

# Insulin Inhibits Growth Hormone Signaling via the Growth Hormone Receptor/JAK2/STAT5B Pathway\*

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**Insulin is important for maintaining the responsiveness of the liver to growth hormone (GH). Insulin deficiency results in a decrease in liver GH receptor (GHR) expression, which can be reversed by insulin administration. In osteoblasts, continuous insulin treatment decreases the fraction of cellular GHR localized to the plasma membrane. Thus, it is not clear whether hyperinsulinemia results in an enhancement or inhibition of GH action. We asked whether continuous insulin stimulation, similar to what occurs in hyperinsulinemic states, results in GH resistance. Our present studies suggest that insulin treatment of hepatoma cells results in a time-dependent inhibition of acute GH-induced phosphorylation of STAT5B. Whereas total protein levels of JAK2 were not reduced after insulin pretreatment for 16 h, GH-induced JAK2 phosphorylation was inhibited. There was a concomitant decrease in GH binding and a reduction in immunoreactive GHR levels following pretreatment with insulin for 8–24 h. In summary, continuous insulin treatment in rat H4 hepatoma cells reduces GH binding, immunoreactive GHR, GH-induced phosphorylation of JAK2, and GH-induced tyrosine phosphorylation of STAT5B. These findings suggest that hepatic GH resistance may develop when a patient exhibits chronic hyperinsulinemia, a condition often observed in patients with obesity and in the early stage of Type 2 diabetes.**

Growth hormone (GH)<sup>1</sup> is one of the prime regulators of body composition (1). Along with other hormones and growth factors it increases muscle mass and decreases subcutaneous and visceral fat (2, 3). 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-Willi syndrome in which there is diminished circulating GH (4, 5). Abdominal obesity is also associ-

ated with human peripheral insulin resistance, hyperinsulinemia, and Type 2 diabetes (2, 6). Common to all of these conditions is an increase in the ratio of insulin to GH (1, 2, 6).

The GHR belongs to the superfamily of cytokine receptors and in humans and rabbits the full-length GHR is translated from a single mRNA (7). Circulating GHBP results from proteolytic cleavage of the plasma membrane-associated GHR (7). However, a recent study suggests that primate GHBP may also arise from an alternatively spliced mRNA, a mechanism first indicated in rodents (7, 8). 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) (9, 10). Activation of JAK2 by GH, and by other cytokines and growth factors, leads to phosphorylation and activation of one or more signal transducers and activators of transcription (STAT) (10–12). The JAK-STAT pathway, is a major pathway for GH regulation of gene transcription. Although GH promotes activation of STAT1, STAT3, STAT5A, and STAT5B, gene disruption experiments indicate that STAT5B is necessary for GH regulation of sexually dimorphic hepatic genes (10, 13).

*In vivo* insulin appears to be necessary for normal liver GH responsiveness, probably by maintaining liver GHR levels (14–17). In Type 1 diabetic patients and streptozotocin-treated rodents, insulin deficiency is correlated with hepatic GH resistance which, in most studies, is associated with reduced levels of circulating GHBP in patients or decreased liver GHR in rodents (14–24). In streptozotocin-treated rats, circulating insulin-like growth factor 1 (IGF-1), whose mRNA expression is regulated by GH, is reduced as is GH binding capacity. Insulin treatment restores IGF-1 levels and in some, but not all experiments, restores GH binding (14, 17, 25–27). In Type 1 diabetic patients intraperitoneal insulin administration restores GHBP levels better than subcutaneous insulin treatment (19, 20, 23). This suggests that peripheral (subcutaneous) insulin administration may result in portal insulin concentrations insufficient, compared with intraperitoneal insulin infusion, to properly regulate hepatic GHR expression and therefore circulating GHBP. Patients with Type 2 diabetes and peripheral insulin resistance also exhibit reduced circulating IGF-1 levels, possibly due to a decrease in GH responsiveness, but it has not yet been studied whether liver GHR or circulating GHBP levels are reduced accordingly (18, 23).

*In vitro* studies are also inconsistent concerning whether insulin can increase GH binding and GHR mRNA. For example, in a study with primary cultures of rat hepatocytes, insulin treatment increases GH binding 4-fold with no significant effect on GHR mRNA expression (28). In a study measuring the subcellular localization of GHR in osteoblasts, continuous insulin treatment decreases the fraction of cellular GHR presented at the plasma membrane via inhibition of surface translocation of GHR with no effect on the total cellular content of

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<sup>1</sup> The abbreviations used are: GH, growth hormone; GHR, growth hormone receptor; JAK, Janus activating kinase; STAT, signal transducers and activators of transcription; PRL, prolactin; IGF, insulin growth factor; GHBP, growth hormone binding protein; h, human; o, ovine; b, bovine.

GHR (29). However, changes in GH-induced signaling pathways have not been investigated in these studies measuring insulin-induced changes in GHR and GH binding.

There is impaired GH action after 60 years of age in humans, possibly resulting from decreases in circulating GHBP levels, and therefore most likely hepatic GHR levels (30). Also, there may be defects in GH stimulation of the JAK-STAT signaling pathway in aging humans, as there are in aging mice (31). Therefore, investigation of factors that affect GH responsiveness may help in the understanding of aging-related changes in GH action.

In the present study, insulin pretreatment for 8–24 h was found to reduce the acute effect of GH on STAT5B phosphorylation in rat H4 hepatoma cells. The GH-induced tyrosine phosphorylation of JAK2, immunoreactive GHR, and binding of  $^{125}\text{I}$ -hGH were also reduced following insulin pretreatment. Inhibition of GH-induced STAT5B phosphorylation, immunoreactive GHR, and GH binding were all reduced by insulin pretreatment with similar kinetics. Our study indicates an extensive reduction in hepatoma cell GH responsiveness following conditions that may mimic the chronic hyperinsulinemia observed in some obese patients and patients in the early stages of Type 2 diabetes.

#### EXPERIMENTAL PROCEDURES

**Materials**—Bovine GH (bGH; lot number APF11182B) and ovine prolactin (oPRL, NIDDK-oPRL-21; lot number AFP-10692C) were kindly provided by Dr. A. F. Parlow, Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center (Torrance, CA) and the NIDDK, National Institutes of Health National Hormone & Pituitary Program. The  $^{125}\text{I}$ -human GH ( $^{125}\text{I}$ -hGH) was purchased from NEN<sup>TM</sup> Life Science Products. Porcine sodium-insulin was a gift from Dr. Ron Chance (Eli Lilly, Co., Indianapolis, IN) and unlabeled hGH was also kindly provided by Eli Lilly, Co. Fetal bovine serum, calf serum, and horse serum were purchased from Life Technologies, Inc. (Grand Island, NY). Protein G-Sepharose was obtained from Pharmacia Biotech Inc. (Uppsala, Sweden) and ECL detection reagents were obtained from Amersham Corp. Other materials were purchased from Sigma and Fisher (Pittsburgh, PA) unless otherwise noted.

**Antibodies**—Anti-STAT5 monoclonal antibody (raised against amino acid 451–649 of sheep STAT5A) was purchased from Transduction Laboratories (Lexington, KY). Mouse anti-STAT5A (raised against the unique C terminus of murine STAT5A) and mouse anti-STAT5B (raised against the unique C terminus of murine STAT5B) monoclonal antibodies and rabbit anti-phosphotyrosine-STAT5 polyclonal antibodies (raised against the phospho-peptide around C-terminal Y694 of murine STAT5A that is conserved in both STAT5A and STAT5B of human, sheep, and rat) were obtained from Zymed Laboratories Inc. (San Francisco, CA). Rabbit anti-JAK2 peptide antiserum, directed at residues 758–776 of murine JAK2 (called anti-JAK2UBI), and 4G10 mouse monoclonal anti-phosphotyrosine antibody were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). A second anti-JAK2 serum (anti-JAK2AL33) was raised in rabbits against a glutathione *S*-transferase fusion protein incorporating residues 746–1129 of the murine JAK2, as described (32, 33). Two anti-GHR antisera were raised in rabbits against peptides of the cytoplasmic domain of the GHR: anti-GHRcyt3728 against residues 317–620 of the human GHR cytoplasmic domain and anti-GHRcytAL37 against residues 271–620 fused to glutathione *S*-transferase as described (33, 34). Secondary antibodies including the peroxidase-linked sheep anti-mouse serum and the peroxidase-linked donkey anti-rabbit serum were obtained from Amersham Life Science.

**Cell Culture**—Rat H4-II-E (H4) hepatoma cells were cultured in Swim's medium supplemented with 10% serum mixture (5% horse serum, 3% newborn calf serum, and 2% fetal calf serum) and 2  $\mu\text{g}/\text{ml}$  gentamycin sulfate (35). At about 50% confluence, cells were removed from serum and maintained in serum-free media for 24 h prior to protein extraction.

**Protein Extraction**—Hormone treatment (detailed in the text) was terminated by rinsing the cells once with TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) and cells were collected in 100  $^{\circ}\text{C}$  SDS-lysis buffer (1% SDS, 10 mM Tris-HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, 0.5 mM  $\text{Na}_3\text{VO}_4$ ) and boiled for 5 min. One volume of 4  $\times$  Laemmli sample buffer (8% SDS, 250 mM Tris-HCl, pH 6.8, 40%

glycerol, 4%  $\beta$ -mercaptoethanol, 0.02% bromphenol blue) was added to 3 volumes of whole cell lysates, the solution was boiled for an additional 5 min and stored at  $-80^{\circ}\text{C}$  until subjected to polyacrylamide gel electrophoresis.

In an alternative method, cells were scraped into 4  $^{\circ}\text{C}$  TBS plus 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, 0.5 mM  $\text{Na}_3\text{VO}_4$  and cell pellets were collected by centrifugation ( $800 \times g$  for 2 min at 4  $^{\circ}\text{C}$ ). Cell pellets were solubilized with mild agitation for 1 h at 4  $^{\circ}\text{C}$  in Triton-lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 1.5 mM  $\text{MgCl}_2$ , 1 mM EDTA, 10 mM  $\text{Na}_4\text{O}_7\text{P}_2$ , 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, 1 mM  $\text{Na}_3\text{VO}_4$ , 10 mM benzamide, 2  $\mu\text{g}/\text{ml}$  aprotinin). After centrifugation at  $15,000 \times g$  for 15 min at 4  $^{\circ}\text{C}$ , the supernatants were immediately subjected to either immunoprecipitation or were boiled for 5 min in sample loading buffer, and stored at  $-80^{\circ}\text{C}$  until electrophoresis.

**Immunoprecipitation, Electrophoresis, and Immunoblotting**—For immunoprecipitation in nonreducing conditions, cells were lysed in Triton-lysis buffer. Protein G-Sepharose was used to adsorb immune complexes and after extensive washing with Triton-lysis buffer precipitated proteins were eluted by boiling in sample loading buffer for 5 min. The proteins were then subjected to 5–9% gradient SDS-polyacrylamide gel electrophoresis. Western transfer of proteins were performed as described previously, except for the use of Protran membrane from Schleicher & Schuell (BA 85) (34). Immunoblotting was performed with the antibodies at the following dilutions: anti-STAT5 (1:500), anti-STAT5A (1:5000), anti-STAT5B (1:5000), anti-PY-STAT5 (1:5000), 4G10 (1:2500), anti-GHRcyt3728 and anti-GHRcytAL37 (1:2000), or anti-JAK2 (1:2000) with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:2000). Incubation with rabbit primary antiserum was at 4  $^{\circ}\text{C}$  overnight, while 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 45 min, respectively, unless specified. Washing times after anti-GHRcyt3728 and anti-rabbit IgG were at room temperature for 90 and 20 min, respectively. All antibodies are used in 0.7% Tween 20 in TBS, pH 8.0, with 0.4% milk, 2% bovine serum albumin, and 0.04% azide added to the primary antibodies except for 4G10, in which milk was excluded. Detection of bound antibodies by ECL and stripping and reprobing of blots were accomplished according to the manufacturer's suggestions.

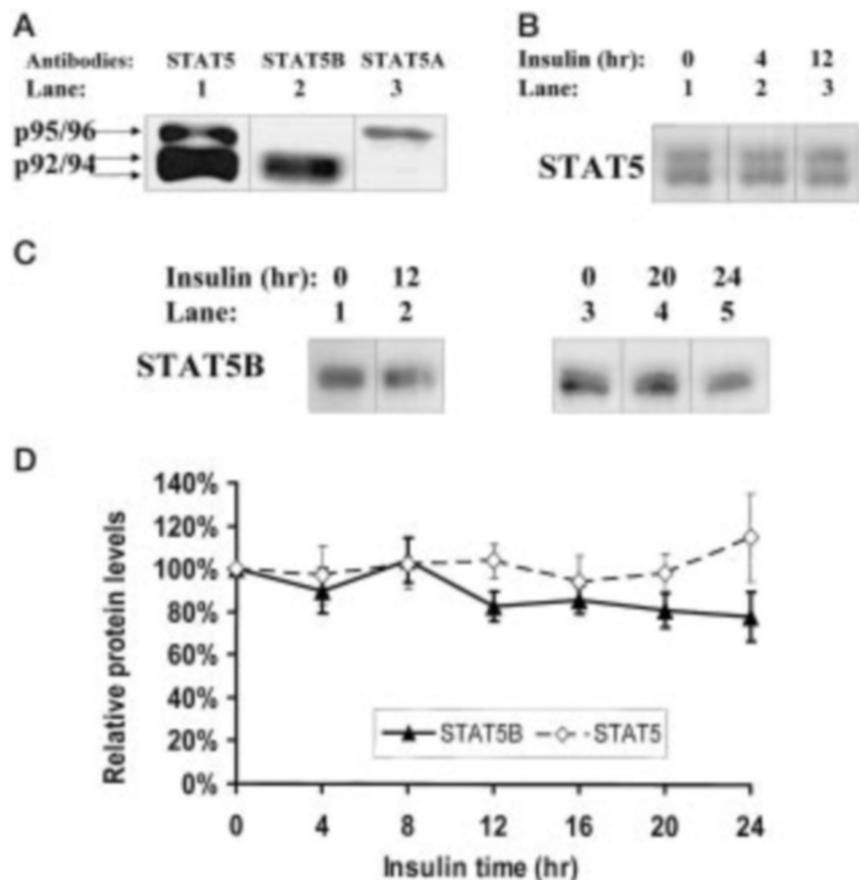
**$^{125}\text{I}$ -hGH-binding Assay**—H4 cells were grown to 50% confluence in 60-mm plates and treated as indicated in the text before partial withdrawal of media to reduced volume to 1 ml. Cells were then incubated with a constant amount of  $^{125}\text{I}$ -hGH within a given experiment (between  $6 \times 10^5$  and  $9 \times 10^5$  cpm per plate in the separate experiments) with or without 2  $\mu\text{g}/\text{ml}$  unlabeled hGH, bGH, or oPRL at room temperature for 2 h with gentle agitation (36, 37). Following incubation, binding was measured by washing the cells twice with 2 ml of 4  $^{\circ}\text{C}$  binding buffer (25 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 1.2 mM  $\text{MgCl}_2$ , 0.1% bovine serum albumin), harvesting the cells in a buffer of 1% SDS, 0.1 N NaOH and counting in a  $\gamma$ -counter (Packard Instrument Co., Meriden, CT).

**Densitometric and Statistical Analysis**—ECL images of immunoblots were scanned and quantified with a Scion-image analysis program (release beta 2) from Scion Corp. (Frederick, MD). All data was analyzed by ANOVA using the InStat statistical program (version 3) by GraphPad Software, Inc. (San Diego, CA).

#### RESULTS

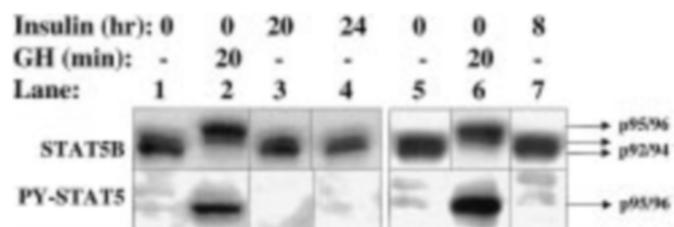
**STAT5A and STAT5B Were Expressed in H4 Cells at Comparable Levels**—We first asked whether the STAT5 proteins were expressed at appreciable amounts in rat H4 hepatoma cells. Using a pan-STAT5 antibody, the immunoreactive STAT5 bands from whole cell extracts from untreated H4 cells migrated as at least 3 bands. From previously published studies, and as confirmed here with isoform-specific antibodies, the upper band that runs as p95 or p96, depending upon the study (and referred to as p95/p96) was specific for STAT5A (Fig. 1A, lanes 1 and 3). The lower p92/p94 doublet was STAT5B with the doublet arising because a fraction of the STAT5B is serine-phosphorylated, even in quiescent cells, and thus displays retarded migration (Fig. 1A, lanes 1 and 2, and Refs. 38 and 39). As observed in Fig. 1A, lane 1, using the pan-STAT5 antibody, there was less STAT5A than STAT5B (approximately 70% as much STAT5A as STAT5B by densitometry) in H4 cell extracts.

**FIG. 1. Effects of insulin treatment on STAT5, STAT5A, and STAT5B levels.** H4 cells were serum starved for 48 h and left untreated or treated with insulin (100 nM) for the time indicated. *A, B,* and *C,* representative experiments are presented with cells harvested, whole cell lysates prepared, and subjected to Western blot analysis as described in the text with antibodies specific for STAT5, STAT5A, and STAT5B as indicated either *above* the lane in *A* and to the *left* in *B* and *C*. *D,* densitometric analysis of autoradiographs from four similar experiments were performed to quantify changes in STAT5 and STAT5B and are expressed as the mean  $\pm$  S.E. of the mean. The levels of STAT5 or STAT5B in control (untreated) cells was arbitrarily set to 100% within each experiment and the levels following various times of insulin pretreatment are expressed as a percentage of expression measured in control cells. All insulin time points are not statistically significant compared with the zero time by ANOVA.



**Effect of Insulin on STAT5 Levels**—We then asked whether insulin altered the cellular levels of the STAT5 isoforms. Using the pan-STAT5 antibody, there was no appreciable change in cellular STAT5 levels following insulin treatment of H4 cells for 4 and 12 h (Fig. 1*B*, lanes 1–3). Using the STAT5B-specific antibody, a slight reduction of STAT5B was sometimes observed following insulin treatment for 12, 20, and 24 h (Fig. 1*C*, lanes 1–5). When multiple experiments were quantified by densitometry (Fig. 1*D*), total cellular levels of STAT5 were unchanged by insulin treatment and STAT5B levels were reduced to approximately 80% of that measured in untreated, control H4 cells after insulin treatment for 12 or more hours. However, this small reduction of STAT5B was not statistically significant.

**Continuous Insulin Treatment Did Not Alter STAT5 Phosphorylation**—Previous studies demonstrated that unphosphorylated STAT5B migrates faster on polyacrylamide gels and the migration of tyrosine and/or serine-phosphorylated STAT5B is retarded (38, 39). In the present studies using rat H4 hepatoma whole cell extracts, and using an antibody specific only to STAT5B, in the absence of GH treatment STAT5B migrated as a doublet of p92/p94 (Fig. 2, top row, lanes 1 and 5) which were previously shown to be specific for unphosphorylated STAT5B (p92) and STAT5B phosphorylated only on serine residues (p94) (38, 39). Using the STAT5B-specific antibody, that does not cross-react with STAT5A, bGH treatment (20 min) of rat H4 hepatoma cells induced tyrosine phosphorylation of STAT5B (PY-STAT5B), as suggested by the retarded mobility of STAT5B (Fig. 2, top row, lanes 2 and 6 versus lanes 1 and 5, respectively). This 95–96-kDa band (p95/p96) is thought to be specific for STAT5B phosphorylated on both serine and tyrosine residues (PY-STAT5B (38, 39)). Thus, when phosphorylated on both tyrosine and serine, STAT5B migrates at approximately the same size as STAT5A (Fig. 1*A*, lane 3).

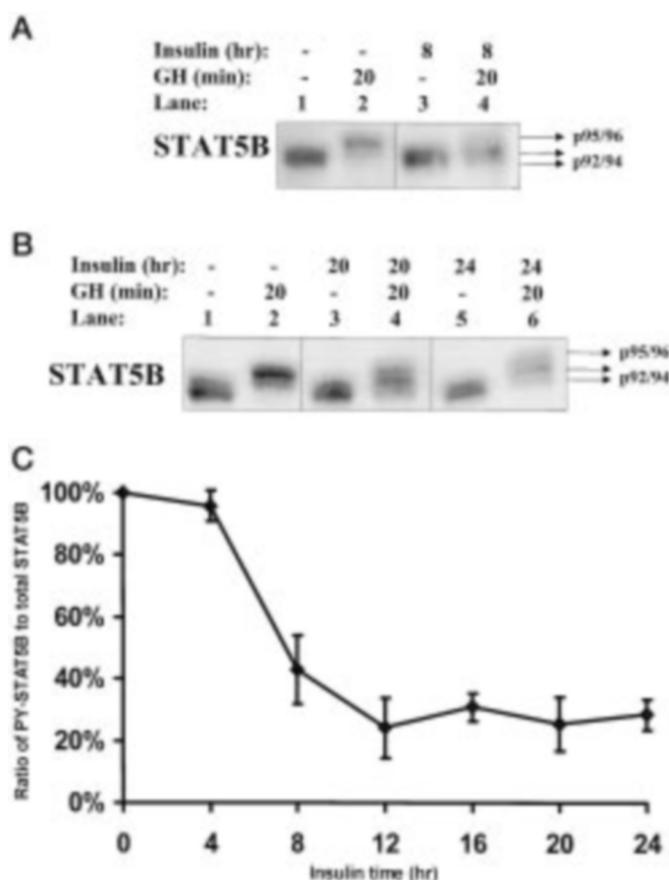


**FIG. 2. Insulin did not effect STAT5B migration and tyrosine phosphorylation of STAT5.** Western blots were performed as described in the legend for Fig. 1 and in the text except that some H4 cells were treated with bovine growth hormone (bGH; 500 ng/ml) for 20 min and the antibodies used (as indicated to the left) included that for STAT5B and phosphorylated tyrosine STAT5. A representative experiment is presented.

Insulin pretreatment of H4 cells for 8, 20, and 24 h did not alter the mobility, and therefore the phosphorylation, of STAT5B (Fig. 2, top row, lanes 3, 4, and 7).

Tyrosine phosphorylation of STAT5 was then measured by using a phosphotyrosine-specific antibody and, consistent with the mobility shift using the STAT5B antibody, bGH-induced the phosphorylation of STAT5 on tyrosine (PY-STAT5; Fig. 2, bottom row, lanes 2 and 6 versus lanes 1 and 5, respectively). Also consistent with the mobility shift using the STAT5B antibody, insulin for 8, 20, and 24 h did not alter the tyrosine phosphorylation of STAT5B (PY-STAT5; Fig. 2, bottom row, lanes 3, 4, and 7). Thus, continuous insulin treatment at the times studied had little effect on the cellular content or phosphorylation state of STAT5B.

**Insulin Inhibited GH-induced STAT5B Phosphorylation and STAT5 Tyrosine Phosphorylation**—Experiments were then designed to examine whether insulin affected GH-stimulated phosphorylation of STAT5B. The ability of bGH for 20 min to



**FIG. 3. Insulin reduced GH-induced STAT5B phosphorylation.** A and B, Western blots were performed as described in the legend for Fig. 1 and in the text except that some groups of H4 cells were pretreated with insulin (100 nM) for the indicated durations prior to addition of bGH (500 ng/ml) for 20 min. Representative Western blots are presented. C, densitometric analysis of autoradiographs from four similar experiments (except  $n = 3$  at insulin 20 h) were performed to quantify the proportion of tyrosine/serine-phosphorylated STAT5B following bGH addition and the indicated times of insulin pretreatment. The data is expressed as the mean  $\pm$  S.E. The ratio of densitometric measurements of the p95/96 band of STAT5B following bGH (500 ng/ml) for 20 min to that of all STAT5B bands was arbitrarily set to 100% in samples from H4 cells without insulin pretreatment (control cells) within each experiment. The corresponding ratio in whole cell extracts from H4 cells treated with insulin (100 nM) for the indicated times prior to bGH addition for 20 min is expressed as a percentage of expression measured in control cells. The 4-h insulin time point is not statistically significant compared with the zero time, whereas the 8-, 12-, 16-, 20-, and 24-h insulin time points are significant ( $p < 0.001$ ) by ANOVA.

induce PY-STAT5B was again observed by the retarded mobility of the STAT5B using the STAT5B isoform-specific antibody (Fig. 3, A and B, lanes 1 versus 2). After insulin pretreatment of H4 cells for 8, 20, or 24 h, there was a reduction of the bGH stimulation of STAT5B retardation. This is most likely due to a smaller fraction of STAT5B being phosphorylated (a smaller fraction migrates at the higher p95/96 PY-STAT5B band) upon bGH addition following insulin pretreatment compared with untreated cells (compare Fig. 3, A, lanes 4 versus 2; and B, lanes 4 versus 2 and 6 versus 2). There was no significant inhibition of bGH-stimulated STAT5B phosphorylation by 4 h of insulin pretreatment, but this inhibition became highly significant between 8 and 24 h of insulin pretreatment ( $p < 0.001$  by ANOVA), with maximum inhibition to approximately 25–30% of control at all times tested between 12 and 24 h (Fig. 3C).

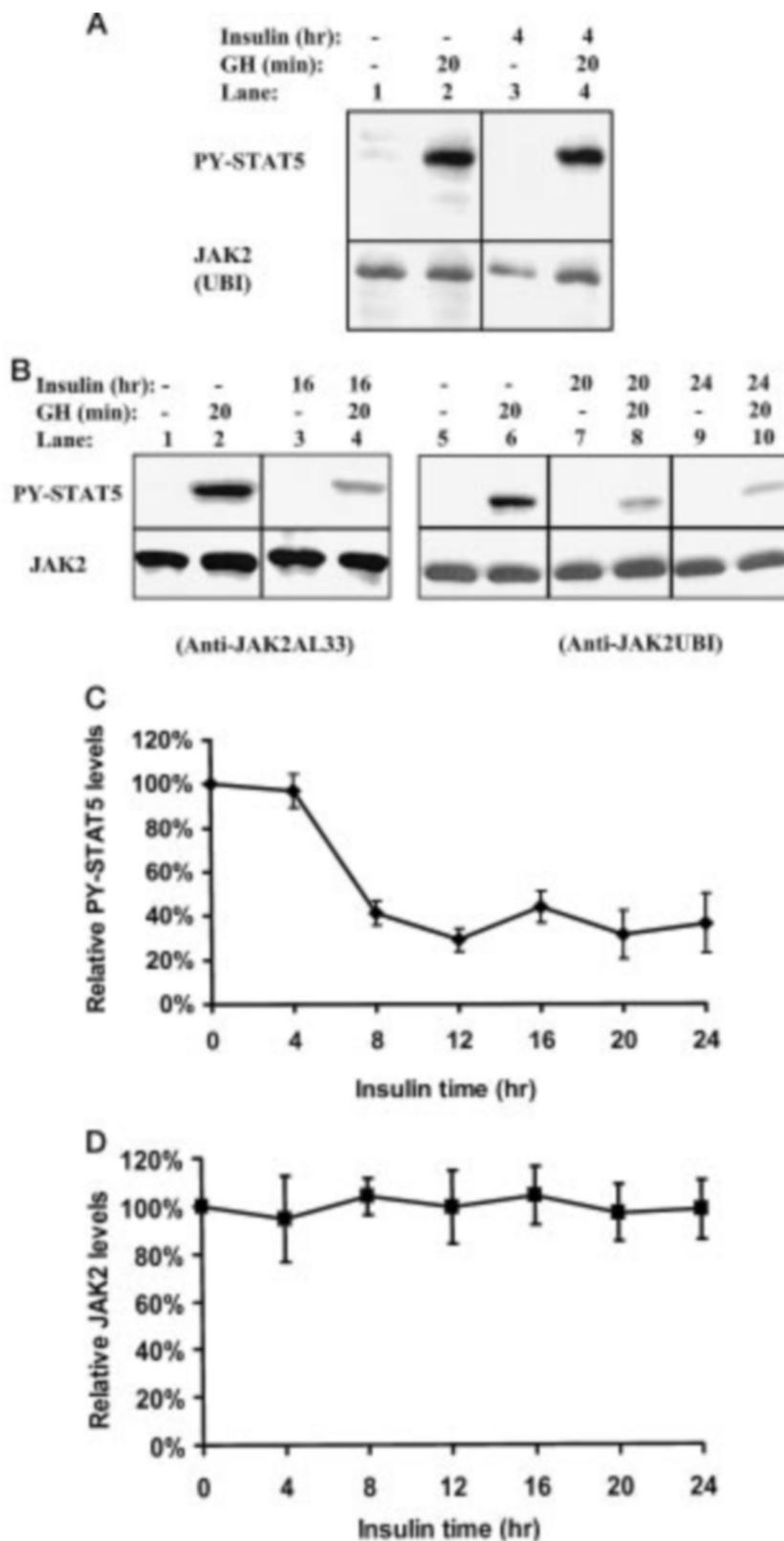
Using an antibody specific for tyrosine-phosphorylated STAT5, no pretreatment or pretreatment with insulin for 4 h resulted in similar levels of bGH-induced PY-STAT5 (Fig. 4A,

top row, lanes 2 and 4). However, following insulin pretreatment for 16, 20, and 24 h, bGH-induced PY-STAT5 was reduced (Fig. 4B, top row, lanes 2 versus 4; and 6 versus 8 and 10). JAK2 protein levels were unaltered at any period of insulin pretreatment when the same Western blots were re-probed with a JAK2-specific antibody (Fig. 4, A, bottom row, lanes 1–4; and B, bottom row, lanes 1–10). When multiple experiments were quantified by densitometry, there was a clear and highly significant reduction ( $p < 0.001$  by ANOVA) in bGH-induced PY-STAT5 by insulin pretreatment using the PY-STAT5-specific antibody at the 8–24-h insulin time points (Fig. 4C). The extent and kinetics of reduction of the bGH stimulation of STAT5 phosphorylation following insulin pretreatment was almost identical when using the PY-STAT5-specific (Fig. 4C) and STAT5B-specific antibody (Fig. 3C). Again, there was no change in JAK2 protein levels at any period of insulin pretreatment when the same Western blots were re-probed with a JAK2-specific antibody (Fig. 4D). From the use of 2 different antibodies measuring the change in mobility of STAT5B and tyrosine-phosphorylated STAT5 directly, we conclude that the effects of insulin are to reduce the ability of GH to stimulate the phosphorylation of STAT5B.

**Insulin Reduced GH-induced JAK2 Tyrosine Phosphorylation**—Multiple possibilities exist as to the mechanism by which insulin pretreatment could inhibit STAT5B phosphorylation. Since STAT5B is phosphorylated by JAK2, the simplest possibility is that there is a reduction in JAK2 levels or activity. As indicated in Fig. 4, A, B and D, there were no measurable changes in the cellular levels of JAK2 at any time following insulin addition. Thus, the next logical question was whether insulin pretreatment altered GH-induced JAK2 activity. Since phosphorylation of JAK2 on tyrosine is required for its activation and ability to tyrosine phosphorylate STAT5 (9), we proceeded to determine the effect of insulin pretreatment on JAK2 tyrosine phosphorylation. Consistent with the data described above with whole cell lysates, there was no effect of acute bGH or 16 h of insulin pretreatment on the levels of immunoprecipitable JAK2 protein (Fig. 5, top row). Following 10 min of bGH, a fraction of the immunoprecipitated JAK2 has been phosphorylated on tyrosine residues as measured by a mouse monoclonal antibody raised against phosphorylated tyrosine residues (Fig. 5, bottom row, lanes 3 versus 1). Insulin pretreatment for 16 h significantly inhibited the ability of bGH to induce tyrosine phosphorylation of immunoprecipitated JAK2 in the absence of any changes in the cellular levels of immunoprecipitable JAK2 (Fig. 5, bottom row, lanes 4 versus 3). Thus, chronic insulin pretreatment inhibits both GH-stimulated JAK2 and STAT5B phosphorylation.

**Insulin Reduced  $^{125}\text{I}$ -hGH Binding to H4 Cells**—The next question was to determine whether the effect of insulin was even further upstream in the GH signaling pathway, at the GHR itself. The published results on insulin regulation of hepatic GH binding are contradictory (14, 24, 25, 28) and no studies have been performed using H4 or related rat hepatoma cell lines. Radiolabeled hGH was used to measure GH binding and in our studies, the total binding of  $^{125}\text{I}$ -hGH binding to H4 cells was approximately 3.0% of the total counts/min of  $^{125}\text{I}$ -hGH added (Fig. 6A). Since hGH is capable of binding to rat somatogenic and lactogenic sites, both unlabeled hGH and bGH were used in competition experiments to determine the level of radiolabeled hGH binding to somatogenic binding sites. Excess unlabeled hGH or bGH competed for  $^{125}\text{I}$ -hGH binding to H4 cells to a similar degree, with a slightly better competition by hGH, resulting in specific binding of approximately 1.6–1.7% (Fig. 6A). This suggests that there was little  $^{125}\text{I}$ -hGH binding to lactogenic binding sites in H4 cells. This was expected since

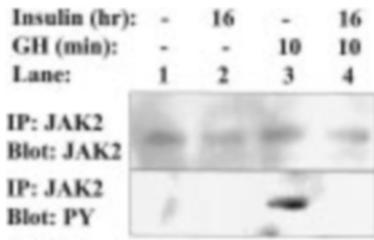
**FIG. 4. Insulin reduced GH-induced STAT5 phosphorylation without changing JAK2 levels.** A and B, Western blots were performed as described in the legend for Fig. 1 and in the text except the PY-STAT5 and anti-JAK2UBI or anti-JAK2AL33 antibodies were used as indicated. Insulin (100 nM) and hGH (500 ng/ml) treatments were for the times indicated. Representative Western blots are presented. C, densitometric analysis of autoradiographs from four similar experiments (except  $n = 3$  at insulin 20 and 24 h) were performed to quantify the changes in PY-STAT5 following various times of insulin pretreatment. The data is expressed as the mean  $\pm$  S.E. The levels of PY-STAT5 immunoreactivity in control (untreated) cells was arbitrarily set to 100% within each experiment and the levels following various times of insulin pretreatment are expressed as a percentage of that in control cells. The 4-h insulin time point is not statistically significant compared with the zero time, whereas the 8-, 12-, 16-, 20-, and 24-h insulin time points are significant ( $p < 0.001$ ) by ANOVA. D, densitometric analysis of autoradiographs from three similar experiments were performed, using the anti-JAK2UBI and anti-JAK2AL33 antibodies to quantify the changes in JAK2 following various times of insulin pretreatment. The data is expressed as the mean  $\pm$  S.E. The levels of JAK2 immunoreactivity in control (untreated) cells was arbitrarily set to 100% within each experiment and the levels following various times of insulin pretreatment are expressed as a percentage of expression measured in control cells. All insulin time points are not statistically significant compared with the zero time by ANOVA.



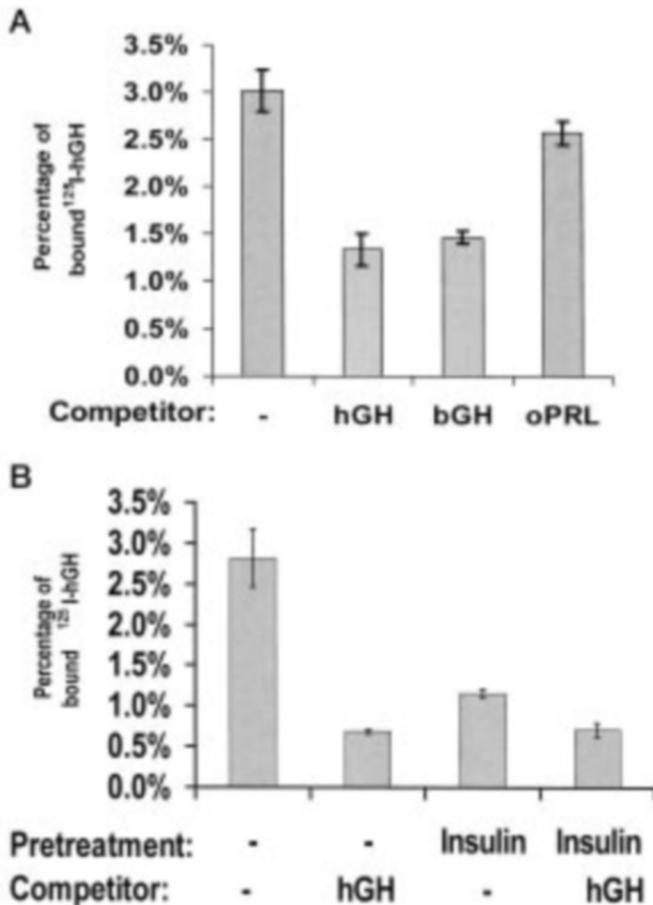
H4 cells are derived from a hepatocellular carcinoma of a male rat (40). Male rats normally express low levels of hepatic lactogenic binding sites (14). To further test this, the ability of unlabeled oPRL to compete for  $^{125}\text{I}$ -hGH binding was measured and found to reduce  $^{125}\text{I}$ -hGH binding to only a small degree suggesting few lactogenic binding sites (Fig. 6A). Thus, the

majority of  $^{125}\text{I}$ -hGH binding is to somatogenic sites and the use of  $^{125}\text{I}$ -hGH binding is an accurate estimate of binding to H4 cell GHR.

In a study to examine the effect of insulin pretreatment on GH binding to H4 cells, specific binding of  $^{125}\text{I}$ -hGH, when using hGH as competitor, was determined to be approximately

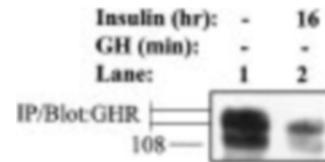


**FIG. 5. Insulin for 16 h reduced JAK2 tyrosine phosphorylation.** H4 cells were treated with insulin (100 nM; 16 h) and bGH (500 ng/ml; 10 min) as indicated and harvested to obtain detergent extracts for immunoprecipitation as described in the text. *Top row*, JAK2 proteins were immunoprecipitated with one JAK2 antiserum, anti-JAK2AL33, and then immunoblotted with JAK2 antiserum from Upstate Biotechnology, Inc. (anti-JAK2UBI). *Bottom row*, the same blot was stripped and re-probed with anti-phosphotyrosine antibody 4G10 (PY antibody).



**FIG. 6. Insulin for 16 h reduced specific <sup>125</sup>I-hGH binding to H4 cells.** The binding assays were performed as described in the text to examine <sup>125</sup>I-hGH binding to H4 cells using 40,000–60,000 cpm of <sup>125</sup>I-hGH per well in the separate experiments. In A, <sup>125</sup>I-hGH binding to H4 cells not pretreated with insulin and either no addition (*first bar*; -) or 2 mg/ml unlabeled hGH, bGH, or oPRL were added as competitors, as indicated. In B, H4 cells were not pretreated (*first and second bars*) or were treated with insulin (100 nM) for 16 h as indicated (*third and fourth bars*). Unlabeled hGH was added to obtain nonspecific binding in the second and fourth groups as indicated. Specific binding is computed as the difference between no unlabeled hGH and when unlabeled hGH is added. The data are the mean ± S.E. of triplicate observations.

2.1% of the total radioactivity added (Fig. 6B). When H4 cells were pretreated with insulin, total binding of <sup>125</sup>I-hGH was diminished from 2.8 to 1.2%. Upon competition with an excess of unlabeled hGH, nonspecific binding was found to be equal to that observed prior to insulin pretreatment. When specific



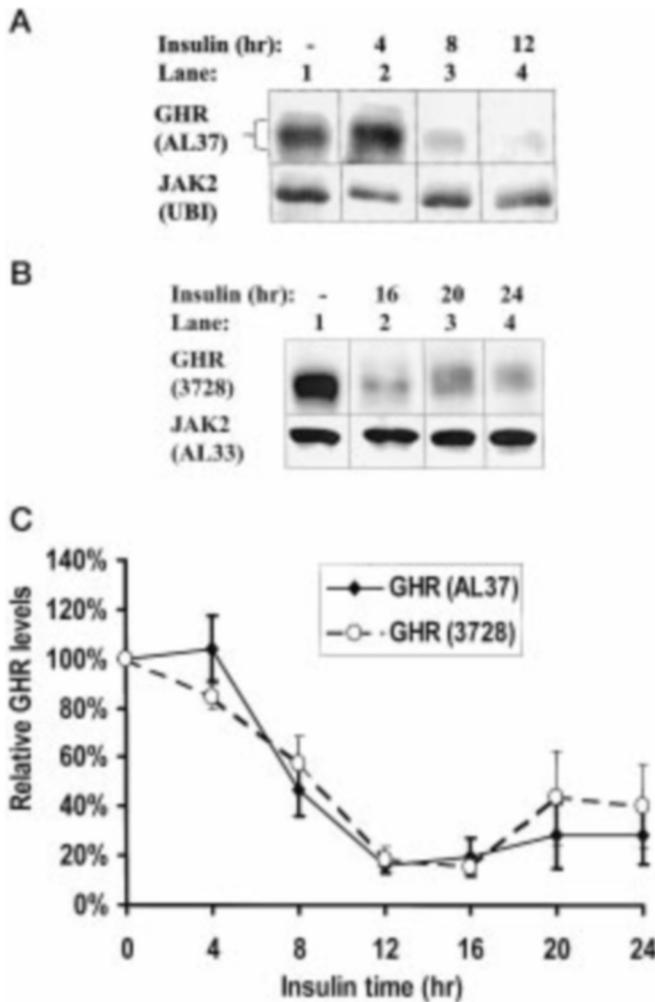
**FIG. 7. Insulin for 16 h reduced the amount of immunoprecipitable GHR.** H4 cells were treated with insulin (100 nM; 16 h) as indicated and harvested to obtain detergent extracts for immunoprecipitation as described in the text. GHR were immunoprecipitated with anti-GHRcytAL37 antiserum and then immunoblotted with the same antiserum. In a parallel lane size markers were run, and the location of one size marker (108 kDa) is shown.

binding was calculated, insulin pretreatment reduced specific <sup>125</sup>I-hGH binding from 2.1 to 0.55% of the total counts added (Fig. 6B). Thus, insulin pretreatment for 16 h reduced specific hGH binding, the vast majority of which is to somatogenic binding sites, by over 70%, to approximately 26% of the specific GH binding obtained prior to insulin pretreatment.

*Insulin Pretreatment Reduced Immunoreactive GHR in Whole Cell Lysates of H4 Cells in a Time- and Dose-dependent Manner*—To further extend our studies of the effects of insulin pretreatment on the GHR, immunoreactive GHR was studied using 2 antibodies raised against the intracellular domain of the GHR. In the first experiments, GHR was immunoprecipitated as described in the experiments for JAK2 (see Fig. 5), except using the anti-GHRcytAL37 antibody for both immunoprecipitation and immunoblotting. Immunoreactive GHR migrated as a broad band with *M<sub>r</sub>* 110–125 due to its complex and variable state of glycosylation (41). Insulin pretreatment for 16 h significantly reduced the amount of immunoprecipitable GHR (Fig. 7, lane 2 versus lane 1).

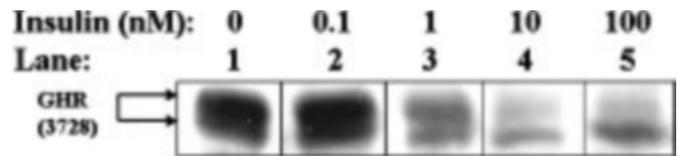
We also measured by immunoblotting the effects of insulin on the abundance of immunoreactive GHR in rat H4 hepatoma whole cell lysates. Using the anti-GHRcytAL37 antibody, immunoreactive GHR was largely unchanged following insulin for 4 h (Fig. 8A, top row, lanes 1 and 2) but by 8 h, and even more so by 12 h, there were significant decreases in immunoreactive GHR as measured by the anti-GHRcytAL37 antibody (Fig. 8A, top row, lanes 3 and 4). There were no consistent changes in total JAK2 protein levels in the same blot that was stripped and re-probed with a anti-JAK2UBI antibody (Fig. 8A, bottom row, lanes 1–4). There were also large reductions in the amount of immunoreactive GHR following insulin pretreatment for 16, 20, and 24 h when using a second polyclonal antibody, anti-GHRcyt3728, to measure changes in GHR expression (Fig. 8B, top row, lanes 2, 3, and 4 compared with lane 1). Again, there were no significant changes in JAK2 levels in the same blot that was stripped and re-probed with the second JAK2 antibody, anti-JAK2AL33 (Fig. 8B, bottom row, lanes 1–4). When several experiments were quantified by scanning densitometry of the autoradiographs, it was clear that there was little or no change in GHR abundance following insulin treatment for 4 h when measured by with either anti-GHR serum (anti-GHRcyt3728 or anti-GHRcytAL37; Fig. 8C). However, by 8 h of insulin pretreatment, immunoblotting with either of the two antisera revealed a significant reduction in immunoreactive GHR, with maximum decreases to only 20% of control values by 12–16 h and was still significantly reduced at 20 and 24 h (*p* < 0.05 to *p* < 0.001; see figure legends for exact *p* values at each time point; Fig. 8C).

When various concentrations of insulin were added to H4 cells for 16 h, a dose-dependent decrease in immunoreactive GHR was observed. There was little effect of 0.1 nM insulin for 16 h compared to untreated cells and a moderate but significant decrease in immunoreactive GHR following 16 h of 1 nM insulin (Fig. 9, lanes 1–3). Insulin was only added once in these



**FIG. 8. Time-dependent effect of insulin on GHR levels.** A and B, Western blots were performed as described in the legend for Fig. 1 and in the text. Some H4 cells were left untreated or were treated with insulin (100 nM) for the times indicated and the antibodies used (as indicated to the left) included 2 for GHR (anti-GHRcyt3728 or anti-GHRcytAL37) and 2 for JAK2 (anti-JAK2UBI and anti-JAK2AL33). Representative Western blots are presented. C, densitometric analysis of autoradiographs from three or four similar experiments were performed, using two antisera of GHR (anti-GHRcyt3728 or anti-GHRcytAL37) following different times of insulin pretreatment (100 nM). The data is expressed as the mean  $\pm$  S.E. The levels of GHR immunoreactivity in control (untreated) cells was arbitrarily set to 100% within each experiment for each of the two antibodies and the levels following various times of insulin pretreatment are expressed as a percentage of expression measured in control cells. For the anti-GHRcytAL37 antibody, the 4-h insulin time point is not statistically significant compared with the zero time whereas the 8-, 20-, and 24-h insulin time points are significant ( $p < 0.01$ ) and the 12- and 16-h insulin time points are significant ( $p < 0.001$ ) by ANOVA. For the anti-GHRcyt3728 antibody, the 4-h insulin time point is not statistically significant compared with the zero time, whereas the 8- and 20-h insulin time points are significant ( $p < 0.05$ ), the 12- and 16-h insulin time points are significant ( $p < 0.001$ ), and the 24-h insulin time point is significant ( $p < 0.01$ ) by ANOVA.

studies, so this may be an underestimation of the sensitivity of the response, since insulin may have been degraded over the extended pretreatment period. In previous studies, 80–98% of insulin (0.02–5 nM) added to H4 cells is still present 6 h later when measured by radioimmunoassay (42). However, the present experiments were performed at 16 h following insulin addition which may have resulted in further degradation. Clearly, insulin at concentrations or 10 and 100 nM reduced GHR to a similar extent, comparable to that shown in Fig. 8C (Fig. 9, lanes 4 and 5). Thus, insulin pretreatment results in



**FIG. 9. Dose-dependent effect of 16-h insulin on GHR levels.** Western blots were performed as described in the legend for Fig. 7 and in the text using the anti-GHRcyt3728 antibody to GHR and no insulin treatment (lane 1) or insulin for 16 h at the indicated concentrations (0.1–100 nM; lanes 2–5). A representative Western blot is presented.

70–80% reductions in GH binding, immunoreactive GHR, JAK2 phosphorylation, and STAT5B phosphorylation.

#### DISCUSSION

Although usually thought of as a “counter-regulatory” hormone, with many actions opposing those of insulin, GH can have acute insulin-like effects, especially in the setting of GH deficiency (1). In contrast, GH excess induces insulin resistance (43) but little is known about the ability of insulin to promote GH resistance. A significant fraction of the adult population exhibit peripheral insulin resistance and hyperinsulinemia. This is true both of Type 2 diabetic patients and a large number of individuals, many of which are obese, but do not exhibit overt diabetes (6). In the present study, prolonged treatment of hepatoma cells with high insulin concentrations, similar to those in the hepatic portal circulation in patients with hyperinsulinemia (see below), resulted in reduced GH binding and a severe diminution of GH-induced STAT5B phosphorylation.

There are two closely related STAT5 isoforms, STAT5A and STAT5B (44). They clearly mediate different functions and the loss of either cannot be compensated by the other isoform *in vivo*, as indicated by studies with knockout mouse models (13, 45). Acute insulin treatment stimulates STAT5B tyrosine phosphorylation in Chinese hamster ovary cells overexpressing either the insulin receptor or STAT5B (46, 47). This change in phosphorylation is not observed in native Chinese hamster ovary cells or hepatoma cells expressing the normal complement of insulin receptors (46). In the present work, it is shown that continuous insulin treatment does not significantly alter STAT5B protein concentrations, STAT5B phosphorylation, or STAT5 tyrosine phosphorylation in rat H4 hepatoma cells.

Patients with peripheral insulin resistance and hyperinsulinemia often exhibit abdominal adiposity, a morphology similar to that found in patients or animal models with GH deficiency or a disruption of GH signaling, such as STAT5B knockout mice (4–6, 13, 48). This lead us to hypothesize that hyperinsulinemia may result in GH resistance. This hypothesis is consistent with our presented results indicating that continuous treatment with high concentrations of insulin reduced the ability of GH to stimulate phosphorylation of STAT5B in hepatoma cells. As determined with two different antibodies measuring a GH-induced mobility shift in STAT5B due to phosphorylation and directly measuring tyrosine phosphorylation of STAT5, GH-induced STAT5B phosphorylation was severely reduced by insulin pretreatment for 8–24 h. In addition, the GH-induced tyrosine phosphorylation of immunoprecipitated JAK2 was almost completely inhibited by insulin pretreatment with little change in JAK2 protein levels.

Since a reduction in GH binding could contribute to the reductions in STAT5B and JAK2 phosphorylation, GH binding was measured using  $^{125}\text{I}$ -hGH. Ovine PRL was ineffective in competing for  $^{125}\text{I}$ -hGH, so we conclude that there were few, if any, lactogenic binding sites on rat H4 hepatoma cells, a cell line derived from an hepatocarcinoma of a male rat (40), and the specific  $^{125}\text{I}$ -hGH binding was to somatogenic sites. Due to the low levels of GH-specific binding in H4 cells, we choose to

measure binding for 2 h at room temperature. The measured specific binding is likely due to both cell surface-binding sites and a small amount of internalized  $^{125}\text{I}$ -hGH-GHR complexes. The percentage that is degraded or released into the media is thought to be minimal due to the room temperature incubation and relatively short time of incubation (49, 50). The low amount of total and specific GH binding in this cell line is not surprising since GHR mRNA levels in H4 cells are 40% or less of those in rat liver (51). To our knowledge these represent the first experiments to demonstrate GH binding in H4 cells.

Insulin pretreatment for 16 h reduced specific  $^{125}\text{I}$ -hGH specific binding to somatogenic binding sites to 26% of the specific binding prior to pretreatment, a decrease in GH binding comparable to the reduction of GH-induced STAT5B phosphorylation. Previous studies are inconsistent on whether insulin increases GH binding and GHR mRNA or not (14–28). This may be due to multiple, and sometimes separate, controlling factors for GHR expression and for GH binding. For example, while *in vivo* data suggests that insulin treatment of insulin-deficient individuals might increase GHBP (and therefore most likely GHR expression), high physiological levels of GHBP may feedback to decrease GH-receptor mRNA levels and low levels of GHBP may increase GH-receptor mRNA expression (18, 22, 52). Additionally, continuous insulin treatment of osteoblasts decreases the fraction of cellular GHR presented at the plasma membrane via inhibition of surface translocation of GHR with no effect on the total cellular content of GHR (29). Thus, there may be multiple feedback mechanisms controlling GHR expression and the subcellular localization of GHR. Most *in vivo* studies do not maintain high insulin concentrations for extended periods and therefore do not study the effects of insulin under conditions similar to patients with insulin resistance and hyperinsulinemia. The effect of insulin on GHR expression may be dependent on the concentration of insulin, the duration of insulin treatment and may be tissue and cell-type specific (27).

Decreases in GH binding could be due to insulin-dependent changes in GHR synthesis or degradation, or insulin-dependent reduction of cell surface GHR without changes in total GHR expression. GHR normally migrates as a broad band with  $M_r$  110–125 due to its complex and variable glycosylation (41, 53). Using 2 different antibodies that recognize the cytoplasmic domain of the GHR, there was no significant change in immunoreactive GHR during the first 4 h of insulin treatment. However, immunoreactive GHR was reduced by insulin pretreatment for 8–24 h, with a maximum reduction to less than one-fourth of the untreated cells with insulin for 16 h. Densitometric analysis of the broad GH bands indicated a similar reduction of GHR using either antiserum. Insulin treatment (16 h) at 10 and 100 nM reduced GHR to a similar extent, while 1 nM insulin resulted in an intermediate reduction and 0.1 nM insulin did not alter the amount of immunoreactive GHR, respectively. Thus, chronic hyperinsulinemia resulted in a similar reduction in GH binding, whole cell immunoreactive GHR, and the ability of GH to induce JAK2 and STAT5B phosphorylation.

The finding of reduced GHR by both binding studies and Western analysis suggests that insulin might reduce expression of GHR, thereby decreasing the number of available somatogenic binding sites and decreasing the levels of immunoreactive GHR. A second, more remote possibility, is that insulin pretreatment results in a post-translational alteration of the GHR resulting in decreased GH binding. This modification would then also have to result in a reduced immuno-detectability by Western blot analysis, possibly due to a conformational change in the cytoplasmic region of the GHR, to which the GHR antibodies were raised. Since two separate polyclonal antibodies

were used, it is unlikely that a covalent modification would result in such similar decreases in immunoreactivity of the GHR and in GH binding. However, we cannot exclude this possibility.

In a number of species, IGF-1 may have some actions similar to those of insulin. Since GH is one of the main regulators of IGF-1 expression, elevated IGF-1 may also act to reduce GHR and GH responsiveness as part of a negative feedback system. However, in the present studies the effects of insulin are through the insulin receptor since H4 hepatoma cells, like the H35 cells from which H4 cells were derived and rat liver, contain few if any IGF-1 receptors (54, 55).

The similarity between the timing and percentage decrease in GHR levels and STAT5B phosphorylation following insulin pretreatment suggests that the decrease in GHR is the cause of the reduction of GH-induced STAT5B phosphorylation. The remaining GHR are still functional since a small amount of GH-induced STAT5B phosphorylation is observed even following extended insulin pretreatment. The reduction of GHR levels and GH-induced STAT5B phosphorylation required more than 4 h of elevated insulin and the insulin concentration needed to be 1 nM or greater. In normal human subjects basal insulin concentrations in the hepatic portal circulation are approximately 0.2 nM, severalfold higher than peripheral insulin concentrations (56). Peak post-prandial insulin concentrations reach 0.5–1.0 nM in the peripheral circulation and approximately 3 nM in the portal circulation (56, 57). However, in obese individuals with peripheral insulin resistance, peak post-prandial systemic insulin concentrations reach 3–7 nM and this increase in insulin concentration is more prolonged than in normal subjects (58). Portal circulation insulin concentrations were not measured in these obese individuals, but if the ratio of peripheral to portal insulin remains consistent, portal circulation concentrations of insulin would be expected in the range of 20–50 nM. Therefore, normal postprandial concentrations of insulin may be insufficient to result in substantial or prolonged GH resistance. However, in patients with peripheral insulin resistance, where there are elevated basal insulin levels and extended postprandial peripheral insulin concentrations above 1 nM, peripheral GH resistance may develop. In these same individuals, where insulin concentrations in the hepatic portal circulation may rise to well above 10 nM for extended periods, there is an even greater possibility for the development of hepatic GH resistance. Thus, prior to commencement of exogenous GH administration, knowledge of a patient's insulin sensitivity and circulating insulin concentrations may be warranted.

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