Growth Hormone-induced Differential Desensitization of STAT5, ERK, and Akt Phosphorylation*

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Secretion of growth hormone (GH) in adult male rats is characterized by high peak and undetectable trough levels, both of which are required for male-specific pattern of liver gene expression and GH-induced phosphorylation of STAT5. The present study suggests that regulation of GH receptor (GHR) levels in rat hepatoma cells by repeated GH stimulation determines GH responsiveness via the JAK2/STAT5 pathway. A short exposure to GH rapidly reduced GHR levels which resulted in an equal desensitization of the JAK2/STAT5 pathway. Recovery of GH-induced STAT5 phosphorylation correlated with the time-dependent recovery of GHR levels during incubation in the absence of GH. Acute GH also induced phosphorylation of ERK1/2 and Akt, and this induction was also inhibited by prior exposure to GH. However, unlike the JAK2/STAT5 pathway, the effect of GH to activate the MEK/ERK and phosphatidylinositol 3-kinase/Akt pathways did not recover following prolonged incubation in the absence of GH. Thus, GH administration desensitizes the JAK2/STAT5 pathway, possibly because of down-regulation of GHR, whereas an additional post-receptor mechanism is required for the prolonged refractoriness of the MEK/ERK and phosphatidylinositol 3-kinase/Akt pathways toward a second GH stimulation. Our study suggests that both receptor and post-receptor mechanisms are important in GH-induced homologous desensitization.

It is still controversial whether growth hormone (GH)-induced desensitization of GH signaling is the result of down-regulation of GH-receptor (GHR) levels or post-receptor signaling pathways. Elucidation of the processes of this homologous desensitization may further our understanding of the regulation of GH action, as well as the possible effects of GH on intracellular signaling molecules that are also utilized by other hormones, cytokines, and growth factors.

GH is secreted in a pulsatile fashion to promote growth and diverse metabolic actions (1, 2). In young adult male rats, GH is released in ~1-h pulses with peak serum concentrations of 150–400 ng/ml, and interpulse intervals of 2 h or more, where serum GH concentrations are negligible (3). In female rats, GH is secreted more frequently, resulting in the continuous presence of GH in the circulation, with peaks of 50–150 ng/ml (2–5). The sexually dimorphic pattern of GH secretion provides an important mechanism for transcriptional regulation of sexually dimorphic genes in the liver.

Growth hormone signaling cascades include Janus kinase 2 (JAK2) and signal transducers and activators of transcription (STAT) family transcription factors (including STAT1, STAT3, STAT5A and STAT5B; Refs. 6–9), phosphatidylinositol 3-kinase (PI 3-kinase) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) (10–14). GH may induce phosphorylated (P)-ERK1/2 via the indirect association of JAK2 to growth factor binding protein 2 (Grb2)-Son of Sevenless (SOS) complexes through SH2 containing Shc proteins (12, 15, 16) or via trans-phosphorylation of the epidermal growth factor receptor by JAK2 (17), which can then recruit the Grb2-SOS complex. These pathways can then lead to activation of the Ras-Raf-MEK-ERK pathway (18). GH may also activate ERK1/2 via a PI 3-kinase activity (10, 19), which can be activated by JAK2 via both insulin receptor substrate 1-dependent and -independent mechanisms (19, 20). Although GH activates ERK in the hepatocyte when either injected into animals or when added directly to cultured hepatocytes (18, 21), it is not known whether repeated GH pulses, as is normally present in vivo, cause multiple spikes of activated (phosphorylated) ERK.

Desensitization and resensitization are critical processes regulating GH actions on target tissues. In hypophysectomized rats, a second GH exposure can trigger a full hepatic response of STAT5 tyrosyl phosphorylation (PY-STAT5) if it follows the first injection by 4 h (7, 22). In a rat hepatocyte-derived cell line, full responsiveness to succeeding GH stimulation via the JAK2/STAT5 pathway requires a minimum of 2.5 h without GH (23). These studies indicate that the liver and liver-derived cells undergo an obligatory recovery period after stimulation by a GH pulse (23).

Two general mechanisms may underlie the requirement for a recovery period for resensitization of GH-induced signaling. First, the recovery period could allow restoration of a short term decrease of cell-surface GHR following a cycle of GH binding, receptor internalization, and degradation or recycling. Alternatively, the period may be required to reset GH-activated intracellular signaling pathways.

Important post-receptor mechanisms of desensitization of
GH may vary in the cellular expression of members of the family of suppressor of cytokine signaling, or cytokine-inducible SH2 proteins (24–30). Another post-receptor mechanism for the post-GH pulse recovery period may be the regulation of phosphatase activity (31). However, for this to be an operative mechanism, a time-dependent induction followed by reduction of phosphatase activity coincident with the inhibition and then recovery of GH responsiveness would be required, and has not yet been demonstrated.

It is clear that GH induces rapid internalization of GHR, but there is conflicting evidence concerning whether GHR is degraded following internalization (23, 32–35). The GHR may either be recycled back to the cell surface or be degraded in the proteosome or lysosome (36–38). In the present study, using rat hepatoma H4IIE (H4) cells, both desensitization of the JAK2/STAT5 pathway following initial GH stimulation and the recovery of GH sensitivity of this pathway during a GH-free incubation period were strongly correlated with reduction and recovery, respectively, of GHR levels. Therefore we hypothesize that, with repeated GH applications, a major mechanism of desensitization and resensitization of GH signaling and JAK2/ STAT5 activation was the result of reduction and recovery of GHR levels. However, following a 1-h exposure to GH, even 16 h in its absence was insufficient to obtain a recovery of the GH-induced pathways of MEK/ERK and PI 3-kinase/Akt. This lack of recovery of MEK/ERK and PI 3-kinase/Akt signaling indicates that recovery of GHR and JAK2/STAT5 signaling was insufficient for recovery of all GHR signaling capabilities. There must be at least one additional post-receptor desensitization mechanism that results in reduced GH signaling even when GHR levels are restored.

**EXPERIMENTAL PROCEDURES**

**Materials—**Bovine GH (bGH; lot APF10325C) was kindly provided by Dr. A. F. Parlow, Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center (Torrance, CA) and the National Institutes of Health NIDDK National Hormone & Pituitary Program. Fetal bovine serum, calf serum, and horse serum were purchased from Invitrogen. Other materials were purchased from Sigma Health NIDDK National Hormone & Pituitary Program. Fetal bovine serum, calf serum, and horse serum were purchased from Invitrogen.

**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, except for the use of Protran membrane from Schleicher & Schuell (BA 85; Ref. 40). The membranes were blocked in 0.4% milk, 5% bovine serum albumin in TBS, 0.7% Tween 20, pH 8.0. Immunoblotting was performed with the antibodies at the following dilutions: anti-STAT5 (1:500), anti-STAT5B (1:5000), anti-PY-STAT5 (1:2000), anti-JAK2 (1:2000), anti-PI 3-kinase/Akt (1:2000), anti-ERK1/2 (1:1000), anti-Akt (1:1000), anti-MEK1/2 (1:1000), anti-P-ERK1/2 (1:1000), anti-P-Akt (1:1000), anti-P-MEK (1:1000) with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:2000). Incubation with rabbit primary antisera was at 4 °C overnight, 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 reprobed at least twice, the second probing using an antibody to detect total amounts of phosphorylated (phosphorylated and non-phosphorylated) of the same protein. These repeat probing 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.

**Genometric 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). All data were analyzed by analysis of variance using the InStat statistical analysis program (version 3) from Graphpad Software, Inc. (San Diego, CA).

**RESULTS**

**Kinetics of GH-induced Phosphorylation of STAT5—**The kinetics of GH-induced tyrosyl phosphorylation of STAT5 (PY-STAT5) in H4 cells was examined. Because plasma GH levels can vary between 0 and 400 (or higher) ng/ml in young adult male rats and between 50 and 150 ng/ml in young adult female rats (4, 5), bGH at concentrations of 50–500 ng/ml were used. The highest concentration of bGH, 500 ng/ml, resulted in a large induction of PY-STAT5 at all points tested between 5 and 60 min following its addition to the cell culture media (Fig. 1A), compared with vehicle-treated control cells (lane 10). This large induction diminished after 60 min, returning toward basal values by 90 min (Fig. 1A). Addition of bGH at various doses resulted in maximum levels of PY-STAT5 that were clearly dose-dependent. Designating the 30-min time point of the 500 ng/ml concentration of bGH as 100%, the maximal effects of bGH at 100 and 50 ng/ml at 30 min were +65 and +50%, respectively (Fig. 1B; additional data not shown). When multiple time-course experiments were averaged, it is evident that peak PY-STAT5 was achieved most rapidly at 500 ng/ml and was slowest at 50 ng/ml (Fig. 1C).
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antibody against the cytoplasmic domain of human GHR (GHR3728), which cross-reacts with rat GHR (42), treatment with bGH (500 ng/ml) for 0–60 min resulted in reduction of GHR levels by ~75% (Fig. 2A). Using a second, independently raised antibody against a different yet overlapping cytoplasmic domain of human GHR (GHR-AL37; Ref. 42), the levels of GHR following 0–60-min treatment with bGH at different doses were also determined. These experiments confirmed the earlier studies that GHR levels were reduced following bGH at 50, 100, and 200 ng/ml, as well as with 500 ng/ml for 5–60 min, with maximum reductions to ~20–30% of the control levels at 60 min at all concentrations of bGH tested (Fig. 2B). Similar to induction of PY-STAT5, the fastest reduction of GHR levels occurred in H4 cells treated with the highest concentration (500 ng/ml) of bGH.

Reduction in GH Signaling following 1-h GH Pretreatment followed by a 3-h Wash-out Period to Mimic the in Vivo Intermulse Interval—Growth hormone is secreted in ~1-h pulses in young adult male rats, with interpulse intervals of non-detectable GH greater than 2 h. To mimic this temporal pattern of GH exposure, H4 cells were pretreated with bGH at 50, 100, 200, and 500 ng/ml for 1 h, after which the GH was washed away and the cell incubated for 3 h in GH-free, serum-free medium. Compared with untreated cells, those treated with bGH (500 ng/ml) for 1 h followed by 3 h in GH-free, serum-free medium possessed levels of immunoreactive GHR that were ~50% of that in the untreated sample (Figs. 3 (row 1, lane 7 versus lane 6) and 4A). Because this is higher than the amount following GH for 60 min without the 3 h in GH-free, serum-free medium (Figs. 2B and 3 (row 1, lane 7 versus lane 5)), it suggests that there was a partial recovery of GHR levels during the 3-h wash-out period (see the next section). At the beginning of the second bGH application, and at each treatment time point thereafter, there were lower GHR levels following the second versus the first exposure to bGH for 5–60 min. Thus, after a 1-h GH treatment and 3 h in GH-free, serum-free media, there was a lower level of GHR, and cellular GHR could be further decreased by a second exposure to 500 ng/ml bGH (Figs. 3 and 4A). Similar results were obtained with bGH at 50 ng/ml (Fig. 4B) and at 100 and 200 ng/ml (data not shown). The GH-induced reduction in GHR levels was faster at 500 versus 50 ng/ml, but achieved a similar percentage reduction by 60 min (Fig. 4, A versus B). Additionally, following the first and the second GH applications at 500 ng/ml, the GH-induced reduction of GHR resulted in lines that were parallel to each other (Fig. 4, A and B) suggesting that the rate of loss of GHR was determined by the concentration of GH in the media, regardless of initial levels of GHR. Finally, when the same experiments were evaluated for JAK2 protein levels, they remained approximately constant (Fig. 3, row 2).

Because repeated applications of GH are capable of activating multiple cycles of STAT5 phosphorylation in vivo, we studied whether GH-induced changes in the levels of GHR affected the ability of a second exposure of GH to induce PY-STAT5. Following a 1-h exposure to 500 ng/ml bGH followed by a 3-h incubation in GH-free, serum-free medium, PY-STAT5 levels returned to basal (Fig. 3, row 3, lane 7 versus lane 6). The second application of 500 ng/ml bGH for 5 min induced tyrosyl phosphorylation of STAT5 to ~60% of that induced by 5 min GH.
bGH alone (Figs. 3 (row 3) and 5). The decreased ability of the second bGH exposure to induce PY-STAT5 compared with the first bGH was evident at all bGH concentrations tested (500 ng/ml shown in Fig. 5; data not shown for 50, 100 and 200 ng/ml bGH). Even though there was a reduced maximal effect of the second exposure to bGH on PY-STAT5, there were similar kinetics of bGH-induced tyrosyl phosphorylation of STAT5 and PY-STAT5 dephosphorylation for both exposures to GH (Fig. 5). This suggests no significant changes in the activity of a phosphatase to dephosphorylate STAT5B between the first and the second exposure to GH.

The same cell extracts were analyzed for changes in protein levels and mobility of STAT5B. The STAT5B protein normally appeared as a doublet in the Western blot because of serine phosphorylation of STAT5B resulting in retarded mobility (Fig. 3, row 4) (23). GH-induced STAT5B activation is associated with dual phosphorylation of STAT5B at both serine and tyrosine residues, resulting in a third band (highest band) with the slowest mobility and a loss of the lowest band (23). Following the first bGH treatment, the intensity of the highest band became greater than that of the second band; by 3 h following GH removal, STAT5B had been dephosphorylated and had returned to the basal state (Fig. 3, row 4). Consistent with the PY-STAT5 data, following the second GH exposure for 5–60 min, the intensity of the highest band was approximately equal to or only slightly greater than that of the second band (Fig. 5, row 4) instead of the preponderance of the highest (tyrosyl-phosphorylated STAT5B) band following the initial GH application. This implies, by measurement of gel retardation, a lesser ability of GH to induce phosphorylation of STAT5B following the second versus the first GH exposure, similar to the lesser induction of PY-STAT5 as measured by phosphorylation-specific antibodies.

Total STAT5B protein levels were the same or increased, and certainly not decreased, following the second versus the first GH exposure (Fig. 3, row 4). Thus, the lower GH-induced PY-STAT5 resulting from the second versus the first GH exposure could not be explained by loss of STAT5B protein.

**Time-dependent Recovery of GHR and GH-induced PY-STAT5**—When H4 cells were treated with bGH (500 ng/ml) for 1 h, the bGH was washed away, and the cells were incubated in GH-free, serum-free media for various time intervals, there
was a recovery of both GHR levels and the ability of GH to induce PY-STAT5. As described previously, GHR levels were reduced to $\frac{1}{2}$ – $\frac{1}{3}$ of the control levels following GH treatment (Fig. 2B). Following a 1-h interval in GH-free, serum-free media, GHR levels increased negligibly and were $\sim$30% of that in untreated cells (Fig. 6B). Following 2, 3, 6, and 16 h in GH-free, serum-free media, the cellular levels of GHR recovered to $\sim$40, 60, 80, and 100%, respectively, of that in cells not treated with GH (Fig. 6B).

There was little remaining PY-STAT5 after 1 h of GH followed by 1 h in GH-free, serum-free media, and phosphorylation of STAT5 could still be induced by 20 min of GH, but only to $\sim$20% of that induced by 20 min of GH without GH pretreatment and washing (Fig. 6, A and B). The ability of GH for 20 min to induce PY-STAT5 increased with increasing times of GH-free, serum-free incubation. Following 2, 3, 6, and 16 h, the ability of GH to induce PY-STAT5 recovered to $\sim$40, 60, 80, and 90%, respectively, of that induced by 20 min of GH without GH pretreatment and washing (Fig. 6, A and B). Thus, the time course of recovery of GHR was nearly identical to the recovery of the ability of GH to induce PY-STAT5 (Fig. 6B).

As a washing control, H4 cells were washed without any prior GH exposure and then incubated for 3 h in GH-free, serum-free media. Simply washing alone, without GH pretreatment, had no significant effect on GHR levels and the ability of GH to induce PY-STAT5 (Fig. 6A and C). Conversely, when GH was maintained in the medium for 4 h, with no recovery period in GH-free, serum-free media, there was no recovery in GHR levels, which were reduced to 10% of that in untreated cells, or in the ability of a second GH exposure to induce PY-STAT5, which was also reduced to $\sim$10% (Fig. 6, A and C).
GH at 500, 200, and 100 ng/ml, or 30 min of GH at 50 ng/ml, were the effects of different times of GH treatment were expressed compared to insulin (100 ng/ml). Western blots with the bGH concentrations and treatment times indicated were performed as described in Fig. 1 and as indicated, except for the Western blots from three independent experiments using different doses of bGH (bGH concentrations indicated). The data are expressed as mean ± S.E. The maximal effect for each concentration of bGH was set to 100%, and the effects of different times of GH treatment were expressed compared with that concentration's maximal effect. The levels following 20 min of GH at 500, 200, and 100 ng/ml, or 30 min of GH at 50 ng/ml, were the times for maximal P-ERK1 levels at that concentration and were set to 100%.

Kinetics of GH-induced Phosphorylation of ERK1/2, MEK1/2, and Akt—The kinetics of GH-induced phosphorylation of ERK (P-ERK), MEK (P-MEK), and Akt (P-Akt) were also examined in H4 cells. Following addition of bGH (500 ng/ml), P-ERK1/2 was detected within 5 min, was maximal at 10–20 min, and decreased by 30 min, returning to basal, unmeasurable levels by 60 min (Fig. 7A, lane 1). At lower concentrations of bGH, P-ERK1/2 levels were increased to a lesser extent and not as rapidly. When comparing different concentrations of GH, the maximal induction of P-ERK1 was 29, 44, and 82% at 50, 100, and 200 ng/ml, respectively, of the P-ERK1 induced by 500 ng/ml bGH (Fig. 7A and data not shown). When multiple time course experiments were averaged, it is evident that P-ERK was achieved most rapidly at 500 ng/ml and was slowest at 50 ng/ml (Fig. 7B). Therefore, bGH at 50–500 ng/ml induced P-ERK1/2, with greater maximum phosphorylation, and shorter times to maximum phosphorylation at the higher doses of GH.

Addition of bGH at 100, 200, or 500 ng/ml to H4 cells also induced phosphorylation of Akt (P-Akt; Fig. 7C; data shown for 500 and 100 ng/ml), whereas induction of P-Akt by 50 ng/ml bGH was barely detectable (data not shown). Because MEK1/2 are direct upstream kinases for ERK1/2, the kinetics of P-MEK1/2 induced by bGH were, as expected, similar to that of P-ERK1/2, although induction of P-MEK1/2 was not as extensive. GH-induced P-MEK1/2 was maximum between 10 and 20 min, and decreased after 30 min (Fig. 8, row 3). Reduction in GH-induced Phosphorylation of ERK1/2, MEK1/2, and Akt following 1-h GH Pretreatment followed by a 3-h Wash-out Period to Mimic the in Vivo Interpulse Interval—When H4 cells were pretreated with bGH for 1 h followed by 3 h in GH-free, serum-free media, the GH-induced phosphorylation of both ERK1/2 and MEK1/2 (Fig. 8, rows 1 and 3, respectively) were greatly diminished compared with that induced by bGH prior to any pretreatment. When multiple similar experiments were analyzed, a more rapid increase in phosphorylation of ERK1 was evident with increasing bGH concentrations for the first addition of bGH (Fig. 9, A–D). In addition, all bGH concentrations (50, 100, 200, and 500 ng/ml) resulted in desensitization of the ERK1/2 pathway to a second bGH exposure. The second exposure to bGH never achieved greater than 10–15% of the P-ERK1/2 induced by the first exposure (Fig. 9, A–D).

To control for equal loading and to determine whether the effects on P-ERK1/2 and P-MEK1/2 were dependent upon changes in the total amounts of ERK1/2 and MEK1/2 protein, all blots were re-analyzed for the total cellular amounts of ERK1/2 and MEK1/2 protein that were not significantly affected, and certainly were not reduced (Fig. 8, rows 2 and 4, respectively). Thus, the decreased ability of the second GH exposure to induce phosphorylation of ERK1/2 and MEK1/2 was not the result of decreases in the amounts of these proteins, but of desensitization of GH signaling through the MEK-ERK pathway.

Growth hormone induction of PI 3-kinase activity may be important for ERK1/2 induction (19), so the ability of multiple GH exposures to activate (phosphorylate) the PI 3-kinase substrate Akt was determined. The first exposure to GH induced P-Akt, which was most noticeable at 5 and 10 min (Fig. 10, row 1). Following exposure to bGH for 1 h, followed by 3 h in GH-free, serum-free medium, there was no significant induction of P-Akt by a second exposure to bGH. There may have been a slight increase in total cellular Akt protein following GH pretreatment and the 3-h wash-out period, but there was certainly no reduction of total Akt (Fig. 10, row 2). Insulin, which can induce P-Akt via the PI 3-kinase pathway in H4 cells (Fig. 10, lane 14), was fully capable of stimulating phosphorylation of Akt at these time points (Fig. 10, lane 13). Therefore, similar to the MEK/ERK pathway, the first GH application followed by a 3-h incubation period, led to refractoriness of P-Akt induction by a second exposure to bGH.

Lack of a Time-dependent Recovery of GH-induced P-ERK—There was no significant recovery of GH-induced ERK1/2 phosphorylation, even following many hours in GH-free, serum-free medium, following the initial GH exposure. As indicated in Fig. 11A, GH administration for 20 min resulted in induction of P-ERK1/2 whether the cells were not previously washed (Fig. 11A, lane 1) or whether the cells had been washed but without GH pretreatment, and then placed into serum-free medium for 3 h (Fig. 11A, lane 9). However, following 1 h of GH, washing and 1, 2 (Fig. 11A), or 3 h (Fig. 8) of GH-free, serum-free medium, GH was unable to induce phosphorylation of ERK1/2. Surprisingly, even after 6 or 16 h in GH-free, serum-free medium, GH was still unable to significantly induce P-ERK1/2 (Fig. 11A). There were no changes in total ERK1/2 protein at any of the times tested. Additionally, even at these longer time points, insulin, another inducer of P-ERK1/2 in these cells, is fully active in inducing phosphorylation of ERK1/2 (Fig. 11B). Thus, this lack of recovery was not the
result of changes in ERK1/2 protein levels, the ability of ERK1/2 to be phosphorylated by other hormones/growth factors, in this case insulin, or the washing procedure. It was the prior exposure to GH that resulted in refractory GH signaling. Compared with the recovery of GH-inducible PY-STAT5 (data from Fig. 6C shown again in Fig. 11C for comparison), this suggests that the recovery of GH-induced signaling through the ERK1/2 pathway was much delayed.

**DISCUSSION**

The data presented in this study suggest that addition of GH to H4 hepatoma cells induces degradation of GHR, which was resynthesized during the GH-free period. This degradation and resynthesis of GHR was paralleled by desensitization and resensitization, respectively, of GH-induced STAT5 phosphorylation. In the first experiments, as expected, an initial exposure to GH resulted in a time- and concentration-dependent increase in PY-STAT5. Clearly shown here is that GH addition also resulted in a rapid loss of immunoreactive GHR and the rate of this loss was dependent upon the concentration of GH added. However, by 60 min following GH addition, GHR was reduced to ~25% of untreated levels by all concentrations of GH used (50, 100, 200, or 500 ng/ml).

Several recent studies focused on the mechanisms of desensitization of GH signaling by either pulsatile or continuous GH stimulation (7, 27, 38, 43, 44). Because of difficulties in directly measuring GHR levels, it has not been clear whether or not short periods of GH exposure (0–60 min) rapidly reduce GHR levels. Using Western blot analysis, the altered intensity of GHR may be caused by reasons other than a change in total cellular GHR protein levels. For example, spreading of the GHR protein band on Western blots may be caused by GH tyrosyl phosphorylation and gel retardation of a fraction of GHRs, which occurs rapidly following GH stimulation. Additionally, in IM-9 lymphocytes, GH mediates internalization and translocation of GHR from a detergent soluble to an insoluble cellular fraction (45). This results in a loss of GHR detectable by Western blot analysis of the detergent extract, making it difficult to determine whether there is reduction in total GHR or loss of the detergent-extractable GHR (45). The present study may have avoided these problems by boiling the harvested cells in 2% SDS buffer, thereby including all subcellular compartments containing GHR (34).

GH-induced covalent modification of GHR could affect binding of GHR antibodies. However, our two polyclonal antibodies for GHR were raised against two overlapping, large segments of the cytoplasmic domain of human GHR. Therefore, it is unlikely that GH-induced receptor phosphorylation affects binding affinity of these two antisera against GHR. Even if there were reduced binding affinity of GHR antibodies to the phosphorylated GHR, it is unlikely that the reduced intensity of GHR bands in samples treated for 45–90 min with GH would be the result of this decreased affinity. Most of the tyrosyl-phosphorylated GHR should have been dephosphorylated by 30–60 min (46), and inducible serine/threonine phosphorylation of GHR has not been demonstrated. Thus, the present study clearly indicates that GH exposure rapidly reduced total GHR levels, as detected by Western blot analysis with our two separate GHR antibodies, and that the rate of this reduction is dependent upon the concentration of GH added.

It is not clear whether a GH-inducible or a constitutive phosphatase is responsible for dephosphorylating tyrosyl-phosphorylated GHR/JAK2 and PY-STAT5. It is proposed that a GH-inducible, labile phosphatase may regulate the process of desensitization and resensitization (31). Other studies suggest that desensitization of the GH-induced JAK2/STAT5b pathway requires de novo synthesis of an inhibitory protein, possibly a phosphatase (31). In IM-9 lymphocytes, desensitization of GH-stimulated JAK2 tyrosyl phosphorylation does not affect interferon-γ (IFN-γ)-stimulated phosphorylation of JAK2 (47), suggesting that if there is a de novo synthesized inhibitor or phosphatase, it must be specific for the GHR/JAK2, but not the IFN-γ/JAK2 pathway. Alternatively, if GH-induced desensitization of its own pathway is achieved by down-regulating GHR, it is simple to envision how the JAK2 response to IFN-γ can be preserved. The present data demonstrate a rapid down-regulation of GHR by GH exposure and a constitutive, rather than GH-inducible, protein-tyrosine phosphatase is probably sufficient to dephosphorylate and inactivate this pathway. Thus, our work supports the hypothesis that desensitization following a GH pulse is the result of the reduction of GHR at the cell surface following GH binding and receptor internalization/degradation.

In the present study, a 10-fold higher concentration of bGH (500 versus 50 ng/ml) induced a 10-fold higher PY-STAT5 levels at 5 min, and also reduced GHR levels more rapidly, likely by more rapid binding and increased GHR saturation at the higher GH concentration. The slower reduction of GHR levels following a lower GH dose was also supported by the corresponding delay in peak levels of P-ERK1. The data presented here indicate that GH treatment reduced GHR levels to ~20% of that in the untreated cells following a 60-min treatment with GH, and GHR remained low (~10%) with constant GH for 4 h. Consistent with the loss of GHR, PY-STAT5 levels following 4-h treatment with GH were ~10% of the peak PY-STAT5 levels induced by 20-min bGH treatment. Thus, there was an approximately equal decrease in GHR levels and in GH-inducible STAT5 tyrosyl phosphorylation, implying a cause and effect relationship.

When hepatoma cells were exposed to GH for 60 min, washed, and allowed to recover in GH-free, serum-free media, immunoreactive GHR was still decreased to ~50% and a second application of GH further reduced GHR levels. The rate of loss of GHR was greater in both the first and second exposures...
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Fig. 9. Induction of P-ERK1/2 following GH pretreatment plus a 3-h wash-out period. A–D, densitometric analysis of autoradiographs from multiple experiments (n = 3) similar to that represented in Fig. 8 were performed to quantify P-ERK1 levels following different concentrations of GH. GH1 (solid line), first GH exposure; GH2 (dashed line), second GH exposure after the first (at the same concentration) for 1 h followed by 3 h in GH-free, serum-free media. The data are expressed as mean ± S.E. The P-ERK1 levels following 30 min of GH at 50 ng/ml, and 20 min of GH for all other doses, were arbitrarily set to 100%.

The rates of increase of PY-STAT5 following addition of GH were approximately equivalent for both the first and the second GH exposures. The slopes of the lines obtained from the rates of decrease of PY-STAT5, presumably because of dephosphorylation of PY-STAT5, were also approximately equal. Thus, although there is less GHR present at the beginning of the second GH exposure, these data imply that the rates of STAT5 phosphorylation and dephosphorylation were equivalent following the first and the second exposures to GH. This suggests that the phosphatase(s) necessary for PY-STAT5 dephosphorylation was present in approximately equivalent amounts in untreated cells or in cells treated with GH for 60 min and then allowed to recover in GH-free, serum-free media.

The rate-limiting step(s) for resensitization of hepatocytes following stimulation by a GH pulse is not well defined (23, 31). In the present study, following a 1-h GH application, subsequent incubations with GH-free, serum-free media for 1–16 h resulted in a time-dependent recovery of immunoreactive GHR levels. This is most likely because of an increase in total cellular GHR levels, probably by de novo synthesis. Covalent modifications such as phosphorylation and ubiquitination may be less likely to be found in GHR following extended GH-free intervals. Therefore, the increase in immunoreactive GHR levels in whole cell lysates should accurately reflect total cellular GHR levels. Although interpulse intervals with non-detectable GH levels in the young adult male rats are ~3 h, GHR levels in the H4 cells recovered to only approximately half of the control levels following a 3-h incubation in GH-free, serum-free media. We speculate that the rate of in vivo GHR synthesis may be high enough for a full recovery during a 3-h period. With cultured H4 cell incubations in GH-free, serum-free media, the lack of hormones may result in a slower synthesis rate of GHR, resulting in a longer time course of recovery. Additionally, in vivo exposure to GH following a pulse of secretion is most likely different from the full 1-h constant exposure used in the present studies. In vivo peaks of GH are just that, peaks, unlike the “plateau” of constant GH used in the present studies.

Significant to this discussion, the gradual increase in GHR levels correlated well with the recovery of GH signaling as measured by GH-induced PY-STAT5. This suggests that resensitization of the GH/JAK2/STAT5 pathway may be dependent on the recovery of GHR levels during the GH-free interval. The JAK2/STAT5 pathway is one of the major signaling pathways utilized by GH in the liver, and we have now found that under several different experimental conditions (the data presented here and in Ref. 42), there is a direct 1:1 correlation in the amount of immunoreactive GHR and the ability of GH to induce tyrosyl phosphorylation of STAT5.

However, when GH-induced phosphorylation of ERK1/2 is examined in H4 cells, the correlation between GHR levels and GH-induced signaling is limited. Like induction of PY-STAT5, GH induction of P-ERK1/2 is greater in magnitude and reaches a peak more rapidly when using higher concentrations of GH.

to GH when 500 ng/ml GH was used as compared with 50 ng/ml. The slopes of the lines obtained from the rates of decrease of GHR were approximately equal, suggesting that the rates of loss of GHR in response to GH addition were equivalent following either the first or the second exposure to GH. Thus, although there was less GHR at the beginning of the second GH exposure, the mechanism of GHR loss seemed to be similar based on the equal rates of GHR loss.

Significantly, when hepatoma cells were exposed to GH for 60 min, washed, and allowed to recover in GH-free, serum-free media, the reduction of GHR resulted in reduced GH-stimulated tyrosyl phosphorylation of STAT5, which can be measured by a phosphorylated tyrosine-specific STAT5 antibody. Less PY-STAT5 was induced by the second GH exposure than by the first. GH-stimulated tyrosyl phosphorylation of STAT5 can also be measured by a change in mobility of STAT5 on polyacrylamide gels. Retardation of STAT5B is a sensitive indicator of PY-STAT5. In the first exposure to GH, a larger percentage of STAT5B was retarded, whereas following the second exposure to GH, a lesser percentage of total STAT5B was retarded.

The rates of increase of PY-STAT5 following addition of GH were approximately equivalent for both the first and the second GH exposures. The slopes of the lines obtained from the rates of decrease of PY-STAT5, presumably because of dephosphorylation of PY-STAT5, were also approximately equal. Thus, although there is less GHR present at the beginning of the second GH exposure, these data imply that the rates of STAT5 phosphorylation and dephosphorylation were equivalent following the first and the second exposures to GH. This suggests that the phosphatase(s) necessary for PY-STAT5 dephosphorylation was present in approximately equivalent amounts in untreated cells or in cells treated with GH for 60 min and then allowed to recover in GH-free, serum-free media.

The rate-limiting step(s) for resensitization of hepatocytes following stimulation by a GH pulse is not well defined (23, 31). In the present study, following a 1-h GH application, subsequent incubations with GH-free, serum-free media for 1–16 h resulted in a time-dependent recovery of immunoreactive GHR levels. This is most likely because of an increase in total cellular GHR levels, probably by de novo synthesis. Covalent modifications such as phosphorylation and ubiquitination may be less likely to be found in GHR following extended GH-free intervals. Therefore, the increase in immunoreactive GHR levels in whole cell lysates should accurately reflect total cellular GHR levels. Although interpulse intervals with non-detectable GH levels in the young adult male rats are ~3 h, GHR levels in the H4 cells recovered to only approximately half of the control levels following a 3-h incubation in GH-free, serum-free media. We speculate that the rate of in vivo GHR synthesis may be high enough for a full recovery during a 3-h period. With cultured H4 cell incubations in GH-free, serum-free media, the lack of hormones may result in a slower synthesis rate of GHR, resulting in a longer time course of recovery. Additionally, in vivo exposure to GH following a pulse of secretion is most likely different from the full 1-h constant exposure used in the present studies. In vivo peaks of GH are just that, peaks, unlike the “plateau” of constant GH used in the present studies.

Significant to this discussion, the gradual increase in GHR levels correlated well with the recovery of GH signaling as measured by GH-induced PY-STAT5. This suggests that resensitization of the GH/JAK2/STAT5 pathway may be dependent on the recovery of GHR levels during the GH-free interval. The JAK2/STAT5 pathway is one of the major signaling pathways utilized by GH in the liver, and we have now found that under several different experimental conditions (the data presented here and in Ref. 42), there is a direct 1:1 correlation in the amount of immunoreactive GHR and the ability of GH to induce tyrosyl phosphorylation of STAT5.

However, when GH-induced phosphorylation of ERK1/2 is examined in H4 cells, the correlation between GHR levels and GH-induced signaling is limited. Like induction of PY-STAT5, GH induction of P-ERK1/2 is greater in magnitude and reaches a peak more rapidly when using higher concentrations of GH.
GH addition also results in stimulation of the kinases upstream of ERK1/2, MEK 1/2. Similarly, GH addition results in stimulation of a separate signaling pathway, the PI 3-kinase pathway or a similar pathway, resulting in phosphorylation of Akt. Additionally, like the decrease in GH-induced PY-STAT5, when H4 cells were pretreated with GH for 60 min, washed, and allowed to recover for 3 h in GH-free, serum-free media, the ability of GH to induce P-ERK1/2, P-MEK1/2, and P-Akt was greatly diminished. This was true at all concentrations used for the first and the second GH exposures (50, 100, 200, and 500 ng/ml).

Unlike GH-induced PY-STAT5, the present study demonstrated persistent homologous desensitization of GH-induced phosphorylation of ERK1/2 and Akt, and possibly PI 3-kinase activity, which is one pathway resulting in phosphorylation of Akt (48). When H4 cells were pretreated with GH, washed, and allowed to recover in GH-free, serum-free media, the ability of GH to induce P-ERK1/2, P-MEK1/2, and P-Akt were decreased to a much greater extent than the reduction of GHR. Additionally, the desensitization of these signaling pathways to a second GH exposure persisted for as long as 16 h in GH-free, serum-free media, long after GHR had returned to pretreatment levels and at a time that GH-induced PY-STAT5 had fully recovered. Insulin, another inducer of both ERK1/2 and Akt activity in H4 cells, was still able to stimulate phosphorylation of ERK1/2 and Akt following removal of GH and incubation in GH-free, serum-free media. Thus, GH homologous desensitization of MEK/ERK and Akt activation was profoundly less able to recover as compared with GHR and the JAK/STAT5B pathway. For the loss of GH induction of P-ERK1/2 and P-Akt, there is the requirement of prior exposure to GH, which causes the desensitization, rather than just the washing, of cells. The MEK/ERK and PI 3-kinase/Akt pathways are shared pathways with insulin, whereas the JAK/STAT5B pathway is more specific to GH. It is therefore interesting to speculate whether the insulin-like effect of GH, which only occurs after long periods of GH deficiency and is lost after a single expo-

**FIG. 10.** Induction of P-Akt following GH pretreatment plus a 3-h washout period. Western blots were performed as described in the legend for Fig. 1, except for the use of the antibodies for P-Akt and total Akt. GH1, first GH exposure at 500 ng/ml; GH2, second exposure to GH (at the same concentration, 500 ng/ml and the times indicated) following the 1-h first exposure to bGH and 3 h in GH-free, serum-free media. Insulin was added for 5 min at $1 \times 10^{-7}$ M.

**FIG. 11.** Induction of P-ERK1 and PY-STAT5 following GH pretreatment and various durations of GH-free incubation. A, representative Western blot performed as described in the legend for Fig. 1, except for the use of the antibody for P-ERK1/2. GH1, first GH exposure at 500 ng/ml for 20 or 60 min; GH2 (when applicable), second exposure to GH for 20 min following the 1-h first exposure and varying times in GH-free, serum-free media (SFM). B, representative Western blot performed as described in A, except comparing the effects of GH alone, insulin ($1 \times 10^{-7}$ M) alone, or insulin following GH pretreatment for 60 min and then 16 h in GH-free, serum-free media. C, densitometric analysis of Western blots from three similar experiments, as represented in A, with incubation in GH-free, serum-free media for 1, 2, 3, 6, and 16 h. The P-ERK1 levels induced by 20 min of 500 ng/ml bGH following 1-h pretreatment with 500 ng/ml bGH and the various periods of incubations in GH-free, serum-free media (x axis) were expressed as a percentage of the normal P-ERK1 induction by 20 min bGH in non-bGH-pretreated cells. The data are expressed as mean ± S.E. and indicated by the filled triangles and dashed line. Additionally, replicated from Fig. 6C for comparison, PY-STAT5 levels following 20 min of bGH and the various periods of incubations in GH-free, serum-free media are indicated by the open circles and solid line.
sure to GH (49–53), may be related to this loss of GH inducible MEK/ERK and PI 3-kinase/Akt signaling.

Our study suggests that repeated GH stimulation may induce desensitization via the JAK2/STAT5 pathway by downregulation of GHR, and that resensitization of the JAK2/STAT5 pathway paralleled the recovery of GHR levels. However, other post-receptor mechanisms may be important in the refractoriness of the ERK1/2 and Akt pathways to a second GH stimulation. This refractoriness is not a deficiency in activable GHR, JAK2, or STAT5B, because they are fully activable by 6–16 h, whereas P-ERK1/2 and P-Akt are still resistant to a second GH activation. Thus, both receptor and post-receptor mechanisms are important in GH-induced homologous desensitization, but these mechanisms differ for different GH-sensitive signaling pathways.

Acknowledgments—We thank A. Keeton, M. Amsler, W. Bennett, and J. Jiang for helpful and insightful discussions and suggestions. We are grateful to A. F. Parlow (Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center, Torrance, CA) and to the National Institutes of Health NIDDK National Hormone & Pituitary Program for the gift of bGH.

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