Desensitization of the JAK2/STAT5 GH signaling pathway associated with increased CIS protein content in liver of pregnant mice

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Miquet, Johanna G., Ana I. Sotelo, Andrzej Bartke, and Daniel Turyn. Desensitization of the JAK2/STAT5b GH signaling pathway associated with increased CIS protein content in liver of pregnant mice. Am J Physiol Endocrinol Metab 289: E600–E607, 2005.—Chronic exposure to growth hormone (GH) was related to the desensitization of the JAK2/STAT5 signaling pathway in liver, as demonstrated in cells, female rats, and transgenic mice overexpressing GH. The cytokine-induced suppressor (CIS) is considered a major mediator of this desensitization. Pregnancy is accompanied by an increment in GH circulating levels, which were reported to be associated with hepatic GH resistance, although the molecular mechanisms involved in this resistance are not clearly elucidated. We thus evaluated the JAK2/STAT5b signaling pathway and its regulation by the suppressors of cytokine signaling (SOCS)/CIS family and the JAK2-interacting protein SH2-Bß in pregnant mouse liver, a model with physiological prolonged exposure to high GH levels. Basal tyrosyl phosphorylation levels of JAK2 and STAT5b in pregnant mice were similar to values obtained for virgin animals, in spite of the important increment of GH they exhibit. Moreover, these signaling mediators were not phosphor-ylated upon GH stimulation in pregnant mice. A 3.3-fold increase of CIS protein content was found for pregnant mice, whereas the abundance of the other SOCS proteins analyzed and SH2-Bß did not significantly change compared with virgin animals. The desensitiza-tion of the JAK2/STAT5b GH signaling pathway observed in pregnant mice would then be mainly related to increased CIS levels rather than to the other regulatory proteins examined.

pregnant mice; growth hormone signaling; growth hormone receptor; suppressors of cytokine signaling/cytokine induced suppressors; signal transducer and activator of transcription

GROWTH HORMONE (GH) IS CONSIDERED the central regulator of postnatal growth, but it is also involved in the coordination of a wide range of biological processes in vertebrates. In particu-lar, GH has roles in reproductive function, including sexual differentiation, pubertal maturation, and fertility. GH is also required for fetal nutrition and growth during pregnancy and for mammary development and lactation. These actions may reflect endocrine roles of pituitary GH, directly or via induction of hepatic or local IGF-I, as well as autocrine or paracrine roles of GH produced in reproductive tissues. It may also act by increasing gonadotropin secretion and responsiveness (13, 37, 38, 39).

There is a strong correlation between GH secretion and reproductive status (39). Maternal GH levels increase progres-sively throughout gestation, in correlation with fetal growth (38, 44, 65). Because GH does not cross the placental barrier, it indirectly enhances fetal growth by increasing placental size and modulating maternal metabolism, resulting in an increase in availability of nutrients to the fetus (27, 35, 38). In primates, this increase is due to the expression of the placental GH variant gene (39), whereas, in rodents, the rise in plasma GH levels during late pregnancy is a consequence of increased activity of the pituitary gene (11, 18, 39). During late pregnancy in the rat, circulating IGF-I levels decline despite the increment of GH levels (19, 24, 25, 28, 61). This decrease is due, at least in part, to lower hepatic synthesis of IGF-I (25), as hepatocytes are the primary source of circulating IGF-I (9). This state of hepatic GH resistance was related to decreased GH receptor (GHR) gene expression in rat liver (25). However, other authors reported unchanged or higher GHR expression during pregnancy (18, 43, 60, 61), suggesting that the GH resistance associated with late pregnancy occurs at the postreceptor level.

GH binds to two GHR molecules, leading to the activation by trans-phosphorylation of the constitutively associated tyrosine-kinase JAK2, which then phosphorylates the receptor and different signaling mediators (12, 66). In liver, the signal transducer and activator of transcription (STAT) 5b is a major signaling molecule for GH, implicated in the transcription of the gene encoding for IGF-I, among others (36, 63, 64). The JAK/STAT pathway activated by GH is negatively regulated by the suppressors of cytokine signaling (SOCS)/cytokine-induced suppressors (CIS) proteins and by tyrosine phospho-rases such as SHP-1 and SHP-2 (31, 34, 59). GH induces the expression of CIS and SOCS-1, -2, and -3. CIS has been implicated in the desensitization of GH-STAT5b signaling by continuous GH levels (30, 40, 45, 51). The kinase activity of JAK2 can also be modulated by the adapter protein SH2-Bß (53, 54).

Pregnant mice present increased serum GH-binding protein (GHBP) concentration and hepatic GH and membrane-assoc-iated GHBP (MA-GHBP) levels, which are probably caused, at least in part, by the higher GH circulating concentration (10, 18). However, little is known about GH signal transduction during pregnancy and the molecular mechanisms implied in hepatic GH resistance that accompany this state. Comparative studies have been performed assessing GH signal transduction status in the late gestational fetus and male adult rat, demonstrating intact fetal hepatic GH signaling, but the mother has

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not been evaluated (49). To investigate the hepatic GH signal transduction during pregnancy, we studied the JAK2/STAT5b GH signaling pathway and its regulation by SOCS/CIS proteins and SH2-Bβ in 15- to 16-days-pregnant mice, when maximum levels of circulating GH are achieved (18). Because hepatic GH resistance was reported in pregnant rats (19, 61), GH desensitization in liver of pregnant mice could be expected.

MATERIALS AND METHODS

Animals. Female 15- to 16-days-pregnant Swiss-Webster mice, 3 mo of age, and virgin mice as normal controls were used. The day that a plug was found was considered day 0 of pregnancy. Animals were housed in a controlled environment with a photoperiod of 12 h light-12 h dark (lights on from 0600 to 1800) and a temperature of 20 ± 2 °C. Sanitary controls were performed for all major murine pathogens, and the results of the tests were uniformly negative. Animals were given free access to a nutritionally balanced diet and tap water. Housing, handling, and experimental procedures followed the guidelines of the National Institutes of Health for Care and Use of Laboratory Animals (Publication no. 85–2, revised 1985). The protocols were approved by the Animal Studies Committee of the School of Pharmacy and Biochemistry, University of Buenos Aires.

Materials. Ovine GH (oGH) and murine GH (mGH) were obtained through the National Hormone and Pituitary Program (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health). BSA (fraction V), HEPES, Tris, phenylmethylsulfonyl fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF, and the precipitate was washed three times with washing buffer (50 mM Tris, 10 mM vanadate, and 1% Triton X-100, pH 7.4). The final pellet was resuspended in 50 μl Laemmli buffer, boiled for 5 min, and stored at −20 °C until electrophoresis.

Western blotting. Samples (25 μl of the supernatant obtained from the immunoprecipitation procedure or 60 μg solubilized liver protein) were resolved by SDS-PAGE under reducing conditions with a Bio-Rad Mini Protein apparatus (Bio-Rad Laboratories). Electrophoresis of proteins to nitrocellulose membranes was performed for 1 h at 100 volts (constant) with the Bio-Rad mini transfer apparatus in 25 mM Tris, 192 mM glycine, 20% (vol/vol) methanol, and, for proteins with a molecular mass higher than 100 kDa, 0.02% SDS. To reduce nonspecific antibody binding, the membranes were incubated for 2 h at room temperature in blocking buffer (10 mM Tris·HCl, 150 mM NaCl, and 0.02% Tween 20, pH 7.6) containing 3% BSA. The membranes were then incubated overnight at 4°C with the primary antibody (data not shown). Immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) using preflashed Kodak XAR film (Eastman Kodak, Rochester, NY). Band intensities were quantified by optical densitometry (Densitometer model CS-930; Shimadzu) of the developed autoradiographs. For reblotting, membranes were stripped by incubation for 30 min at 50°C in buffer containing 62.5 mM Tris·HCl, pH 7.6, 2% SDS, and 0.7% mercaptoethanol. Blots were washed, reblocked, and immunolabeled as described above. Additional membranes were analyzed by chemiluminescence before incubation with the primary antibody to determine that the reactive band observed in the immunoblotting corresponds to a protein recognized specifically by the primary antibody (data not shown).

Statistical analysis. Results are presented as means ± SE of n, the number of samples indicated. Experiments analyzed all groups of animals in parallel, and the number of separate experiments performed is indicated for each case. Statistical analyses were performed by ANOVA followed by the Tukey-Kramer test using the Instat statistical program by GraphPad Software (San Diego, CA). Student’s t-test was used when two groups were analyzed. Data were considered significantly different at P < 0.05.

RESULTS

Animal characteristics. Data on mGH, glucose, and insulin circulating levels are shown in Table 1. Pregnant mice displayed an increase in GH levels compared with virgin mice (P < 0.0001), in accordance with the increase in GH concentration that occurs during pregnancy in the mouse. Pregnant mice displayed an increment in serum insulin levels, whereas glucose levels did not vary, compared with virgin animals.
GHR protein level. GHR content was evaluated by immunoprecipitation and subsequent Western blotting of solubilized livers with αGHR antiserum. Densitometric analysis of autoradiographs showed a fourfold increase in GHR content in pregnant mice compared with virgin animals (Fig. 1; \( P < 0.0001 \), 6/group).

JAK2 tyrosine phosphorylation and protein levels. Tyrosine phosphorylation of JAK2 was evaluated by immunoprecipitation with anti-phosphotyrosine antibody (αPY) and Western blotting with αJAK2 antibody. As shown in Fig. 2A, basal tyrosine phosphorylation levels of JAK2 were similar in virgin and pregnant mice. After GH administration, JAK2 tyrosine phosphorylation increased ∼4.4-fold in virgin animals (Fig. 2A, \( P < 0.001 \), \( n = 5 \)), whereas for pregnant mice the difference between basal and GH-stimulated conditions was not statistically significant. JAK2 protein content was evaluated by Western blotting of liver extracts. Densitometry of autoradiographs showed that JAK2 concentration is not significantly different between virgin and pregnant mice (Fig. 2B).

STAT5b tyrosine phosphorylation and protein levels. Tyrosine phosphorylation of STAT5b was evaluated by immunoprecipitation with αSTAT5b antibody and Western blotting with αPY antibody. Tyrosine phosphorylation of STAT5b was approximately sevenfold increased upon GH stimulation in virgin mice (Fig. 3A, \( P < 0.0001 \); \( n = 11 \)/group). GH stimulation did not significantly change the phosphorylation levels of STAT5b in pregnant mice, although some animals partially responded to GH administration (2 of 11 animals). No significant differences in the basal phos photyrosine level of STAT5b in pregnant vs. virgin mice was observed (Fig. 3A). The protein content of STAT5b was analyzed by stripping and reblotting the membranes with αSTAT5b antibody; the concentration of this protein is similar in pregnant and virgin mice (Fig. 3B).

SOCS and CIS protein levels. CIS protein content was evaluated by immunoprecipitation and Western blotting of liver homogenates with αCIS antibody. Pregnant mice displayed a 3.3-fold increase in CIS concentration compared with virgin animals (Fig. 4A; \( P < 0.0001 \); \( n = 8 \)/group). SOCS-1, -2, and -3 protein content was analyzed by Western blotting of liver homogenates with specific antibodies. There were no significant differences between the values obtained for pregnant and virgin mice in any of these proteins (Fig. 4, B–D).

SH2-Bβ protein level. The protein content of SH2-Bβ was determined by immunoprecipitation with αSH2-B antibody and Western blotting with an αSH2-B antibody. No significant difference was observed in the level of this protein between virgin and pregnant mice (Fig. 5).

### Table 1. Characteristics of experimental animals

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<tr>
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<th>Virgin</th>
<th>Pregnant</th>
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<tr>
<td>mGH, ng/ml</td>
<td>2.8±0.3 (8)</td>
<td>101±8* (7)</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>129±13 (7)</td>
<td>136±22 (7)</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.3±0.2 (6)</td>
<td>1.6±0.4† (9)</td>
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Values are means ± SE; no. of mice in parentheses. Determinations were made after 8 h of fasting. Murine growth hormone (mGH) was measured by specific RIA, glucose by the glucose oxidase procedure, and insulin by an ultrasensitive ELISA kit. * \( P < 0.0001 \) and † \( P < 0.05 \) vs. virgin mouse values.

DISCUSSION

In this report, we describe the desensitization of the GH JAK2/STAT5b signaling pathway in pregnant mouse liver, which is associated with increased hepatic CIS protein content. Pregnant mice at 15–16 days of gestation were used, as in this period GH circulating levels are maximal (18). Despite the very important increase in serum GH levels in the pregnant animals used, no higher basal tyrosine-phosphorylated levels of STAT5b or JAK2 were found when compared with virgin mice; moreover, GH stimulation did not induce a significant increase in the phosphorylation of these signaling mediators. These results reflect a lack of response to GH in pregnant mouse liver. A state of hepatic GH resistance associated with late pregnancy has been described in pregnant rats, since serum IGF-I concentration dramatically decreases despite the elevated GH levels (19, 24, 25, 28, 61). This hepatic resistance can occur at the plasma membrane or at the intracellular level. Escalada et al. (25) related the decline in serum and hepatic IGF-I content with decreased liver mRNA GH levels in the pregnant rat. However, other authors found similar or higher GH content in rodent liver during pregnancy (10, 18, 43, 60, 61). Camarillo et al. (10) described an increase in GHR content in pregnant mice, accompanied by a substantially higher increment in MA-GHBP levels, which could act as dominant-negative forms of the receptor at the membrane level (52). Because we found increased GHR protein content in pregnant mouse liver, we decided to explore intracellular mechanisms of signaling downregulation, which can contribute to liver GH resistance. The desensitization of the JAK2/STAT5b GH pathway described here may account for the decreased IGF-I levels reported during late pregnancy, since this signaling pathway is...
Fig. 2. A: equal amounts of solubilized livers from virgin and pregnant mice, stimulated with ovine growth hormone (oGH: +) or nonstimulated (−), were immunoprecipitated with anti-phosphorylated tyrosine (αPY) antibody and subjected to Western blotting with anti-JAK2 (αJAK2) antibody. Tyrosyl phosphorylation was quantified by scanning densitometry and expressed as a percentage of the mean value measured for growth hormone (GH)-stimulated virgin mice. In the control, a sample incubated with protein A-Sepharose in the absence of the immunoprecipitating antibody, no immunoreactive band was observed, indicating that the precipitation was specific. B: JAK2 protein content was analyzed by Western blotting of solubilized livers with αJAK2 antibody. Quantification was performed by scanning densitometry and expressed as a percentage of the mean value measured for virgin mice. Data are means ± SE of 5 (A) and 6 (B) samples/group, each one representing a different animal, run in 4 (A) and 2 (B) separate experiments. *P < 0.001 vs. nonstimulated virgin mice. Blots shown are representative of different experiments.

Fig. 3. A: liver extracts of GH-stimulated (+) or nonstimulated (−) virgin and pregnant mice were prepared, and equal amounts of the solubilized tissue were immunoprecipitated with αSTAT5b antibody, separated by SDS-PAGE, and subjected to Western blot analysis with αPY antibody. Protein phosphorylation was quantified by scanning densitometry and expressed as a percentage of the mean value measured for GH-stimulated virgin mice. B: for the evaluation of STAT5b protein content, the membranes used for the detection of tyrosine phosphorylation were stripped and reblot with the same IP antibody. The control, a sample incubated with protein A-Sepharose in the absence of the immunoprecipitating antibody, displays no reactive band, indicating that the precipitation was specific. Quantification was performed by scanning densitometry and expressed as a percentage of the mean value measured for virgin mice. Data are means ± SE of 11 (A) and 6 (B) samples/group, each one representing a different animal, run in 9 (A) and 2 (B) separate experiments. *P < 0.0001 vs. nonstimulated virgin mice. Blots shown are representative of different experiments.
a key component of GH-stimulated IGF-I gene transcription (63).

Continuous GH secretion profile was associated with desensitization of the JAK2/STAT5b GH signaling pathway, as demonstrated in the female rat and in hepatocytes continuously exposed to GH (14, 15, 29, 62). Pregnancy is a condition characterized by prolonged elevated GH levels, among many other physiological changes. In rats, it was demonstrated that the increment in GH circulating levels that occurs during pregnancy reflects an increase in basal plasma GH levels and GH pulse amplitude, but not in pulse frequency (11). The cytokine-induced suppressor CIS was shown to be a key mediator of the STAT5b desensitization that occurs in cells and tissues chronically exposed to GH (30, 40, 45, 51). It competes with STAT5b for common tyrosine-phosphorylated residues on the cytoplasmic tail of GHR, and it may also mediate proteasome-dependent degradation of GHR/JAK2 complexes (41, 51). The results presented in this work are in accordance with the JAK2/STAT5b desensitization that occurs under chronic GH stimulation and its association with CIS. Previous

Fig. 4. A: liver extracts of virgin and pregnant mice were prepared, and equal amounts of the solubilized tissue were immunoprecipitated with anti-cytokine-induced suppressor (αCIS) antibody, separated by SDS-PAGE, and subjected to Western blot analysis with the same antibody. Data are means ± SE of 8 samples/group, each one representing a different animal, run in 3 separate experiments. *P < 0.0001 vs. virgin mice. The control, a sample incubated with protein A-Sepharose in the absence of the immunoprecipitating antibody, displays no immunoreactive band, indicating that the precipitation was specific. B-D: suppressor of cytokine signaling (SOCS)-1, -2, and -3 protein content was analyzed by Western blotting of equal amounts of solubilized livers of virgin and pregnant mice with the indicated antibodies. Data are means ± SE of 4 (B), 6 (C), and 7 (D) different individuals/group run in 1 (B) or 2 (C and D) separate experiments. Quantification was performed by scanning densitometry and expressed as a percentage of the mean value measured for virgin mice. Blots shown are representative of different experiments.
reports from our laboratory described increased liver C1R
protein content in two models of transgenic mice overexpress-
ing GH, in which the hepatic JAK2/STAT5b GH-signal trans-
duction pathway was also suppressed (30, 45).

The content of the other SOCS proteins analyzed was not
altered in pregnant mice, although GH was described to induce
their synthesis (31, 41). Although SOCS-1 and SOCS-3 inhibit
JAK2 activity (31, 41), SOCS-2 was proposed to have dual
effects on GH signaling (26, 32, 33). We have previously
reported that SOCS-2 levels were not altered and that SOCS-3
content was decreased in GH-overexpressing transgenic mouse
liver, although the mechanism involved and the physiological
relevance of this finding were not completely elucidated, sug-
gesting that these proteins were not directly involved in the GH
signal desensitization observed (30, 45). SH2-Bβ is a JAK2
activity modulator that may bind to the phosphorylated kinase,
serving as an activator, or, alternatively, it may bind to non-
phosphorylated kinase, preventing its abnormal activation (53,
54). GH-overexpressing transgenic mice exhibited an increase
in SH2-Bβ content that would be bound to nonphosphorylated
JAK2, possibly accounting for its desensitization (46), whereas
the protein abundance of SH2-Bβ was not significantly differ-
ent between pregnant and virgin female mice. The discrepan-
cies observed between these two models of GH increment may
be attributed to the fact that GH levels present in pregnant mice
are not as elevated as in GH transgenic mice, which express
very high GH concentrations since birth (45). Moreover, other
hormonal and metabolic alterations accompany pregnancy,
which may be responsible for the differences found between
these GH-overexpression models. During the second half of
mouse pregnancy, large amounts of placental lactogens and
other prolactin (PRL)-related proteins are produced by the
placenta. These hormones bind to the PRL receptor and are
believed to activate the signaling pathways used by PRL (57).
PRL signals via JAK2/STAT5b and induces the expression of the
same SOCS/CIS proteins that GH does (6, 48). However,
the contribution of these PRL-like placental hormones to the
observed liver GH desensitization during mouse pregnancy is
doubtful, since it was reported that GH desensitization is
caused by homologous stimulation with continuous GH but not
by heterologous stimulation by PRL in pancreatic islets of
Langerhans (8). Moreover, apart from the high GH levels,
other hormonal changes related with gestation could also
contribute to the regulation of hepatic GHR levels (5, 17, 47).

Chronic elevation of GH is known to induce insulin resis-
tance and hyperinsulinemia in humans and animals (16, 20,
23). Insulin levels were increased in the pregnant animals used
in this work, whereas glucose serum concentration did not
significantly change, in accordance with the known insulin
resistance that accompanies this period (3, 4, 42, 44, 55, 56).
Previous studies from our laboratory showed transgenic mice
overexpressing bovine GH to be insulin resistant (2, 21, 22),
and they also present hepatic GH resistance associated with the
molecular alterations described for pregnant mice in the
present work (45). Conditions of chronically elevated GH are
thus associated with both insulin resistance and hepatic GH
resistance, but the molecular mechanism involved has not been
elucidated yet.

The hepatic GH resistance in pregnant mice described here,
which occurs at the postreceptor level, suggests that liver is not
a major GH target during late pregnancy in the mouse. The
metabolic actions that GH induce in maternal metabolism
occur in other tissues. It was proposed that the hepatic resis-
tance described in mid- and late pregnancy would permit the
selective activation of gonadal responses to GH, without acti-
vating the systemic responses mediated by IGF-I (39). It would
be interesting then to evaluate GH signaling and its regulation
in other potential GH-responsive tissues. It is important to note
that, even when maternal GH does not cross the placental
barrier, GH signaling is intact in the developing fetus in the rat,
suggesting that fetal GH has physiological roles in late peri-
natal life (49).

In summary, the elevation of GH circulating levels that
occurs during pregnancy is accompanied by a desensitization
of the JAK2/STAT5b pathway of GH signal transduction in
pregnant mouse liver, which is associated with an increase in
the protein content of the suppressor of cytokine signaling CIS.
These results further corroborate previous reports relating CIS
to the STAT5b desensitization produced by chronically ele-
vated GH levels in vivo and propose a molecular mechanism
implicated in the hepatic GH resistance during late pregnancy.

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REFERENCES


