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Inadequacy of the Janus Kinase 2/Signal Transducer and Activator of Transcription Signal Transduction Pathway to Mediate Episodic Growth Hormone-Dependent Regulation of Hepatic CYP2C11

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ABSTRACT

CYP2C11, the most commonly expressed hepatic cytochrome P450 isoform in male rats, is induced by the masculine "episodic" secretory growth hormone profile. A considerable number of reports have indicated that episodic growth hormone effects are mediated by the activation of the Janus kinase 2 (Jak2)/signal transducer and activator of transcription (Stat)5B signal transduction pathway. We observed that restoration of the normal masculine plasma growth hormone pulse in hypophysectomized male rats did indeed rapidly activate (phosphorylate) Jak2, shortly followed by activation and nuclear translocation of Stat5B. Infusion of a growth hormone pulse with an amplitude that was 10% of the normal height induced a dramatic overexpression of CYP2C11, had little effect activating Jak2, but induced a more rapid and greater accumulation of activated nuclear Stat5B. Restoration of a growth hormone pulse with an amplitude of only 1% of normal had little effect phosphorylating Jak2, activated and translocated to the hepatic nucleus ~70% of the normally induced levels of Stat5B, but had no inductive effect on CYP2C11. Last, the hypophysectomized male rat receiving no growth hormone replacement expressed 25 to 35% of normal concentrations of CYP2C11 despite no measurable activation of either Jak2 or Stat5B. These results raise concerns regarding the requisite role of the Jak2/Stat5B pathway in mediating episodic growth hormone regulation of CYP2C11. However, accumulation of activated extracellular signal-regulated kinase (ERK)1 and ERK2 were the only transducers measured in the study not affected by the 1% replacement pulse of growth hormone and were elevated 2- to 3-fold above normal when the pulse was renaturalized to 10% of physiological amplitude, suggesting the possible involvement of mitogen-activated protein kinase in episodic growth hormone regulation of CYP2C11.

CYP2C11 is the most abundant male-specific isoform of cytochrome P450, comprising ~50% of the total hepatic cytochrome P450 in male rat liver (Morgan et al., 1985). Expression of CYP2C11 is regulated by the masculine growth hormone (GH) plasma profile, characterized by episodic bursts (~200–300 ng/ml plasma) every 3.5 to 4 h, which are separated by undetectable interpulse GH periods (Legraverend et al., 1992; Shapiro et al., 1995). Infusion of a GH pulse (40 μg/kg b.wt.) every 4 h to hypophysectomized (HYPOX) male rats restores both the normal masculine episodic plasma profile and intact-like expression levels of CYP2C11 (Shapiro et al., 1993; Agrawal and Shapiro, 2000). Renaturalization of just four or even two daily plasma GH pulses of physiological amplitudes is also sufficient to restore normal levels of hepatic CYP2C11 (Waxman et al., 1991; Shapiro et al., 1993). However, when the frequency of pulses is increased to seven per day, and the GH-devoid interpulse period approaches 2.5 h or less, CYP2C11 is completely suppressed (Waxman et al., 1991; Shapiro et al., 1993). GH regulation of CYP2C11 clearly requires a minimum duration of the GH-devoid interpulse period, but what is the importance of the pulse amplitude? Circulating GH pulse amplitudes from 300 to 20% of normal induce physiological expression levels of CYP2C11 (Agrawal and Shapiro, 2000). Hormone pulse heights 10 to 5% of normal induce an overexpression of CYC2C11 mRNA, protein, and catalytic activity (Pampori and Shapiro, 1994; Agrawal and Shapiro, 2000) that is most dramatic at the transcript level in which a portion of mRNA is characterized by retention of its terminal

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ABBREVIATIONS: GH, growth hormone; HYPOX, hypophysectomized; Jak2, Janus kinase 2; Stat, signal transducer and activator of transcription; HRP, horseradish peroxidase; MAP, mitogen-activated protein kinase; rGH, rat growth hormone; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; TBST; Tris-buffered saline/Tween 20; ERK, extracellular signal-regulated kinase.

intron (Pampori and Shapiro, 2000). Pulse amplitudes that decline to ~2% or less of normal become incapable of inducing CYP2C11 expression (Agrawal and Shapiro, 2000). Thus, although a minimal GH-devoid interpulse is required for CYP2C11 expression, the hepatocyte must be able to recognize a pulse, albeit at a subnormal height, to regulate (stimulate) CYP2C11 expression.

Several studies have established a role for the Janus tyrosine kinase/signal transducer and activator of transcription (Jak/Stat) signal transduction pathway in mediating the actions of GH. GH binding to its plasma membrane receptor leads to receptor dimerization (de Vos et al., 1992; Darnell et al., 1994). Next, Jak2 (one possible isoform of the transducer) associates with the dimerized receptor, leading to autophosphorylation of the kinase and phosphorylation of the intracellular domain of the receptor (Leung et al., 1987; Darnell et al., 1994). Subsequent to Jak2 activation, a member of the Stat family associates with the GH-receptor-Jak2 complex and is itself phosphorylated (Darnell et al., 1994). Once activated, Stat dimerizes, translocates to the nucleus where it binds to a regulatory or response element for GH-dependent target genes (Bergad et al., 1995; Ganguly et al., 1997; Subramanian et al., 1998). Reports have identified the transcription factor Stat5B as a key signaling molecule mediating the actions of the masculine episodic GH profile (Waxman et al., 1995). Activation of two other Stat proteins, Stat 1 and Stat 3, were found to be nondiscriminatory between the masculine episodic and feminine continuous GH profile (Ram et al., 1996).

Whereas previous studies have shown that the masculine GH profile regulates expression of murine male-dependent isoforms of cytochrome P450 through the Jak2/Stat5B signal transduction pathway (Udy et al., 1997; Park et al., 1999), a similar mechanism regulating rat CYP2C11 has only been inferred (Choi and Waxman, 2000). Because variations in the pulse amplitude of the circulating masculine GH profile can alter expression levels of rat CYP2C11 (Pampori and Shapiro, 1994; Agrawal and Shapiro, 2000), we have examined the relationship between the GH pulse amplitude, CYP2C11 expression, and the concentrations, activation, and translocation of members of the Jak2/Stat5 signal transduction pathway.

Materials and Methods

Antibodies and Chemicals. Antibodies were purchased against Stat5A, Stat5B, β-actin, HRP-conjugated anti-goat (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Jak2, phosphotyrosine Stat5, anti-phosphotyrosine (4G10), protein A-Sepharose (Upstate Biotechnology, Lake Placid, NY), phospho-Jak2 (Chemicon International, Temecula, CA), activated mitogen-activated protein (MAP) kinase (Cell Signaling Technology Inc., Beverly, MA), CYP2C11 (Oxford Biomedical Research, Oxford, MI), and HRP-conjugated anti-mouse and anti-rabbit (Amersham Biosciences, Piscataway, NJ). Recombinant rat GH and materials used to assay plasma rat GH (rGH) were obtained from the National Hormone and Peptide Program and Dr. A. F. Parlow (Harbor-UCLA Medical Center, Torrance, CA). Other chemicals of molecular biology grade were purchased either from Sigma-Aldrich (St. Louis, MO) or Roche Diagnostics (Indianapolis, IN).

Animals. Animals were housed in the University of Pennsylvania Laboratory Animal Resources Facility under the supervision of certified laboratory animal medicine veterinarian. These animals were treated according to a protocol approved by the University's Institutional Animal Care and Use Committee. HYPOX male [Crl: CD (SD) BR] Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Rats were HYPOX by the supplier at 8 weeks of age and maintained for 4 to 5 weeks on commercial rat pellets and 5% sucrose drinking water. These animals were housed under conditions of regulated temperature (20–23°C) and photoperiod (12 h light-dark cycle; lights on at 8:00 AM). The effectiveness of the surgery was verified by the lack of body weight gain during the observation period and an absence of a pituitary or its fragments at necropsy.

Surgical Implantation of Catheter, GH Treatment, and Assay. Indwelling right atrial catheters were implanted by methods described previously (MacLeod and Shapiro, 1988; Pampori et al., 1991). After 3 days, the unrestrained and unstressed catheterized HYPOX rats were infused with either 40, 4, or 0.4 μg of rGH/kg b.wt. by an external syringe pump apparatus over a 3-min period with a frequency of six pulses per day for 6 days (i.e., one pulse every 4 h). Control rats were similarly infused with vehicle. On the fourth day of infusion, atrial blood samples (12 μl) were collected every 15 min for 8 h. rGH patterns were determined by radioimmunoassay (Shapiro et al., 1989). In another set of experiments, catheterized HYPOX rats were infused with a single dose of 40, 4, or 0.4 μg of rGH/kg b.wt. Rats were decapitated at 5, 15, 30, 45, 60, 120, 180, and 240 min after the rGH infusion. The 0-min rats were given only rGH buffer and euthanized immediately. Blood was collected in heparinized buffer for rGH estimation. Livers were removed immediately and minced into small pieces on ice-chilled Petri dishes. A fraction of minced liver was stored in RNA-Later (Ambion, Austin, TX) at −70°C for RNA extraction.

Preparation of Subcellular Fractions of Liver. Livers were processed by the method of Sierra et al. (1993). In brief, minced livers were homogenized in buffer (10 mM HEPES, 15 mM KCl, and 2.4 M sucrose, pH 7.6) containing 5% nonfat dry milk. The homogenization buffer contained different protease and phosphatase inhibitors: 0.5 mM phenylmethylsulfonyl fluoride, 1 mM DL-dithiothreitol, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovandate, 10 mM sodium fluoride, 0.15 mM spermine, and 0.5 mM spermidine. The homogenized livers were centrifuged over a sucrose cushion for 60 min at 100,000*g* in a precooled rotor. The upper layer of cellular debris was discarded after centrifugation, and the remaining supernatant was designated as the postnuclear fraction. A portion of the postnuclear fraction was further centrifuged at 100,000*g* for 60 min at 4°C, and the resultant supernatant was designated as the cytosol. After the initial centrifugation and removal of the postnuclear fraction, the walls of the centrifuge tubes containing the nuclear pellet were washed with ice-cold normal saline to avoid contamination by other subcellular fractions. The nuclear pellet was lysed with nuclear lysis buffer (10 mM HEPES, 0.1 M KCl, 0.1 mM EDTA, 10% glycerol, and 3 mM $MgCl_2$) also containing the above-mentioned protease and phosphatase inhibitors. The resultant nuclear suspension was precipitated with 2.0 M ammonium sulfate for 30 min in an ice bath with slow shaking and centrifuged at 100,000*g* for 60 min at 4°C. The tubes were removed immediately and the supernatant was transferred into new tubes. Next, the supernatant was incubated with 3 mg/ml ammonium sulfate for 30 min in an ice water bath and centrifuged at 100,000*g* for 20 min. The pellet was dissolved in a nuclear dialysis buffer (25 mM HEPES, 0.1 mM EDTA, 40 mM KCl, and 10% glycerol, pH 7.4) containing the above-mentioned protease and phosphatase inhibitors. The protein content of the different subcellular fractions was quantified using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

Immunoprecipitation. Rat liver nuclear extract (50 μg) were incubated overnight at 4°C with anti-phosphotyrosine (4G10) antibody in 100 μl of immunoprecipitation buffer (1% Triton X-100, 0.5% Nonidet P-40, 10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovandate, 10 mM sodium fluoride, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). Immunoprecipitates were then bound to 50% of

protein A-Sepharose for 2 h on a rotary mixer at 4°C. Samples were centrifuged for 5 min at 14,000*g* and washed with immunoprecipitation buffer three times. Proteins were eluted by boiling for 10 min in 30 μl of sample buffer. Immunoprecipitates were electrophoresed and immunoblotted with Stat5B antibody.

Western Blot. Different subcellular fractions of liver (postnuclear, cytosol, and nuclear extract) were electrophoresed under denaturating conditions on a SDS-PAGE system. We used 1.5-mm, 10% SDS-polyacrylamide gels for CYP2C11, Stat5A, Stat5B, phospho-Stat5, and activated MAP kinase and 4 to 10% gradient gels (SDS-PAGE) for Jak2 and phospho-Jak2. Electrophoresis was performed at 55 V overnight at room temperature. Electrophoresed protein was transferred to a nitrocellulose membrane (Osmonics, Westborough, MA) using a standard wet transfer method at 400 mA for 6 to 7 h at 4°C. The nitrocellulose membrane was blocked overnight with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) at 4°C with slow shaking. Blocked membranes were washed with TBST and incubated with appropriate primary antibodies for 2 h at room temperature with slow shaking. Thereafter, membranes were washed with TBST to remove unbound primary antibody and incubated for 1 h at room temperature with specific HRP-conjugated secondary antibodies. Extra secondary antibody was washed off using TBST, and membranes were exposed to X-ray films (Eastman Kodak, Rochester, NY) in the presence of an enhanced chemiluminescence reagent (Amersham Biosciences Inc.). The blots were analyzed with an Alpha Innotech FluorChem 8800 gel documentation system using a visible light source. Densitometric units were obtained as integrated density values as calculated by the software supplied with the gel documentation system. Equal loading of protein was confirmed by using Ponceau S staining and Western blot analysis for the expression of β-actin. Furthermore, protein values were normalized to two control samples repeatedly run on all blots.

RNA Extraction. Liver samples stored in RNA-Later were extracted with TRIzol reagent (Invitrogen, Carlsbad, CA). The manufacturer's recommended protocol was followed. In brief, liver was homogenized with the TRIzol and mixed with chloroform (1:5 ratio). This suspension was centrifuged at 4°C for 20 min at 12,000*g*. RNA was precipitated from the aqueous phase by using isopropanol for 30 min at room temperature and pelleted by centrifugation at 4°C for 15 min at 12,000*g*. The RNA pellet was washed with 75% ethanol. The RNA was finally dissolved in diethyl pyrocarbonate-treated distilled water and quantified at 260 nm. The purity of RNA was established by the 260/280-nm spectrophotometric ratio.

Reverse Transcription-Polymerase Chain Reaction and Northern Blot. cDNA synthesis was carried out in a total volume of 20 μl. For the conversion of total RNA to cDNA, a 20-μl reaction mixture was prepared containing 1× reverse transcriptase buffer, 5 mM $MgCl_2$, 1 mM of each dNTPs, 1 unit of RNase inhibitor, 2.5 units of murine leukemia virus reverse transcriptase (Promega, Madison, WI), 2.5 μM oligo-d$(T)_{16}$ (Applied Biosystems, Foster City, CA) and 1 μg of RNA. The mixture was incubated at 42°C for 1 h and stored at −20°C. Subsequent PCR was performed in 100-μl reaction mixture containing 1× PCR buffer, 2 mM $MgCl_2$, 2.5 units of *Taq*DNA polymerase, 1 μl of cDNA, and 150 nM specific sets of primers. The primer sequence and cycling condition for CYP2C11 (Zaphiropoulos and Wood, 1993) and glyceraldehyde-3-phosphate dehydrogenase (Okumoto et al., 2003) were reported elsewhere. PCR was performed in a GeneAmp PCR System 9600 thermocycler (PerkinElmer Life and Analytical Sciences, Boston, MA). The final PCR products were separated electrophoretically on 1.5% agarose gel run with 0.5× Tris borate-EDTA for 90 min at 80 V. Electrophoresed gels were stained for 20 min with 0.5× Tris borate-EDTA containing 1 μg/ml ethidium bromide. The final PCR product was quantified with a FluorChem 8800 gel documentation system (San Leandro, CA) using a UV lamp. Densitometric units were obtained as integrated density values as calculated by the software supplied with the gel documentation system. CYP2C11 mRNA was normalized with glyceraldehyde-3-phosphate dehydrogenase mRNA levels for individual livers. The PCR product for CYP2C11 was purified and sequenced with DNA sequencer model 377 (Applied Biosystems) using the specific primer for CYP2C11. According to a Blast search (www.ncbi.nlm.nih.gov), the purified PCR product exhibited 100% sequence homology with the *CYP2C11* gene (*Rattus norvegicus*) (sequence not presented). The Northern blotting procedure for CYP2C11 was reported previously (Dhir and Shapiro, 2003).

Statistical Analysis. Data were normalized to the same control sample run with every blot. All data were subjected to analysis of variance, and differences were determined with *t* statistics and Bonferroni's procedure for multiple comparison.

Results

Regulation of Hepatic CYP2C11 Expression by the Pulse Amplitudes in the Masculine GH Profile. HYPOX resulted in the absence of circulating GH and a concomitant ~70% decline in CYP2C11 (mRNA and protein) expression that was completely corrected by restoring the physiological episodic profile with the infusion of 40 μg of rGH/kg b.wt. every 4 h for 6 days (Fig. 1). When the renaturalized pulse was administered at 4 μg rGH/kg b.wt. (i.e., 10% of the normal amplitude), both CYP2C11 protein and to a greater extent, mRNA, were overexpressed. A further reduction in the infused rGH pulse to 0.4 μg/kg b.wt. (i.e., 1% of the normal amplitude) for 6 days resulted in barely detectable plasma rGH peaks (~3 ng/ml) having no greater inductive effect on CYP2C11 expression than administration of hormone diluent alone to HYPOX male rats (Fig. 1).

rGH Pulse Replacement. Because CYP2C11 expression levels vary with the pulse amplitudes in the masculine episodic GH profile (Fig. 1), we examined the effects of various pulse heights on the concentrations, activation, and translocation of several signal transducers reported to regulate episodic GH-dependent transcription. First, however, we measured the resulting plasma hormone levels in HYPOX male rats infused with a single pulse of different rGH concentrations (Table 1). A pulse administration of rGH at 40 μg/kg b.wt. produced a physiological-like peak amplitude (~270 ng/ml) and duration (~1 h) in plasma. Administration of 4 μg/kg b.wt. reduced the pulse height by ~90% and its duration by at least one-half. Barely detectable peaks of rGH (i.e., 3 ng/ml) were observed in HYPOX male rats injected with the lowest concentration of rGH (0.4 μg/kg b.wt. or 1% of the physiological replacement dose). The values for plasma rGH among the single pulse treatment groups (Table 1) were somewhat higher in comparison with those rats infused with the episodic profile for 6 days (Fig. 1). In this regard, in the multiple-pulse experiment (Fig. 1), rGH was delivered via an external pulse simulator over a period of 3 min, whereas the single-pulse treatment (Table 1) was administered by injection within 5 to 10 s.

Phospho-Jak2. When measured in the nuclear-free extract, all three concentrations of rGH were found to stimulate Jak2 phosphorylation (Fig. 2). However, the levels of phosphorylation varied significantly between the highest and the other two doses. Whereas all treatments induced a significant peak in phospho-Jak2 within 5 min of hormone infusion, activation of the transducer was ~7-fold higher in the 40 μg/kg b.wt. treatment group compared with the other two treatments. In fact, 15 min after the highest rGH pulse, phospho-Jak2 levels, although declining, still remained sig-

nificantly higher ($p < 0.01$) than that observed in both of the lower treatment doses. The two lower doses of rGH stimulated indistinguishable phosphorylation levels of Jak2 in an apparent biphasic manner; small peak at 5 and 30 min. Regardless of rGH dose, phospho-Jak2 was no longer detectable within 60 min after hormone administration. Although total Jak2 concentrations were considerable in the hepatic nuclei from all treatment groups, no nuclei, at any time point, exhibited detectable levels of the activated (i.e., phosphorylated) form of Jak2 (data not shown).

Stat5A. We found no rGH dose effects on hepatic cytosolic Stat5A concentrations during the 240-min study period (Fig. 3, top). At all rGH doses, Stat5A peaked at 15 min, declined at 30 min, and returned to basal levels at 45 min, where it basically remained for the next 195 min. Time-dependent changes in the levels of postnuclear (i.e., predominantly membrane fraction) Stat5A were minimal and similar at the three different doses of rGH with one exception; Stat5A peaked ($p < 0.01$) at 30 min after only the 40-μg hormone dose (Fig. 3, middle). In contrast to findings in the cytosol and postnuclear fractions, Stat5A was never present in the nucleus at zero time. Presumed nuclear translocation of Stat5A occurred within 5 min of infusion at every rGH dose (Fig. 3, bottom). Thereafter, the time-dependent change in Stat5A accumulation in the nucleus were similar at all rGH doses, although the magnitude of change varied. That is, nuclear Stat5A peaked between 30 and 45 min after all the doses of rGH and returned to baseline (i.e., zero levels) after 60 min. However, because of dose-dependent differences in apex levels of Stat5A, the total amount of nuclear Stat5A found

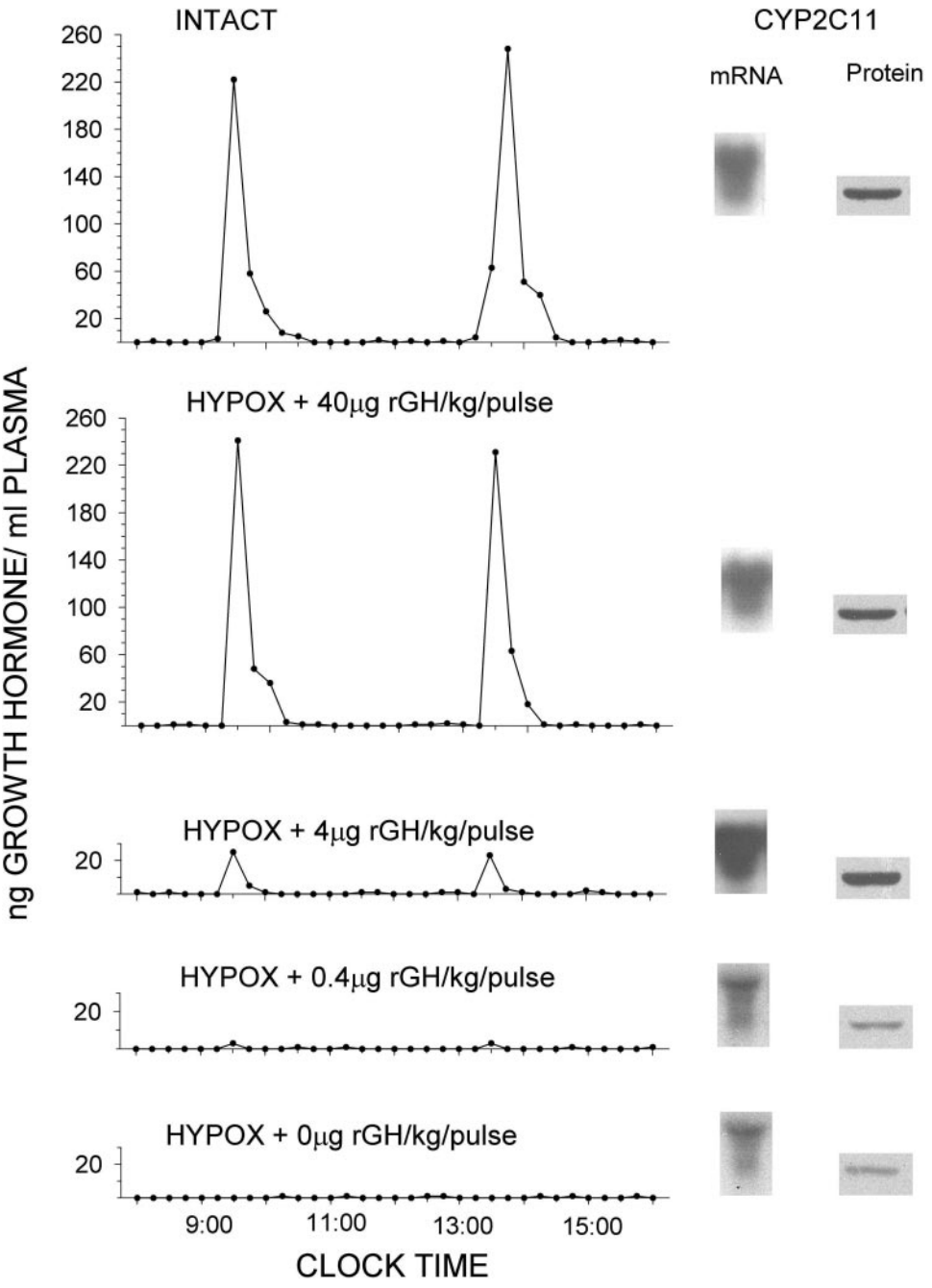

Fig. 1. Regulation of hepatic CYP2C11 expression by pulse amplitudes in the circulating rGH profile. Plasma rGH was monitored for 8 h and renaturalized for 6 days by use of our pulse simulator apparatus described elsewhere (Pampori et al., 1991). First (top) panel, endogenous plasma rGH profile and hepatic CYP2C11 mRNA and protein in intact male rats. Second panel, restored physiological plasma rGH profile and CYP2C11 expression in HYPOX male rats infused with 40 μg rGH/kg b.wt./pulse. Third panel, restored subphysiological (10% of normal pulse amplitude) plasma rGH profile, CYP2C11 mRNA, and protein in HYPOX male rats infused with 4 μg rGH/kg b.wt./pulse. Fourth panel, another restored subphysiological (1% of normal plasma amplitude) plasma rGH profile and CYP2C11 expression in HYPOX male rats infused with 0.4 μg rGH/kg b.wt./pulse. Fifth (bottom) panel, plasma rGH and CYP2C11 expression in HYPOX male rats infused with rGH vehicle. Circulating rGH was determined on day 4 of infusion, whereas CYP2C11 mRNA and protein were measured after euthanasia on day 7. The rGH profiles and CYP2C11 expression levels presented are representative of findings observed in four additional animals in each treatment group.

during the 5- to 60-min plasma pulse was greatest in the nuclei of livers isolated from rats administered 40 μg of rGH, somewhat less in nuclei from rats treated with 4 μg of the hormone and least in nuclear fractions isolated from the lowest rGH-treated rats. Thus, if the total amount of accumulated nuclear Stat5A during the first hour after hormone treatment was 100% at the highest rGH dose, it was ~70% at the medium dose and ~50% at the lowest hormone dose.

TABLE 1

Resulting levels of rat growth hormone in plasma of rGH-treated hypophysectomized male rats

Plasma was collected at different time point after an intra-atrial injection of 40, 4, or 0.4 μg of rGH/kg b.wt., and resulting hormone levels were determined as described under *Materials and Methods*. Values are presented as the mean ± S.D. of two observations.

Time	rGH Dose (μg/kg b.wt.)		
	40	4	0.4
	ng/ml		
0 min	0	0	0
5 min	271 ± 23	29 ± 7	3 ± 1[a]
15 min	109 ± 11	15 ± 3	0
30 min	32 ± 5	2 ± 2	0
45 min	22 ± 3	0	0
60 min	6 ± 1	0	0
120 min	0	0	0
180 min	0	0	0
240 min	0	0	0

[a] At the sensitivity limit of the assay (2–3 ng/ml).

Stat5B. Administration of rGH had little effect on the hepatic cytosolic concentrations of Stat5B during the 240-min observation period (Fig. 4, top). With the exception of a 50% decline in Stat5B levels 30 min after hormone exposure, transducer concentrations remained fairly constant. Moreover, there were no rGH dose effects on the kinetics of cytosolic Stat5B; all doses of rGH produced similar effects. rGH-induced changes in Stat5B in the postnuclear fraction were more dynamic than that observed in the cytosol. Within 5 min of hormone exposure, Stat5B concentrations increased, peaked at 15 min (3 to 4 times above zero time), and declined to baseline by 45 min (Fig. 4, middle). Whereas the 0.4- and 4-μg dose of rGH produced the same kinetic profile in postnuclei Stat5B levels, the higher 40-μg dose of rGH increased transducer concentrations ~35% above that observed at the lower hormone doses. In contrast to the cytosolic and postnuclear fractions, which contained substantial amounts of Stat5B before rGH treatment, there were no detectable levels of Stat5B in the nucleus before hormone infusion (Fig. 4, bottom). Within 5 min of rGH exposure, nuclear Stat5B increased most precipitously at the 4-μg dose. In fact, whereas Stat5B levels peaked between 30 and 45 min and declined to baseline sometime after 60 min in all treatment groups, the medium dose (4 μg/kg b.wt.) produced the fastest increase

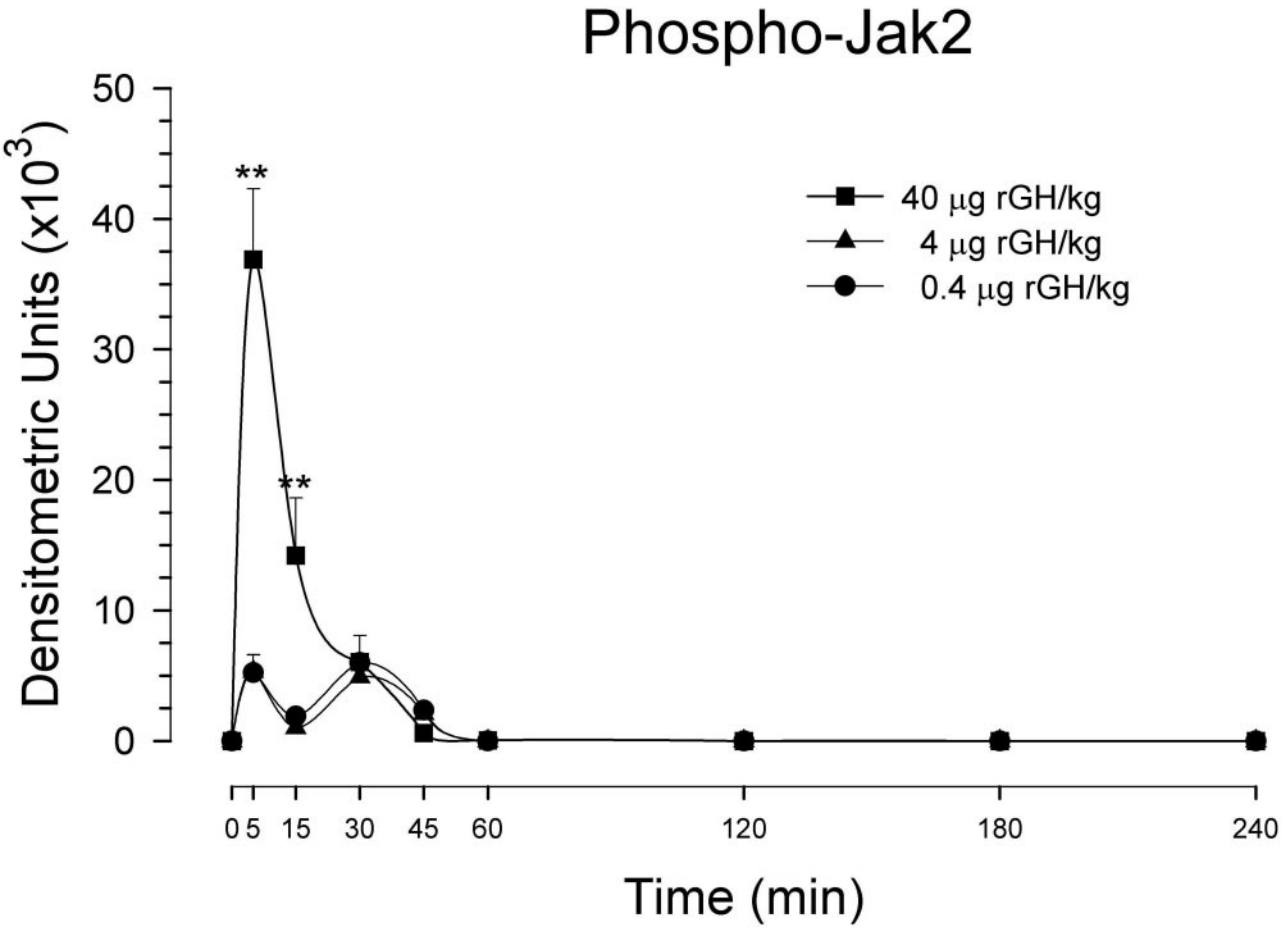

Fig. 2. Phospho-Jak2 levels in HYPOX rats i.v.-administered various pulse doses of rGH. HYPOX male rats were injected, via an intra-atrial catheter, with a single dose (i.e., 40, 4.0, or 0.4 μg of rGH/kg b.wt.) and euthanized at different time points between 0 and 240 min. Phospho-Jak2 was measured in the postnuclear fraction of livers using Western blot analysis as described under *Materials and Methods*. Values presented are the mean ± S.D. of at least four animals at each time point. **, $p < 0.01$ compared with the other two treatments at the same time point.

and greatest accumulation of Stat5B. Compared with the effects of the 4-μg rGH dose, nuclear Stat5B levels during this time period were ~25% lower in the hepatic nuclei from rats treated with the physiological 40-μg rGH dose and about ~50% less when 0.4 μg of the hormone was administered. Our results from immunoprecipitation experiments also confirmed the quantitative differences of activated Stat5B in the nuclei of rats infused with different GH pulse doses (Fig. 4, inset).

Phosphotyrosine Stat5. Because we found that nuclear accumulation of Stat5A and Stat5B was GH-dependent, presumably requiring activation (i.e., phosphorylation) and translocation from extranuclear sites (Argestisnger et al., 1993), we examined the subcellular distribution and kinetics of GH-regulated phosphotyrosine Stat5. (Unfortunately, antibodies against the phosphorylated Stat5A and Stat5B forms were not commercially available.) In contrast to our findings measuring Stat5A and Stat5B, cytosolic and postnuclear phosphotyrosine Stat5 were not detectable before GH treatment. Therefore, within 5 min of treatment, cytosolic levels of the activated transcription factor increased in amounts reflecting the dose of rGH (Fig. 5, top). Thereafter, irrespective of dose, cytosolic phosphotyrosine Stat5 peaked at 15 min, declined slowly, and at similar rates for the two lower rGH doses, to zero at 120 min. Cytosolic phosphotyrosine Stat5 reached a plateau between 15 and 60 min in rats treated with the highest, physiological hormone dose. Thereafter, concentrations declined to zero by 180 min. Thus, exposure to the 4- and 0.4-μg doses of rGH activated similar cytosolic levels of Stat5, which were significantly less (35 and 50%, respectively) than that produced by the 40-μg hormone dose. GH treatment activated postnuclear Stat5 in a typical bell-shaped kinetic curve. Phosphotyrosine Stat5 concentrations increased within 5 min of hormone infusion, peaked at 30 min, and declined to zero levels by 60 min (Fig. 5, middle). The 40- and 4-μg dose of rGH induced the same ($p > 0.07$) activational profile, whereas the lower, 0.4-μg hormone dose stimulated the phosphorylation of ~60% less Stat5. The

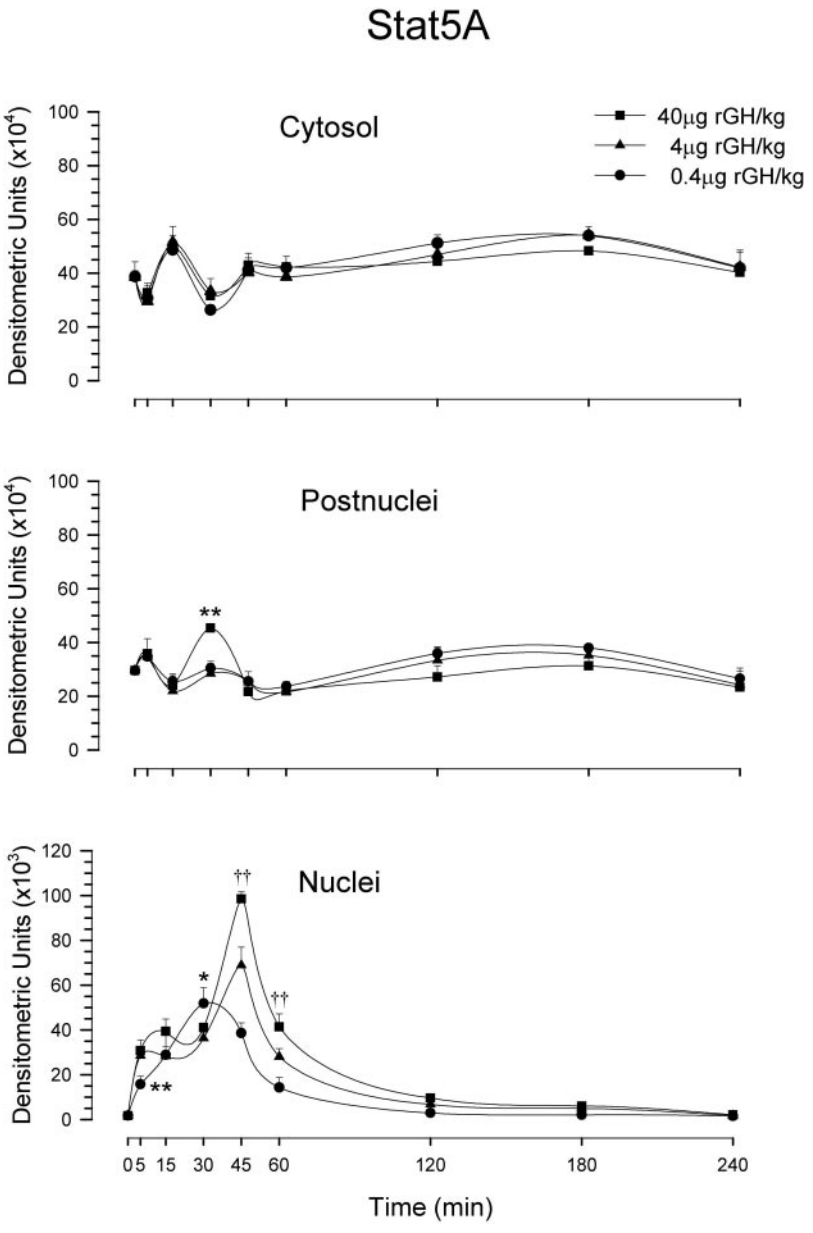

Fig. 3. Total Stat5A levels in the hepatic subcellular fractions of HYPOX rats i.v. administered various pulse doses of rGH. HYPOX male rats were injected, via an intra-atrial catheter, with a single dose of rGH (i.e., 40, 4.0, or 0.4 μg of rGH/kg b.wt.) and euthanized at specific time points between 0 and 240 min. Stat5A was measured using Western blot analysis as described under *Materials and Methods*. Values presented are the mean ± S.D. of at least four animals at each time point. *, $p < 0.05$ and **, $p < 0.01$ compared with the other two treatments at the same time point. ††, $p < 0.01$ when all values are significantly different from each other at the same time point.

amount and speed at which the activated transcription factor accumulated in the nucleus was greatest when rats were injected with 4 μg of rGH/kg b.wt. (Fig. 5, bottom). Thirty minutes after rGH treatment, nuclei exposed to 4 μg of the hormone contained more than twice as much phosphotyrosine Stat5 as the other doses. Although activated Stat5 slowly peaked at 60 min after 40 μg of rGH and at 15 min after 0.4 μg of rGH, the activated transcription factor was no longer detectable in all treatment groups by 120 min. Similar to nuclear Stat5B, total accumulation of nuclear phosphotyrosine Stat5 during the 2-h post-treatment period was greatest after 4 μg of rGH, ~20% less after the physiological 40 μg of rGH and 40 to 50% less after 0.4 μg of rGH.

Phospho-ERK1 and Phospho-ERK2. Activated-MAP kinase is composed of both a 44-kDa protein phospho-ERK1 and a 42-kDa protein phospho-ERK2 that was quantified separately (Fig. 6). Cytosolic phospho-ERK1 and phospho-ERK2 responded similarly to rGH treatment. The physiological rGH dose of 40 μg/kg b.wt. stimulated a rapid and dramatic elevation (~4 fold) in both forms of activated ERK1 and ERK2 in the cytosolic fraction within 5 min of hormone treatment, which fell to baseline by 15 min, where it remained until the conclusion of the study at 240 min. Exposure to the 4-μg dose of rGH induced a slower rise in cytosolic phospho-ERK1 and phospho-ERK2 that peaked after 30 min and permanently declined to baseline at 45 min (Fig. 6, left). The area under the curves indicated that the 4-μg dose resulted in a 3-fold greater accumulation of the transducers than the 40-μg dose. Compared with the two higher rGH doses, 0.4 μg of hormone stimulated a nominal and transient increase of activated ERK1 and ERK2 levels in cytosol. Postnuclear changes in the activated MAP kinase components reflected the changes observed in the cytosol. The 40-μg dose of rGH induced a rapid and short-lived peak of phospho-

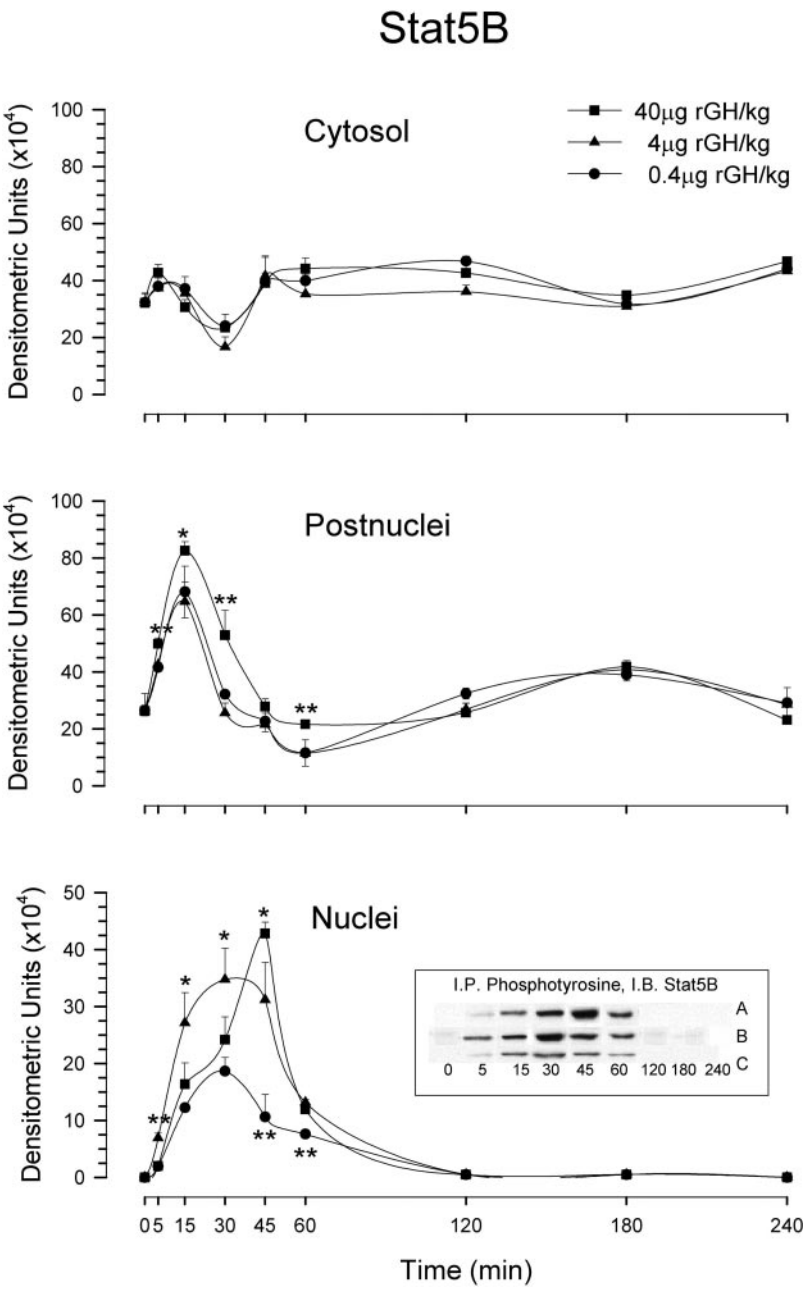

Fig. 4. Total Stat5B levels in hepatic subcellular fractions of HYPOX rats i.v. administered various pulse doses of rGH. HYPOX male rats were injected, via an intra-atrial catheter with a single dose of rGH (i.e., 40, 4.0, or 0.4 μg/kg b.wt.) and euthanized at specific time points between 0 and 240 min. Stat5B was measured using Western blot analysis as described under *Materials and Methods*. Values presented are the mean ± S.D. of at least four animals at each time point. *, $p < 0.05$ and ** $p < 0.01$ compared with the other two treatments at the same time point. Inset, representative blots for the expression levels of activated Stat5B in hepatic nuclei. Phosphotyrosine immunoprecipitates (IP) were immunoblotted (IB) with Stat5B antibody. Treatments were 40 μg/kg b.wt. (A), 4 μg/kg b.wt. (B), and 0.4 μg/kg b.wt. (C) of rGH treatment.

ERK1 and phospho-ERK2 that lasted only 15 min. In contrast, 4 μg of the hormone stimulated a slowly ascending peak that reached its apex at 15 min and declined to baseline at ~60 min (Fig. 6, right). Because of the duration of its peak, the medium dose of rGH resulted in a greater accumulation of the transducer in the postnuclear membrane fraction than did the 40-μg dose. Again, 0.4 μg of rGH induced a very small elevation in the activated MAP kinase components that seemed slightly greater for phospho-ERK2 than phospho-ERK1 (cytosolic as well as postnuclear fraction). Similar to Stat5A and Stat5B, there were measurable concentrations of both activated forms (i.e., ERK1 and ERK2) in cytosolic and postnuclear fractions even before rGH administration (i.e., zero time). We were unable to detect quantifiable levels of either phospho-ERK1 or phospho-ERK2 in any of the hepatic nuclear fractions.

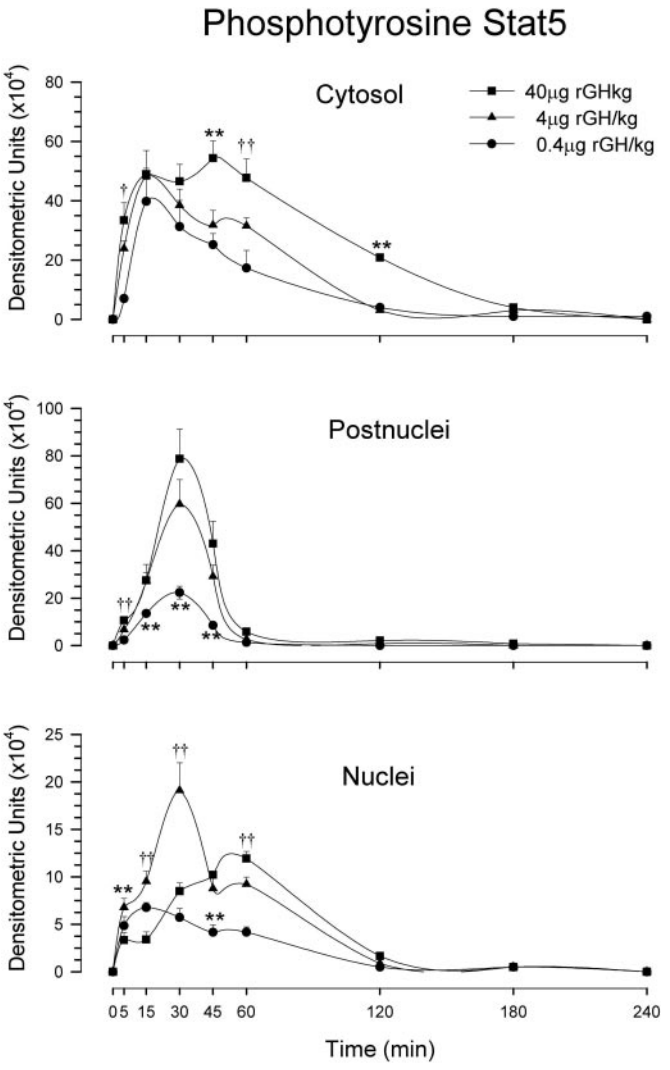

Fig. 5. Phosphotyrosine Stat5 levels in hepatic subcellular fractions of HYPOX rats i.v. administered various pulse doses of rGH. HYPOX male rats were injected, via an intra-atrial catheter with a single dose of rGH (i.e., 40, 4.0, or 0.4 μg/kg b.wt.) and euthanized at various time points between 0 and 240 min. Phosphorylated Stat5 was measured using Western blot analysis as described under *Materials and Methods*. Values presented are the mean ± S.D. of at least four animals at each time point. **, $p < 0.01$ compared with the other two treatments at the same time point. †, $p < 0.05$ and ††, $p < 0.01$ when all values are significantly different from each other at the same time point.

CYP2C11 mRNA. Using reverse transcription-PCR, we investigated the possible induction of CYP2C11 transcription by a single pulse of rGH administered to long-standing HYPOX male rats (Fig. 7). Restoration of a single physiological pulse of plasma GH (40 μg/kg b.wt.) did not increase CYP2C11 expression. In contrast, the lower 4-μg dose of rGH known to be a more effective inducer of CYP2C11 mRNA than the physiologic pulse (Fig. 1), stimulated a small (~20%), but significant ($p < 0.05$) increase in the transcript 240 min after exposure to the hormone. The 0.4-μg dose of rGH inexplicably induced a similar percentage of decline in CYP2C11 mRNA levels.

Discussion

The anterior pituitary secretes about a half-dozen hormones of which GH predominates; comprising ~50% of the total hormone content of the gland. Therefore, GH effects nearly every cell type in the body by regulating the activation and/or suppression of metabolic proteins and the transcription of structural proteins characteristic of the responsive cells (Smith and Thorner, 2000). Whereas GH secretion has been found to be pulsatile in all species examined, the pattern can be further differentiated according to sex. In the case of mammals (e.g., rats, mice, and humans), the masculine secretory plasma GH profile is defined as "episodic" because of its prolonged GH-devoid interpulse periods. In contrast, the so-called "continuous" feminine GH profile is characterized by considerably briefer (2–5-fold) interpulses between peaks, which in the case of the rat, always contains measurable, albeit low, concentrations of GH (Shapiro et al., 1995). This sex difference in the GH secretory profile is responsible for numerous phenotypic sexual dimorphisms ranging from growth rates to metabolic functions (Smith and Thorner, 2000). Regarding the latter, sex differences in the circulating GH profile regulate the sexually dimorphic expression of both rat and murine hepatic isoforms of cytochrome P450 (Legraverend et al., 1992; Shapiro et al., 1995). For example, episodic secretion of GH induces expression of CYP2C11, the predominant isoform found in male rat liver. In contrast, exposure to the continuous GH profile characteristic of the female rat, completely suppresses expression of the isoform. Whereas GH can induce its diverse effects by activating the MAPK pathway, the hepatic nuclear factor pathway, the phosphatidylinositol pathway, the protein kinase C pathway, and/or the Jak/Stat signal transduction pathway, it is the latter pathway that seems to mediate the actions of GH-dependent functions (Herington, 1994; Carter-Su et al., 1996; Lahuna et al., 1997; Waxman and Frank, 2000). Findings using transfected nonhepatocytes, the immortalized CWSV-1 hepatocyte-derived cell line, Stat5A/Stat5B knockout mice, and intact rats (Park et al., 1999; Choi and Waxman, 2000; Waxman and Frank, 2000) indicate, as described in Introduction, that episodic GH mediates its effects, from the very first pulse by activating (phosphorylating) Jak2, which in turn results in the activation of Stat5B. The phosphorylated Stat5B forms a homodimer and translocates to the nucleus where it stimulates transcription of episodic GH-regulated genes. Although not directly examined, the above-mentioned findings have understandably lead to the assumption that episodic GH also regulates

CYP2C11 expression through activation of the Jak2/Stat5B pathway (Choi and Waxman, 2000).

The present findings, however, raise concerns regarding the role of this transduction pathway in regulating CYP2C11 expression. As expected (Waxman and Frank, 2000), restoration of the physiological rGH plasma pulse results in an immediate and dramatic accumulation of activated hepatic Jak2 within 5 min of exposure to the hormone. Thereafter, concentrations of the transducer rapidly decline to undetectable levels in ~45 min. As observed previously (Pampori and Shapiro, 1994; Agrawal and Shapiro, 2000), whereas infusion of this physiological rGH dose (6 pulses/d for 6 days) restores normal-like expression levels of CYP2C11, a similar regimen infusing rGH at only 10% of the physiological pulse amplitude induces a considerable overexpression of the male-specific isoform. In fact, 10% of the normal dose was the only replacement rGH concentration to induce a significant increase in CYP2C11 mRNA levels after exposure to just a single pulse. Nevertheless, this overly effective CYP2C11 induction dose of rGH activated barely detectable levels of Jak2. In fact, Jak2 kinetics induced by rGH pulse amplitudes 10% of normal were indistinguishable from that induced by pulse amplitudes only 1% of physiological and having no effect on CYP2C11 expression. Although these results clearly raise questions regarding the importance of Jak2 activation in mediating GH induction of CYP2C11, it is possible that other requisite events in the transduction pathway, probably downstream from Jak2, were more responsive to the 10% rGH pulse than the 1% dose or for that matter, the physiological (100%) pulse amplitude.

Although we did not measure directly phosphorylated Stat5B (there is no commercially available antibody) in all samples, we believe that our findings of nuclear Stat5B reflect activated levels. First, it has been reported previously (Carter-Su et al., 1996) that for GH to stimulate the translocation of Stat5B to the nucleus, the transducer has to be phosphorylated. In general, nonphosphorylated Stat5B is not found in the nucleus of the unstimulated hepatocyte (Ram et al., 1996). In agreement, we found no detectable concentrations of nuclear Stat5B at zero time. Second, rat liver contains 10 to 20 times more Stat5B than Stat5A (Choi and Waxman, 1999) suggesting that the phosphotyrosine Stat5 levels were most representative of the B form. Indeed, the rGH dose-response curve for nuclear phosphotyrosine Stat5 reflected nuclear levels of Stat5B. Last, we had limited nuclear extract to perform representative immunoprecipitations measuring phosphotyrosine Stat5B and found the result to be in agreement with nuclear Stat5B levels.

Although the 4 μg/pulse/kg b.wt. dose of rGH (restoring the circulating hormonal pulse amplitude to 10% of normal)

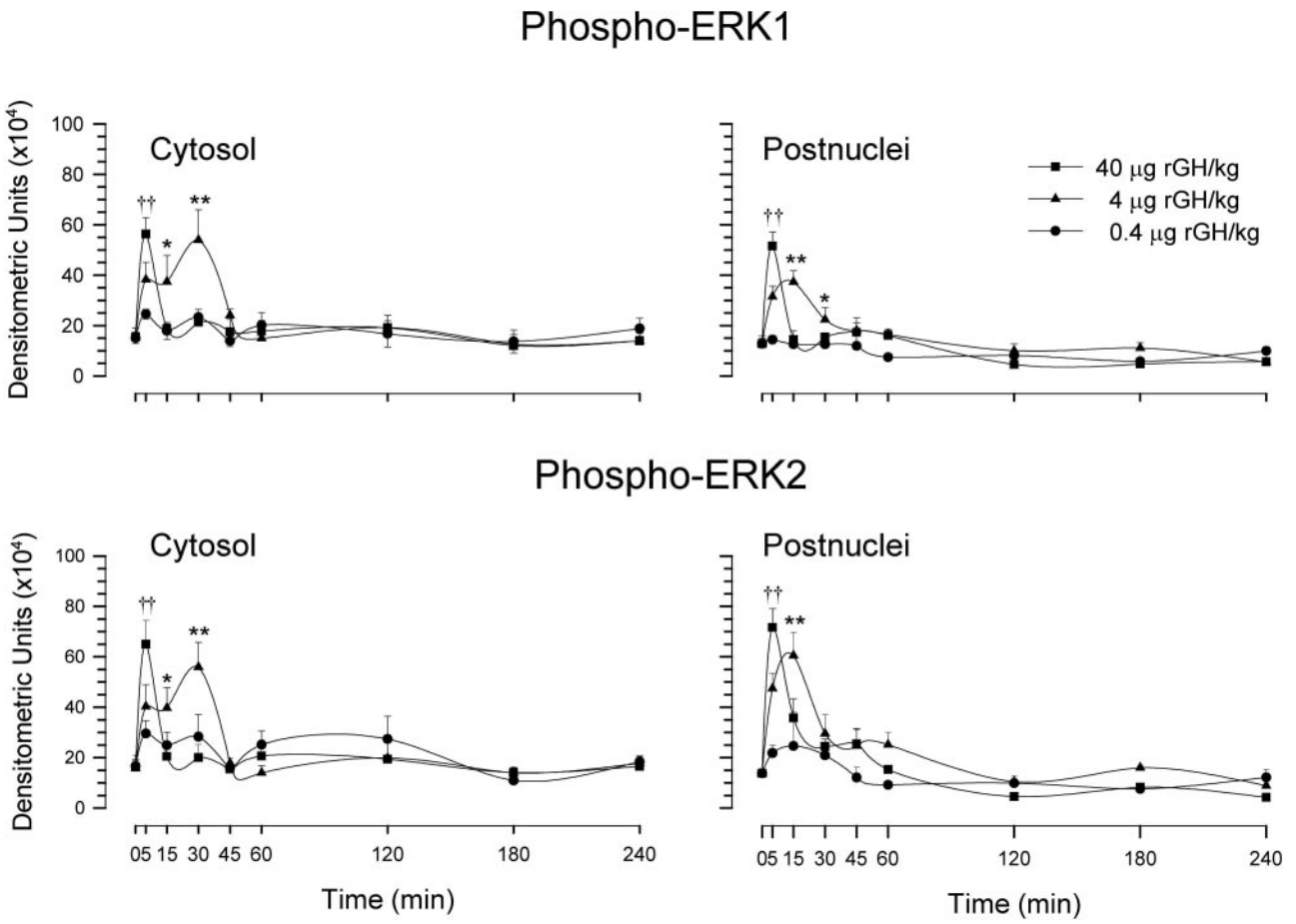

Fig. 6. Phospho-ERK1 and phospho-ERK2 levels in hepatic subcellular fractions of HYPOX rats i.v. administered various pulse doses of rGH. HYPOX male rats were injected, via an intra-atrial catheter with a single dose of rGH (i.e., 40, 4.0, or 0.4 μg/kg b.wt.) and euthanized at various time points between 0 and 240 min. Phospho-ERK1 and phospho-ERK2 were estimated using Western blot analysis described under *Materials and Methods*. Values presented are the mean ± S.D. of at least four animals at each time point. *, $p < 0.05$ and **, $p < 0.01$ compared with the other two treatments at the same time point. ††, $p < 0.01$ when all values are significantly different from each other at the same time point.

stimulated a more rapid elevation and greater total accumulation of nuclear Stat5B than the physiological pulse, the differences were not impressive and were probably within biological variability (Choi and Waxman, 2000). A comparison of peak and total accumulation of nuclear Stat5B revealed a 15-min and <25% difference, respectively, between the two doses of rGH. It is difficult to envision how these small differences in Stat5B kinetics could explain a 200 to 300% overexpression of CYP2C11 mRNA (Pampori and Shapiro, 1994; Agrawal and Shapiro, 2000) induced by the 10% dose of rGH. Equally perplexing were the findings using the 0.4 μg of rGH/pulse/kg b.wt. to renaturalize the circulating masculine episodic profile with a pulse amplitude only 1% of normal. This barely monitorable dose, unable to effect CYP2C11 expression, was 70% as effective activating and translocating Stat5B as the physiological pulse. Although it has been suggested that there may be a threshold level of nuclear phosphorylated Stat5B required to initiate episodic GH-dependent transcription, 70% is considerably above the empirical minimum (Choi and Waxman, 2000). Considering that GH binds to its receptor with a K_d value of 0.1 nM (2.2 ng/ml) (Fuh et al., 1992), it is not surprising that a dose of GH (i.e., 1% of normal) producing plasma concentrations of ~3 ng/ml is capable of activating and translocating Stat5B. However, it is unclear why such high levels of the transducers were unable to initiate CYP2C11 transcription.

As discussed above, GH action can be mediated by numerous signal transduction pathways, all probably interacting at various levels. Therefore, the only signal transducer measured in our study unaffected by the 1% GH dose was phospho-ERK1; phospho-ERK2 was only nominally elevated by the treatment. In fact, the intermediate rGH dose (10% of normal) stimulated a greater (~2–3-fold) accumulation of phospho-ERK1 and phospho-ERK2 in both hepatic cytosol and membranes than the physiological replacement pulse. Although GH can activate Stat5A (Pircher et al., 1997; Park et al., 1999), and unlike Stat5B, it contains a C-terminal putative MAPK phosphorylation site (Smit et al., 1997), its response to the different rGH replacement doses was similar. Of course, it is possible that MAPK (ERK1 and/or ERK2) is involved in GH regulation of CYP2C11 expression through other signal transduction pathways independent of Jak/Stat (Herington, 1994).

Perhaps the most damaging evidence concerning a requisite role for the Jak/Stat pathway in regulating CYP2C11 expression can be found in the HYPOX rat. In the absence of any GH replacement, we, in agreement with others (Ram et al., 1996), observed no detectable activation of hepatic Jak2, Stat5B, or Stat5A. Nevertheless, the HYPOX rat expresses 25 to 35% of normal levels of CYP2C11 mRNA and protein. Of course, the Jak/Stat pathway may only be involved when GH regulates CYP2C11 expression, but the present observations question the unique role of the signal transduction pathway in mediating episodic GH-dependent CYP2C11 expression and raise the possibility that MAPK, which is activated in the absence of GH in HYPOX rats, is involved in CYP2C11 expression.

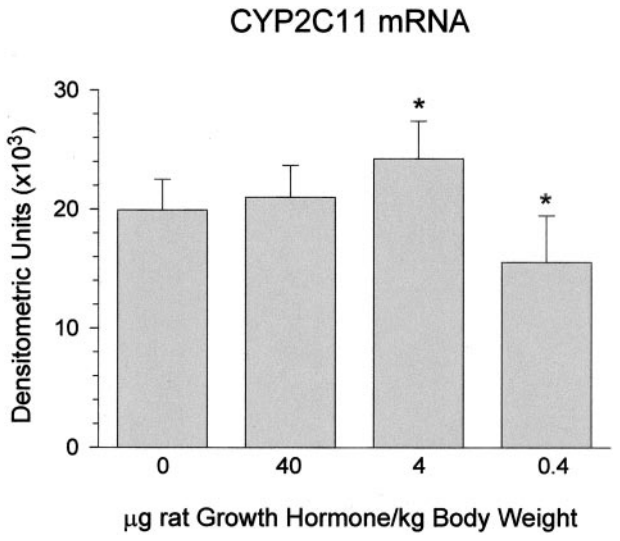

Fig. 7. Hepatic CYP2C11 mRNA expression levels in HYPOX rats i.v. administered various pulse doses of rGH. HYPOX male rats were injected, via an intra-atrial catheter, with a single dose of 40, 4, or 0.4 μg of rGH/kg b.wt. Expression of CYP2C11 mRNA was measured after 0 min (i.e., zero dose) or 240 min of rGH treatment by using semiquantitative reverse transcription-PCR as described under *Materials and Methods*. Expression levels of CYP2C11 mRNA were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA expression levels. Values are presented as the mean ± S.D. of 12 control animals (i.e., 0 dose) and at least four animals for each rGH treatment. *, $p < 0.05$ compared with the control (i.e., 0 dose) treatment group.

Acknowledgments

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