28-fold compared with vehicle-treated control cells (Fig. 1D),
higher than the fold-change of GH-induced PY-STAT3, probably due to the higher levels of basal PY-STAT3 (Fig. 1A and C).

**Prolonged insulin pretreatment reduced GH-induced PY-STAT3 and PY-STAT1 levels in a dose-dependent manner**

Previous studies in our laboratory indicate that GH induction of STAT5B tyrosine phosphorylation is inhibited by prolonged insulin pretreatment probably via downregulation of GHR (Ji et al. 1999). Since GH also can induce tyrosine phosphorylation of STAT3 and STAT1 in H4IIE cells as described above, the effects of insulin pretreatment on subsequent GH-induced tyrosine phosphorylation of STAT3 and STAT1 was investigated. Following 20 min of GH exposure, PY-STAT3 levels were clearly increased (Fig. 2A, row 1, lane 2 vs. lane 1). Following 100 nM insulin for 16 h, unstimulated/basal PY-STAT3 levels were reduced to barely detectable levels, which was about 30% of control (Fig. 2A and B). Moreover, following 16 h insulin pretreatment, GH for 20 min induced PY-STAT3 levels to about 30% of that induced by GH alone.

As shown in Fig. 1, PY-STAT1 levels were substantially induced over basal levels by GH. Unlike PY-STAT3, basal PY-STAT1 levels were not significantly altered by insulin for 16 h (Fig. 2C), even when the autoradiographs were greatly over-exposed. However, after insulin for

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**Figure 2** Prolonged insulin pretreatment reduced GH-induced PY-STAT3 and PY-STAT1 levels. H4IIE cells were treated with or without insulin for 16 h, followed by GH or vehicle for 20 min. Western blot analysis was performed with antibodies for PY-STAT3, PY-STAT1 and PY-STAT5B, followed by stripping and reprobing with antibody for total-(T)STAT5B as a loading control. (A and C) are representative of three experiments. (B and D) show results of densitometric analysis of autoradiographs from three separate experiments performed to quantify PY-STAT3, PY-STAT1 and PY-STAT5B levels. The data are expressed as mean ± S.E. The PY-STAT3, PY-STAT1 and PY-STAT5B levels in GH-treated samples not pre-treated with insulin were arbitrarily set to 1. *P<0.05, ##P<0.01, ####P<0.001 relative to the corresponding control group.
16 h, PY-STAT1 levels were induced following GH for 20 min to only about 1/3 of that following GH for 20 min alone (Fig. 2C and D). The extent of reduction was similar to the reduction of GH-induced PY-STAT5B levels following insulin treatment (Fig. 2C, row 2, Fig. 2D), as also demonstrated previously (Ji et al. 1999).

Various concentrations of insulin were added to H4IIE cells for 16 h, and a dose-dependent decrease of GH-induced PY-STAT3 and PY-STAT1 was observed. Insulin treatment for 16 h at 0·1, 0·33 and 0·67 nM only moderately inhibited GH-induced PY-STAT3 (Fig. 3A and B), while 0·1 nM insulin caused a significant decrease in PY-STAT1 (Fig. 3C and D). This suggests that the mechanisms by which insulin inhibits PY-STAT3 might be different than that for PY-STAT1. Following insulin pretreatment at concentrations of 1, 10 and 100 nM, GH-induced PY-STAT3 and PY-STAT1 were both significantly reduced. Thus, prolonged insulin pretreatment inhibited GH-induced PY-STAT3 and PY-STAT1 in a dose-dependent manner.

Prolonged insulin treatment reduced total STAT3 protein levels without changing total STAT1 and STAT5B protein levels

The inhibitory effect of prolonged insulin on GH-induced PY-STAT3 and PY-STAT1 can be explained, like PY-STAT5B, by the reduction of GHR abundance and GH binding following prolonged insulin treatment (Ji et al. 1999). However, basal PY-STAT3 was also reduced by insulin, so it was then asked whether prolonged insulin treatment affected the protein abundance of STAT3. In H4IIE cells, total STAT3 protein levels decreased by about 40% following 100 nM insulin for 16 h (Fig. 4A, row 1, lane 2 vs. lane 1, Fig. 4B). When the same blot was stripped and reprobed with STAT1 or STAT5B.
antibody, there was no detectable change in total STAT1 or total STAT5B protein levels (Fig. 4A, rows 2–3, lane 2 vs. lane 1, Fig. 4B). Therefore, prolonged insulin treatment specifically reduced total STAT3 protein levels without affecting the total protein levels of STAT1 and STAT5B.

Prolonged insulin treatment reduced basal PY-STAT3 levels more than the reduction in total STAT3 levels and was dependent on the MEK-ERK pathway

As shown in Fig. 2A and B, insulin reduced basal PY-STAT3 levels. Therefore, we further determined whether decreased basal PY-STAT3 resulted from insulin’s inhibition of total STAT3 protein abundance. Unlike basal PY-STAT5B and PY-STAT1, basal PY-STAT3 levels were detectable in serum-starved, untreated H4IIE cells (Fig. 5A, row 1, lane 1). Also, the basal PY-STAT3 levels were reduced by 30–35% following 0·1 and 1 nM insulin, and 80% and 90% following 10 and 100 nM insulin for 16 h respectively (Fig. 5A and B). When the Western blots were stripped and reprobed with STAT3 antibody, following insulin for 16 h, total STAT3 protein levels were not substantially reduced when insulin was added at 0·1 and 1 nM, and were reduced in a concentration-dependent manner when insulin was added at 10 and 100 nM, to about 70% and 60% of the control levels respectively (Fig. 5A and B). Therefore, prolonged insulin treatment reduced basal PY-STAT3 levels more than the reduction in total STAT3 protein levels, suggesting that the reduction of total STAT3 protein levels only partially contributed to the reduction of prolonged insulin on basal PY-STAT3, and that there must be other mechanisms underlying the reduction of basal PY-STAT3. For comparison, the inhibition of GH-induced PY-STAT3 by prolonged insulin at different concentration shown in Fig. 3B was also included in Fig. 5B. Compared with untreated levels, the induction of PY-STAT3 by GH 20 min was about 9 fold (Fig. 5B, right axis). GH-induced PY-STAT3 levels were reduced by 15% following 0·1 nM insulin, and 60%, 70% and 90% following 1, 10 and 100 nM insulin for 16 h respectively (Fig. 3B, Fig. 5B). The dose dependence of insulin’s inhibition of basal PY-STAT3 closely parallels insulin’s inhibition of GH-induced PY-STAT3 and differs from the insulin-induced decrease of total STAT3 levels. Thus, after prolonged insulin treatment, in addition to a reduction of total STAT3 protein, there is an even greater decrease in PY-STAT3 signaling, including reductions of both basal PY-STAT3 and GH-induced PY-STAT3.

Two major cellular signaling pathways activated by insulin are the PI3K-Akt and MEK-ERK pathways. To investigate the pathways involved in the reduction of total STAT3 protein and basal PY-STAT3 levels following prolonged insulin treatment, H4IIE cells were treated with the MEK inhibitors (PD98059 or U0126) or the PI3K inhibitor LY294002 before insulin treatment. Insulin treatment for 16 h reduced total STAT3 protein and basal PY-STAT3 levels (Fig. 5C, rows 1–2, lane 1 vs. lane 2, Fig. 5D-E). This inhibitory effect of insulin was
Prolonged insulin treatment reduced total STAT3 protein and basal PY-STAT3 levels and was dependent on the MEK-ERK pathway. H4IIE cells were treated with vehicle or insulin for 16 h at different concentrations as indicated. Western blot analysis was performed with antibody for PY-STAT3, followed by stripping and reprobing with antibody for total STAT3. (A) is representative of three experiments. (B) shows results of densitometric analysis of autoradiographs from three separate experiments performed to quantify PY-STAT3 and total STAT3 levels. The data are expressed as mean ± s.e. The PY-STAT3 and total STAT3 levels in untreated samples were arbitrarily set to 1 as shown on the left axis. For comparison, quantification of decreased GH-induced PY-STAT3 at different insulin concentrations (from Fig. 3B) is also included. For this dose–response curve, the maximal fold induction is 9-fold by GH alone and can be read from the right axis. (C) H4IIE cells were treated by the MEK inhibitors, PD98059 (PD) or U0126 (U), or in a separate experiment by the PI3K inhibitor LY294002 (LY) for 30 min before 100 nM insulin treatment for 16 h. Western blot analysis was performed by stripping and reprobing with antibodies for PY-STAT3 and total STAT3. A representative blot of three experiments is shown. (D and E) show results of densitometric analysis of autoradiographs from three similar experiments performed to quantify PY-STAT3 and total STAT3 levels. The data are expressed as mean ± s.e. The PY-STAT3 and total STAT3 levels in untreated samples were arbitrarily set to 1. *P<0.05, #P<0.05, **P<0.01, ###P<0.01, $$$P<0.001 relative to the corresponding control group.
significantly reversed by PD98059 or U0126 pretreatment (Fig. 5C, rows 1–2, lane 2 vs. lane 4, lane 2 vs. lane 6, Fig. 5D and E). However, LY294002 did not block the inhibitory effect of insulin on total STAT3 protein and basal PY-STAT3 levels (Fig. 5C, rows 1–2, lane 8 vs. lane 10). This indicates that the MEK-ERK pathway, rather than PI3K pathway, plays a major role in the reduction of total STAT3 protein and basal PY-STAT3 levels by insulin.

Reduction of GH-induced PY-STAT3 and PY-STAT1 by prolonged insulin pretreatment was independent of the MEK-ERK pathway

We also investigated whether the MEK-ERK pathway played a role in the inhibition of GH-induced PY-STAT3 and PY-STAT1 following prolonged insulin treatment. H4IIE cells were treated with the MEK inhibitor PD98059 or U0126 before insulin treatment. As shown in Fig. 2, insulin for 16 h inhibited GH-induced PY-STAT3 and PY-STAT1 (Fig. 6A, rows 1–2, lines 1–2 vs. lane 5, Fig. 6B and C). However, the MEK inhibitors PD 98059 or U0126 failed to block the inhibitory effect of insulin on GH-induced PY-STAT3 and PY-STAT1 (Fig. 6C, rows 1–2, lanes 6–7, Fig. 6B and C). This suggests that, even though the MEK-ERK pathway plays an important role in the reduction of total STAT3 protein and basal PY-STAT3 levels by insulin, it may not be a key pathway mediating the inhibition of insulin on GH-induced PY-STAT3 and PY-STAT1.

Discussion

Growth hormone plays a central role in regulating growth in vertebrates and, in combination with other hormones, in the regulation of protein synthesis and intermediary metabolism (Lewis 1992, Ailhaud et al. 1994). Although GH has many actions opposing those of insulin, it can have acute insulin-like effects, especially when GH is deficient (Ho et al. 1996). In contrast, excessive GH induces insulin resistance (Luger et al. 1990), but little is known about the ability of insulin to promote GH resistance.

Figure 6 Reduction of GH-induced PY-STAT3 and PY-STAT1 by prolonged insulin pretreatment was independent of MEK-ERK pathway. H4IIE cells were treated with the MEK inhibitors PD98059 (PD) or U0126 (U) for 30 min before 100 nM insulin treatment for 16 h. Western blot analysis was performed with antibodies for PY-STAT3 and PY-STAT1. The blot was reprobed with total ERK1/2 (T-ERK1/2) antibody as a loading control. (A) is representative of three experiments. (B and C) show results of densitometric analysis of autoradiographs from 3 separate experiments performed to quantify PY-STAT3 and PY-STAT1 levels. The data are expressed as mean ± se. The PY-STAT3 and PY-STAT1 levels in untreated samples were arbitrarily set to 1. ##P<0.01, ###P<0.001, ####P<0.001 relative to the corresponding control group.
In a previous study, we found that prolonged treatment of H4IIE cells with high insulin concentrations, similar to those in the hepatic portal circulation in patients with hyperinsulinemia, resulted in a severe diminution of GH-induced STAT5B phosphorylation, which correlates with the reduction of GHR protein levels and GH binding (Ji et al. 1999). Like STAT5B, GH also activates STAT3 and STAT1 via the GHR/JAK2 complex. Phosphorylation of the receptor-recruited STAT3, as well as STAT1, leads to STAT protein dimerization and translocation to the nucleus, where it binds to specific gene sequences and in combination with other DNA-binding proteins, modulates gene transcription (Gronowski & Rorwein 1994, Meyer et al. 1995, Campbell et al. 1995, Ram et al. 1996).

GH has been reported to activate STAT3 and STAT1 in mouse 3T3F442A fibroblasts, human skin fibroblasts, cultured hepatocytes and rat liver tissue (Campbell et al. 1995, Han et al. 1996, Ram et al. 1996, Freeth et al. 1998, Phornphutkul et al. 2000, Schaefer et al. 2001). In the present study, we found that GH activated STAT3 and STAT1 in a rat hepatoma cell line.

Although insulin alone is reported to cause acute tyrosine or serine phosphorylation of STAT3 in some cell lines over-expressing the insulin receptor (IR) (Ceresa et al. 1997, Coffet et al. 1997), several studies found that insulin can inhibit STAT3 activation induced by cytokines such as interleukin-6 (IL-6) and leptin. Insulin inhibits the IL-6-induced DNA binding activity of STAT3 and subsequent gene transcription in rat and human hepatoma cells (Thompson et al. 1991, Campos & Baumann 1992, O’Riordain et al. 1995). Besides IL-6, leptin-induced phosphorylation of STAT3 is also attenuated by insulin pretreatment in human hepatoma Huh7 cells and in the rat hypothalamus (Fujita et al. 2003, Kuwahara et al. 2003). Here, we reported that continuous insulin inhibited GH-induced tyrosine phosphorylation of STAT3 and STAT1 in rat hepatoma cells.

The dose-dependent inhibition by insulin on GH-activated STAT3 and STAT1 may be at least partly due to the decreased GHR abundance and GH binding (Ji et al. 1999). However, insulin at low concentration (0·1 nM) was sufficient to significantly reduce GH-induced PY-STAT1, while it did not alter GH-induced PY-STAT3. Since 0·1 nM insulin for 16 h resulted in little reduction of GHR levels (Ji et al. 1999), insulin’s inhibition of PY-STAT1 at this concentration may be due to some post-receptor mechanism. In the present study, insulin also reduced the basal PY-STAT3 without affecting basal PY-STAT1 and PY-STAT5B levels. This cannot be explained by the reduction of GHR and GH binding, but may be caused by the change of STAT3 protein abundance. Therefore, we subsequently determined whether insulin influenced total STAT3 protein levels. In rat H4IIE hepatoma cells, we observed the dose-dependent reduction of total STAT3 protein by prolonged insulin treatment at high concentrations (10 and 100 nM). This is consistent with a previous report that the amount of STAT3 protein decreased after 24 h of insulin treatment as a result of the reduction of STAT3 mRNA (Campos et al. 1996). In contrast, in the present study insulin did not alter the total STAT1 and STAT5B protein levels, indicating that the inhibition by insulin of STAT protein abundance is STAT3-specific. Furthermore, insulin reduced basal PY-STAT3 more than total STAT3 protein levels. The dose-dependence of insulin’s inhibition of basal PY-STAT3 closely parallels the inhibition of insulin on GH-induced PY-STAT3, and was different from the effect of insulin on total STAT3 protein levels. This suggests that, besides reduction of total STAT3 protein abundance, through some separate mechanism insulin may also inhibit the signaling pathway inducing PY-STAT3.

Insulin signaling consists of a cascade of phosphorylation events carried out by protein and lipid kinases (Nakae & Accili 1999, Whitehead et al. 2000). Autophosphorylation of the receptor further phosphorylates insulin receptor substrates (IRS), which serve as docking molecules, favoring the generation of intracellular signals (Myers, Jr et al. 1994, White & Kahn 1994). There are at least two main branches that propagate insulin intracellular signaling: the IRS-PI3K-Akt pathway and the Ras-Raf-MEK-ERK pathway (Maassen & Ouwens 1997, Nakae & Accili 1999). We then determined that the MEK inhibitors PD98059 or U0126 prevented the reduction of total STAT3 protein and basal PY-STAT3 levels while a PI3K inhibitor, LY294002, did not. This indicates that the inhibitory effect of insulin on total STAT3 protein and basal PY-STAT3 levels is specifically via the MEK-ERK pathway. In contrast, MEK inhibitors did not prevent the inhibition of GH-induced PY-STAT3 and PY-STAT1 by prolonged insulin treatment, suggesting that the MEK-ERK pathway may not play a major role mediating insulin’s inhibition of GH signaling via STAT3 and STAT1. Thus, we propose that there are at least two mechanisms by which prolonged insulin treatment inhibits STAT3 activation: 1) reduction of GHR and GH binding, as we previously reported (Ji et al. 1999); and 2) reduction of STAT3 protein abundance and basal PY-STAT3 levels which is dependent on activation of MEK-ERK pathway by insulin.

There may be several consequences resulting from inhibition of insulin on activation of STAT3 and STAT1. First, STAT3 and STAT1 are involved in GH-dependent gene regulation, such as the c-fos gene (Herrington et al. 2000). The physiological relevance of STAT3 and STAT1 in GH-regulated transcription of c-fos is supported by mutational and deletion analysis of the c-fos enhancer (Chen et al. 1995, Robertson et al. 1995). The inhibition of prolonged insulin on activation of STAT3 and STAT1 may result in the reduced expression of c-fos in response to GH.

Secondly, liver-specific STAT3 knockout mice showed impaired acute-phase response in liver (Alonzi et al. 2001).
IL-6 is an important cytokine regulating induction of acute-phase response genes via activation of STAT3 (Lutticken et al. 1994), but little is known about whether GH regulates expression of acute-phase genes. GH secretion increases in response to several types of stress, including early in the acute-phase response, and thus GH may be important in the physiological response to stress (Moore et al. 1995). GH has been reported to induce synthesis of acute-phase proteins, including fibrinogen and α2-globulin, in healthy rat liver (Griffin & Miller 1974). Fibrinogen-β mRNA was also rapidly induced by GH in the liver of GH-deficient dwarf rats (Thompson et al. 2000). Our laboratory recently found that in H4IIE cells, GH induces a Class 2 acute-phase gene, hemopexin (Stred & Messina 2003). The cellular mechanisms by which GH induces acute-phase gene expression is not clear, but activation of STAT3 is a probable pathway. It awaits further study whether GH induces acute-phase genes via activation of STAT3, and whether the inhibition of insulin on STAT3 activation will result in the attenuation of GH-induced acute-phase gene expression.

Lastly, a recent study of liver-specific STAT3 knockout mice also revealed that STAT3 plays an essential role in normal glucose homeostasis by regulating gluconeogenic gene expression (Inoue et al. 2004). In this study, mice with liver-specific deficiency in STAT3 become insulin resistant, with increased expression of gluconeogenic genes, such as phosphoenolpyruvate carboxykinase-1 and glucose 6-phosphatase, resulting in increased gluconeogenesis. Elevated gluconeogenesis and gluconeogenic gene expression can be corrected by adenovirus-mediated restoration of hepatic STAT3 expression in these mice (Inoue et al. 2004). The regulation of hepatic gluconeogenesis is important in the maintenance of blood glucose levels, and insulin is the most important inhibitor of gluconeogenesis. Insulin acts by directly suppressing the expression of the genes for the key gluconeogenic enzymes (Barthel & Schnoll 2003). In contrast, IL-6 is an important cytokine stimulator of gluconeogenesis, both in vitro and in vivo (Blumberg et al. 1995, Klasing 1988). Since insulin normally inhibits IL-6 signaling (Dandonia et al. 2004), insulin may also exert an indirect suppression on gluconeogenesis by inhibiting IL-6 action. An increase in gluconeogenesis in the liver is partially responsible for the enhanced hepatic glucose production and fasting hyperglycemia in individuals with diabetes mellitus (Taylor 1999). When insulin resistance occurs, blood glucose levels will increase resulting from loss of insulin’s direct and indirect inhibition of gluconeogenesis. Furthermore, if the inhibitory effect of insulin on STAT3 that we observed in H4IIE cells is also true in vivo, the impaired STAT3 signaling caused by prolonged hyperinsulinemia may lead to increased gluconeogenesis and even higher blood glucose levels.

In summary, we found that the effects of prolonged insulin treatment in H4IIE cells: 1) inhibited GH-induced tyrosine phosphorylation of STAT3 and STAT1 in a MEK-ERK-independent manner; 2) specifically reduced total STAT3 protein levels and basal tyrosine phosphorylation of STAT3, which was dependent on activation of the MEK-ERK pathway by insulin. The reduction of basal PY-STAT3 exceeded the insulin-induced reduction of STAT3 protein suggesting multiple, separable actions of insulin in reducing basal and GH-stimulated PY-STAT3. The present study combined with a previous one (Ji et al. 1999), leads us to conclude that prolonged insulin treatment inhibits GH signaling via STAT1, STAT3 and STAT5B. However, the underlying mechanism of STAT3 inhibition also involves the reduction of protein abundance, which is different from STAT1 and STAT5B. Our studies indicate that prolonged hyperinsulinemia, such as that found in some obese patients or patients with Type 2 diabetes mellitus may have profound effects on GH signaling. Numerous GH-regulated genes are dependent upon activation of different combinations of STAT1 and STAT3 or on STAT5B. Thus, GH regulation of these genes may be compromised following prolonged hyperinsulinemia due to the reduced ability of GH to phosphorylate/activate STAT1, STAT3 and STAT5B.

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