Noncanonical suppression of GH-dependent isoforms of cytochrome P450 by the somatostatin analog octreotide

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Abstract

Octreotide is a potent somatostatin analog therapeutically used to treat several conditions including hyper GH secretion in patients with acromegaly. We infused, over 30 s, octreotide into male rats every 12 h for 6 days at levels considerably greater than typical human therapeutic doses. Unexpectedly, the resulting circulating GH profile was characterized by pulses of higher amplitudes, longer durations, and greater total content than normal, but still contained an otherwise male-like episodic secretory profile. In apparent disaccord, the normally elevated masculine expression levels (protein and/or mRNA) of CYP2C11 (accounting for > 50% of the total hepatic cytochrome P450 content), CYP3A2, CYP2C7, and IGF1, dependent on the episodic GH profile, were considerably downregulated. We explain this contradiction by proposing that the requisite minimal GH-devoid interpulse durations in the masculine profile that solely regulate expression of at least CYP2C11 and IGF1 may be sufficiently reduced to suppress transcription of the hepatic genes. Alternatively, we observed that octreotide infusion may have acted directly on the hepatocytes to induce expression of immune response factors postulated to suppress CYP transcription and/or upregulate expression of several negative regulators (e.g. phosphatases and SOCS proteins) of the JAK2/STAT5B signaling pathway that normally mediates the upregulation of CYP2C11 and IGF1 by the masculine episodic GH profile.

Key Words

- ▶ cytochrome P450
- ▶ CYP2C7
- ▶ CYP2C11
- ▶ CYP3A2
- ▶ growth hormone
- ▶ IGF1
- ▶ JAK2
- ▶ octreotide
- ▶ sexual dimorphisms
- ▶ somatostatin
- ▶ STAT5B

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Introduction

Octreotide is a potent somatostatin analog most commonly used to reduce blood levels of GH and insulin-like growth factor-1 (IGF1), also known as somatomedin C, in acromegaly patients (Lamberts *et al.* 1993, Yang & Keating 2010). Whereas a single dose of the drug to humans (Marbach *et al.* 1985) or rats (Turner & Tannenbaum 1995) can profoundly reduce plasma GH concentrations for many hours, the therapeutic goal, however, is to

achieve normalization of GH and IGF1 levels in patients (Lamberts *et al.* 1993, Yang & Keating 2010). Although far from conclusive, it has been reported that while octreotide infusion can reduce circulating GH concentrations by 50%, the pulse frequency, characteristic of men, remains undisturbed (Dimaraki *et al.* 2003), suggesting normal expression of GH profile-responsive genes.

In addition to regulating IGF1 expression, GH regulates expression of the constituent cytochromes P450 (CYP) in rat liver as well as in every other species examined. In fact, the sex differences in CYP expression exhibited in rat, mice, and humans appear to be solely regulated by sex differences in secretory GH profiles (Shapiro *et al.* 1995, Dhir *et al.* 2006). Whereas males and females secrete similar daily amounts of GH, females secrete a so-called 'continuous' GH profile comprising numerous daily pulses interrupted by short-lived interpulses of usually low or barely detectable hormone concentrations. By contrast, the 'episodic' masculine GH profile is characterized by significantly fewer secretory bursts of the hormone separated by lengthy interpulses that are invariably devoid of GH. In fact, it is the difference between the continuous (female) and episodic (male) circulating GH profiles and not plasma hormone levels *per se* that are responsible for phenotypic sexual dimorphisms ranging from growth patterns to expression levels of hepatic enzymes (Jansson *et al.* 1985, Shapiro *et al.* 1995).

In the rat, CYP responses to GH regulation are nearly as variable as the number of GH-dependent isoforms. For example, expression of the major female-specific CYP2C12 is dependent on the feminine profile of continuous GH secretion exhibiting measurable hormone levels throughout the interpulses. Exposure to the masculine profile of episodic GH release characterized by GH-devoid interpulses completely prevents expression of CYP2C12 (Pampori & Shapiro 1996, Agrawal & Shapiro 2000). Expression of the major male-specific CYP2C11 requires the episodic 'on/off' masculine profile of secretion while the continuous feminine GH profile is completely suppressive (Pampori & Shapiro 1996, Agrawal & Shapiro 2000). Basically then, it is the continuous exposure to GH, even at concentrations 95% below normal, that induces CYP2C12 expression (Pampori & Shapiro 1996). By contrast, it is a minimum GH-devoid period separated by detectable pulses of as little as 5% of normal that signal CYP2C11 expression (Pampori & Shapiro 1994, Agrawal & Shapiro 2001). Another example of a CYP isoform regulated by yet a different component in the GH profile is male-specific CYP3A2. Unlike CYP2C11, CYP3A2 is maximally expressed in the GH-ablated (e.g. hypophysectomized) rat, disappears when GH is secreted continuously, but is only minimally suppressed under the influence of episodic GH (Waxman *et al.* 1991, Pampori & Shapiro 1996). One more example from several other possibilities is female-predominant CYP2C7 whose maximal expression is dependent on the normal feminine continuous GH profile and is completely suppressed in

the GH-ablated rat (Pampori & Shapiro 1996). Exposure to the masculine episodic GH profile allows expression of CYP2C7 at 25–40% of normal female levels, but the profile must not stray from the physiological, i.e. no alterations in the pulse and interpulse durations, frequencies, and concentrations (Agrawal & Shapiro 2000, 2001).

As each CYP isoform is exquisitely sensitive to the inductive effects of a different component in the secretory GH profile, we measured expression levels of key CYP isoforms as indicators or markers of possibly subtle but physiologically important abnormalities in the GH profiles resulting from octreotide infusion.

Materials and methods

Animals

Male Sprague–Dawley CD rats obtained from Charles River Laboratories (Wilmington, MA, USA) were housed in the University of Pennsylvania Laboratory Animal Resources facility under the supervision of certified Laboratory Animal Medicine veterinarians and were treated according to a research protocol approved by the University's Institutional Animal Care and Use Committee. Rats were housed under conditions of regulated temperature (20–23 °C) and photoperiod (12 h light:12 h darkness cycle; lights on at 0700 h).

Catheter implantation and octreotide treatment

Eight male rats at 10 weeks of age were implanted with our patented indwelling right atrial catheters as described previously (MacLeod & Shapiro 1988, Pampori *et al.* 1991). After 4–5 days, serial blood samples were collected from each rat (40 µl every 15 min for 8 h). Another 4–5 days later, the unrestrained and unstressed catheterized rats were infused, i.v., with 25 µg octreotide (Sandostatin)/kg body weight (Bioniche Pharma, Rosemont, IL, USA) over 30 s and the catheter flushed with diluent. Five minutes later, serial blood samples were again collected from each rat (40 µl every 15 min for 8 h). Twelve hours after the first injection of octreotide, a second 25 µg/kg dose was given as an i.v. infusion over 30 s followed by flushing the catheter. During the next 5 days, rats were treated with octreotide at the same dose every 12 h. Five minutes following the 11th dose of octreotide, serial blood samples were again collected (40 µl every 15 min for 8 h). Twelve hours after the previous dose, the rats received their final evening infusion of octreotide. On the following morning (10 h post-infusion), the rats were quickly decapitated, the

livers quickly excised, weighed, infused with ice-cold physiological saline, and minced; a portion reserved for mRNA determinations was plunged into liquid nitrogen and subsequently stored at -70°C . The remaining minced liver was used for microsome preparation. In an additional experimental group, seven males (10 weeks of age) were implanted with indwelling right atrial catheters and treated with an equivalent amount of diluent (95 μl of physiological saline/infusion) in the same manner as octreotide for 12 infusions. These rats were decapitated 10 h after the last treatment to serve as controls for the biochemical analyses.

GH

Plasma GH was measured using a sensitive sandwich ELISA modified from Steyn *et al.* (2011). A 96-well Costar high binding, type 1, polystyrene EIA/RIA Stripwell flat bottom plate (Corning, Inc., Corning, NY, USA) was coated with 50 μl capture antibody (National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)-anti-rat GH (rGH)-S-5 (monkey), AFP 411S; NIDDK-National Hormone and Pituitary Program, NHPP, Torrance, CA, USA) at a final dilution of 1:40 000 and incubated overnight at 4°C . To reduce nonspecific binding, each well was subsequently incubated for 2 h at room temperature with 100 μl blocking buffer (5% skim milk powder in 0.05% PBS with Tween-20 (PBS-T, 0.05%)) followed by three careful washings for 3 min each time with 100 μl 0.05% PBS-T. A standard curve was generated using a serial dilution of rGH reference preparation-2 (rGH-RP-2; AFP-3190B, NIDDK-NHPP). After 2 h of incubation with samples (50 μl of a 1:60 dilution; plasma: diluent), three careful washings were performed with 0.05% PBS-T for 2 min at each time. The bound samples were then incubated with 50 μl detection antibody (anti-rGH A.S. (rabbit antiserum to rGH, AFP5672099Rb); NIDDK-NHPP) at a final dilution of 1:40 000 for 2 h followed by three careful washings for 2 min each time with 100 μl 0.05% PBS-T. The bound complex was incubated for 90 min with 50 μl HRP-conjugated antibody (anti-rabbit, IgG; Amersham, GE Healthcare Bio-sciences Corp., Piscataway, NJ, USA) at a final dilution of 1:2000 followed by three careful washings for 3 min each time with 100 μl 0.05% PBS-T. Addition of 100 μl O-phenylenediamine (Life Technologies, Grand Island, NY, USA) substrate to each well resulted in an enzymatic colorimetric reaction. This reaction was stopped after 30 min by addition of 50 μl 3 M HCl and the absorbance was read at a wavelength of 490 nm with a M4 microplate reader (Molecular Device, Sunnyvale,

CA, USA). The concentration of rGH in each well was calculated by regression of the standard curve with hypophysectomized male rat plasma used as an appropriate blank and also subtraction of background OD at 650 nm from each reading for the whole assay.

RNA isolation

Total RNA from liver tissue was isolated using Trizol reagent (Life Technologies) purified with the Qiagen RNeasy mini kit and treated with DNase I in order to remove any trace of genomic DNA using RNase-Free DNase Set (Qiagen) according to the manufacturer's protocol. RNA concentrations and purity were determined by u.v. spectrophotometry ($A_{260}/280 > 1.8$ and $A_{260}/230 > 1.7$) and integrity was verified by the intensities of 28S and 18S rRNA bands on a denaturing agarose gel visualized on a FluorChem IS-8800 Imager (Alpha Innotech, San Leandro, CA, USA).

Northern blots

RNA samples (20 μg) were resolved on denaturing 1% agarose gels and transferred onto Nytran N filters from Schleicher and Schuell (Keene, NH, USA). The northern blots were probed with a γ - ^{32}P -labeled oligonucleotide probe for *Cyp2C11* and *Cyp2C12* mRNA (Waxman 1991) using hybridization and high-stringency washing conditions as described previously (Pampori & Shapiro 1996). The consistency of RNA loading between samples was confirmed by ethidium bromide staining of 18S and 28S ribosomal RNAs and was verified using an 18S oligonucleotide probe (Li *et al.* 2000). The hybridized mRNA signals were quantified by scanning the autoradiographs with a FluorChem IS-8800 Imager (Alpha Innotech). The mRNA signals were normalized to the 18S rRNA signals in each lane and demonstrated a mean variation of only $\pm 5\%$, indicating that results were independent of loading errors.

Quantitative RT-PCR

Cyp2C11, *Cyp3A2*, *Cyp2C6*, and *Cyp2C7* gene expressions were determined by quantitative RT-PCR (qRT-PCR) using SYBR green on an Applied Biosystem 7500 Fast Real-Time PCR System (Life Technologies). RNA isolation, concentration, and purity determination were performed as mentioned earlier. cDNA synthesis was completed using the High-Capacity RNA-to-cDNA kit (Life Technologies) as per instructions with appropriate no-RT (–RT) and

nontemplate controls. PCR primers for *Cyp2C11* (Ahluwalia *et al.* 2004), *Cyp3A2* (Kisanga *et al.* 2005), *Cyp2C6* (F: 5'-TCAGCAGGAAAACGGATGTG-3', R: 5'-AATCGTG-GTCAGGAATAAAATAACTC-3'), *Cyp2C7* (Choi *et al.* 2011), and β -actin (F: 5'-CACGGCATTGTCACCAACTG-3', R: 5'-CTGGGTCATCTTTTCACGGT-3') were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). To analyze *Igf1* mRNA expression, an IGF1 TaqMan assay (Rn00710306_m1) was performed using β -actin (Rn00667869_m1) as the housekeeping gene on an Applied Biosystem step-one plus q-PCR instrument as per the manufacturer's recommended protocol (Life Technologies).

Quantitative real-time PCR array

Changes in the expression of genes involved in the JAK/STAT signaling pathway in normal and octreotide-treated rats were determined using real-time PCR array (Rat RT² Profiler PCR Array; SA Biosciences, Qiagen). Isolation, concentration, and purity of total RNA from rat liver were performed as mentioned earlier. The first cDNA strand was synthesized using an RT² First-Strand Kit (SA Biosciences). The cDNA was then applied to the RT² Profiler PCR Array for the JAK/STAT pathway (PARN-039C) according to the manufacturer's guidelines. The qRT-PCRs were run on the Step-one plus q-PCR instrument (Life Technologies) and the data were analyzed using the online RT² Profiler PCR Array Data Analysis (SA Biosciences) program as per instructions.

Western blotting

Hepatic microsomes were prepared from freshly isolated rat livers (Shapiro *et al.* 1989) and then assayed for individual CYP isoforms by western blotting (Pampori *et al.* 1995). STAT5B, phospho-STAT5, and phospho-STAT5B were determined using the cytosolic and/or nuclear fractions (Verma *et al.* 2005). Briefly, 10 μ g microsomal protein, 20 μ g cytoplasmic, or 20 μ g nuclear protein was electrophoresed on 0.75 mm-thick SDS-polyacrylamide (10–12%) gels and electroblotted onto nitrocellulose membranes. The blots were probed with monoclonal anti-rat CYP2C11, anti-rat CYP3A2 (Detroit R&D, Inc., Franklin, MI, USA), anti-rat CYP2C12 (a gift from Dr Marika Rönnholm, Huddinge University Hospital, Huddinge, Sweden), monoclonal anti-rat CYP2B1/B2 (Oxford Biomedical Research, Oxford, MI, USA), monoclonal anti-rat CYP2C6 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-rabbit STAT5B (BD Biosciences,

San Jose, CA, USA) for immunoprecipitation, or anti-mouse STAT5B (Santa Cruz Biotechnology, Inc.), anti-rabbit phospho-STAT5 (Cell Signaling Technology, Inc., Danvers, MA, USA), anti-rabbit phosphotyrosine (EMD Millipore, Billerica, MA, USA) and detected with an enhanced chemiluminescence kit (Amersham, GE Healthcare Bio-sciences Corp.). Signals were normalized to a control sample repeatedly run on all blots and/or to the expression of β -actin (Sigma Chemical Co.). The protein signals were scanned and the densitometric units were obtained as integrated density values quantitated using a FluorChem IS-8800 Imager (Alpha Innotech) software supplied with the gel documentation system.

Testosterone metabolism

The metabolic products of 2 α -hydroxylase (2 α -hydroxytestosterone) and 6 β -hydroxylase (6 β -hydroxytestosterone), indicative of activity levels of CYP2C11 and CYP3A2 proteins respectively (Waxman 1991), were assayed by our previously published method (Agrawal *et al.* 1995a).

Statistical analysis

The ultradian patterns in plasma GH concentrations were analyzed with the aid of the Cluster analysis computer program (Veldhuis & Johnson 1986), as we have reported previously (Agrawal *et al.* 1995b, Dhir & Shapiro 2003). All data, including that obtained from the Cluster analysis program, were subjected to ANOVA and differences were determined using *t* statistics and the Bonferroni procedure for multiple comparisons. For data obtained from PCR array, the *P* values were calculated based on a Student's *t*-test of the replicate $2^{(-\Delta Ct)}$ values for each gene in the control group and treatment groups according to online RT² Profiler PCR Array Data Analysis (<http://pcrdata.analysis.sabiosciences.com/pcr/arrayanalysis.php>).

Results

Circulating GH profile

Plasma GH profiles are presented as schematic representations of the actual circulating profiles (Fig. 1) as well as mathematical analyses of the characteristic components in the profile (Table 1). As controls, the rats secreted fairly 'broad' pulses every 3 h separated by undetectable interpulse concentrations of the hormone somewhat typical of these young adult male rats (Agrawal *et al.* 1995b)

Figure 1

Plasma levels of circulating GH obtained from individual undisturbed catheterized octreotide-infused male rats. Every rat was fitted with a chronic indwelling right atrial catheter for serial blood sampling (MacLeod & Shapiro 1988, Pampori *et al.* 1991). Four to five days following catheter placement, serial blood samples were collected over 8 consecutive h at 15-min intervals (controls). Four to five days later, every rat was injected,

i.v., with 25 µg octreotide/kg bd wt and 5 min later, serial blood samples were again collected. Next, the rats were injected, i.v., every 12 h with the same dose of octreotide for a total of 11 doses. Five minutes following the last injection, serial blood collections were again obtained for 8 h. Similar findings were obtained from six additional animals.

(In contrast to young male rats (~70 days old), males of 150 days of age or older typically secrete GH pulses of around 1 h or less duration (Agrawal *et al.* 1995b, Dhir & Shapiro 2003)). Exposure to a single dose of octreotide had no measurable effects on the GH profiles. When the control rats were infused with an additional ten doses of octreotide for a total of 6 days, the basic masculine GH profile (i.e. episodic pulses interrupted by GH undetectable interpulse periods) was still apparent. However, there was a significant increase in the durations (width),

amplitudes (height), and contents (area) of the GH pulses resulting in a ~35% elevation in the mean concentration of the circulating hormone. Although octreotide produced no calculated statistically significant reductions in the length of the interpulse (valley) periods, one should have expected that the significant increase in the pulse durations, with no change in the interpulse intervals, would have had to result in shorter interpulses, suggesting that the lack of a statistical difference in the interpulse lengths was a spurious finding.

Table 1 Analysis of circulating GH profiles in male rats serving as their own controls and subsequently treated with one dose of octreotide followed by ten additional doses. Every rat was fitted with a chronic indwelling right atrial catheter for serial blood sampling (MacLeod & Shapiro 1988, Pampori *et al.* 1991). Four to five days following catheter placement, serial blood samples were collected over 8 consecutive h at 15-min intervals. Four to five days later, every rat was injected, i.v., with 25 µg octreotide/kg bd wt and 5 min later, serial blood samples were again collected. Next, the rats were injected, i.v., every 12 h with the same dose of octreotide for a total of 11 doses. Five minutes following the last injection, serial blood collections were again obtained for 8 h. Data were analyzed with the aid of the Cluster analysis program for hormonal pulse detection (Veldhuis & Johnson 1986) according to peak interval, period between peaks; width, duration of GH pulses, or interpulse valleys; height, amplitude of hormone peaks; area, integrated area under GH pulses (concentration×duration); and nadir, mean baseline GH concentration. Mean concentration is calculated for the entire 8-h collection period. Values are mean ± s.d. with $n = 7$

Group	Number of doses	Mean concentration (ng/ml)	Peak interval (min)	Pulse			Valley	
				Width (min)	Height (ng/ml)	Area (µg/min per ml)	Width (min)	Nadir (ng/ml)
Control	0	49 ± 8	186 ± 20	105 ± 15	204 ± 18	9.5 ± 1.1	87 ± 17	ND
Octreotide	1	48 ± 7	187 ± 17	116 ± 20	220 ± 27	11.1 ± 1.6	86 ± 13	ND
Octreotide	11	65 ± 9*	202 ± 33	134 ± 13*	253 ± 29*	15.6 ± 2.2*	70 ± 18	ND

ND, not detectable (i.e. ≥ 0.02 ng/ml). * $P < 0.01$ compared with control treatment.

CYP expression

We determined the effects of octreotide on the expression levels of male-specific CYP2C11 and CYP3A2, female-specific CYP2C12, female-predominant CYP2C6 and CYP2C7, and the inducible isoforms CYP2B1 and CYP2B2 (Fig. 2). Six days of octreotide treatment resulted in a dramatic decline in CYP2C11 protein concentrations to ~20% of the control values while mRNA levels as determined by qRT-PCR were reduced to ~4% of normal. To confirm these unexpected results, we performed northern blotting on our samples and found, in agreement with qRT-PCR, a ~90% decrease in hepatic *Cyp2c11* mRNA in males treated with octreotide and in agreement with protein levels, we observed a 65% decline in CYP2C11-dependent testosterone 2 α -hydroxylase in treated rats. Expression levels of CYP3A2, another major male-specific isoform, were similarly reduced by octreotide. CYP3A2 protein, catalytic activity, and mRNA were expressed at ~45, ~55, and ~30% of control levels respectively.

Figure 2

Relative protein concentrations, catalytic enzyme activities, and/or mRNA levels of CYP2C11, CYP3A2, CYP2C6, CYP2C7, CYP2B1, and CYP2B2 in livers from male rats treated with either diluent (controls) or octreotide. Rats fitted with our chronic indwelling atrial catheters (MacLeod & Shapiro 1988, Pampori *et al.* 1991) were injected with either octreotide (25 μ g octreotide/kg bd wt) or an equivalent volume of physiological saline every 12 h for a total of 12 injections. Relative levels were calculated by comparing all values to the control with the highest isoform concentration (i.e. 100%). Values are mean $\pm$ s.d. with $n=7$. * $P<0.01$ compared with saline-treated controls. Comparisons are limited to the effects of octreotide on an individual isoform. Comparisons of expression levels between isoforms are not possible. In this regards, whereas CYP2C11 concentrations represent > 50% of the total CYP in normal male rat liver, CYP2B1 and CYP2B2 are inducible isoforms expressed constitutively at only nominal levels (Ryan & Levin 1993).

CYP2C12 protein and mRNA, the principal female-specific isoform, was undetectable in both control and octreotide-treated rats. By contrast, expression levels of CYP2C6 and CYP2C7, major female-predominant isoforms, were oppositely affected by octreotide. Hepatic CYP2C6 protein and mRNA was increased ~15 and ~25% respectively in octreotide-treated rats. *Cyp2c7* expression, however, was reduced ~50% in the treated rats.

Both CYP2B1 and CYP2B2 are drug-inducible isoforms whose expression levels are constitutively expressed at very low concentrations (Ryan & Levin 1993). Nevertheless, octreotide administration increased CYP2B1 protein concentrations by ~25% and CYP2B2 protein levels were increased ~65%; still contributing to only a small portion of the total hepatic CYP content.

Igf1 expression

One of GH's most important hepatic functions is the upregulation of *Igf1* transcription (Pampori & Shapiro 1996, Davey *et al.* 2001). We found (not presented) that octreotide reduced *Igf1* mRNA levels to 68% of control values ($P<0.01$).

STAT5B expression

Because CYP2C11 expression as well as that of IGF1 is normally dependent upon the activation of the JAK2/STAT5B signal transduction pathway (Verma *et al.* 2005, Waxman & O'Connor 2006), we measured STAT5B protein levels in hepatic cytosol of control and octreotide-treated male rats (Fig. 3). Cytosolic protein levels of the transcription factor were significantly increased in the octreotide-treated rats. As CYP2C11 and IGF1 expression require the activation (i.e. phosphorylation) and nuclear translocation of STAT5B (Waxman & O'Connor 2006, Thangavel & Shapiro 2007), we attempted to measure nuclear phospho-STAT5B by immunoprecipitation with STAT5B antibodies subsequently probing the precipitate with anti-phosphotyrosine as well as in the reverse order, i.e. immunoprecipitation with anti-phosphotyrosine and then probing with antibodies against STAT5B (Thangavel & Shapiro 2007). Unfortunately, neither procedure detected activated STAT5B in the nuclei of control or octreotide-treated rats (Fig. 3). Nevertheless, it was possible that the additional purification step in the immunoprecipitation procedure compromised the ability to detect small amounts of nuclear phospho-STAT5B. Accordingly, we directly measured phospho-STAT5 (though unable to differentiate phospho-STAT5B from

Figure 3

Analyses of cytosolic and/or nuclear STAT5B, phospho-STAT5B, and phospho-STAT5 protein levels in livers from male rats treated with either diluent (control) or octreotide. Rats fitted with our chronic indwelling atrial catheter (MacLeod & Shapiro 1988, Pampori *et al.* 1991) were injected with either octreotide (25 µg octreotide/kg bd wt) or an equivalent volume of physiological saline every 12 h for a total of 12 injections. Relative cytosolic STAT5B levels presented as mean $\pm$ s.d. were calculated by comparing all values to the control liver with the highest isoform concentration (i.e. 100%). * $P < 0.01$ compared with saline-treated controls. (Note: In about half of the samples, the anti-STAT5B reaction produced a double band. According to the vendor and our use of a STAT5B standard, the lower band is STAT5B and the upper band is unknown. Moreover, the relative densitometric values would be unchanged if the upper bands were considered in the calculation as they too exhibit similar treatment differences.) Representative immunoblots are presented for all proteins. Actin levels, not presented, demonstrated equal loading. All transcription factors were measured in seven rats/group. In the case of nuclear phospho-STAT5B, immunoprecipitation (IP) used anti-phosphotyrosine (anti-pY) and immunoblotting (IB) with anti-STAT5B.

the activated 5A form) in the hepatic nuclei of diluent and octreotide-treated rats (Fig. 3). Of the seven animals studied in each treatment group, the hepatic nuclei from just one control rat exhibited a strong reaction to the phospho-STAT5 antibody, another showed a weak reaction, and the remaining five rats had no reaction. Of the octreotide-infused rats, only one exhibited a nuclear reaction, albeit weak, to the antibody. These results are not surprising as phospho-STAT5B *in vivo* is quickly translocated to the nucleus within 15–20 min following the episodic GH pulse where it remains at detectable concentrations for about 40 min (Verma *et al.* 2005). Accordingly, it is very unlikely that our animals were killed during the brief STAT5B-responsive period.

JAK/STAT-dependent genomic expression

Having observed a dramatic downregulation in CYP2C11 expression despite a sizable upregulation of STAT5B, we

investigated whether the expression of other (i.e. non-CYP) JAK/STAT-dependent genes were downregulated by octreotide. On the contrary, however, we found 14 different JAK/STAT-dependent genes upregulated by octreotide treatment, and only two genes significantly downregulated by the treatment (Table 2). Moreover, octreotide increased expression levels of *Jak2*, *Stat3*, *Stat5a*, and *Stat5b* (the latter in agreement with our STAT5B protein findings (Fig. 3)). However, none of the identified genes are members of the CYP superfamily or any of the so-called drug-metabolizing enzymes or drug transporters. Most appear to be genes involved in cell differentiation, growth, or maintenance (i.e. cellular functions), as well as immune-responsive genes. In fact, none of these groups of octreotide-responsive genes are known to be regulated by episodic GH activation of STAT5B. Rather, many of these immune response and cellular function genes are regulated by members of the JAK/STAT families other than JAK2/STAT5B (Imada & Leonard 2000, Kisseleva *et al.* 2002), which, in turn, are activated by non-GH factors (e.g. prolactin, interleukin, cytokines, and lymphokines).

Table 2 Altered *Jak/Stat* and dependent genomic expression levels in livers of male rats treated with octreotide. Rats fitted with our chronic indwelling atrial catheters (MacLeod & Shapiro 1988, Pampori *et al.* 1991) were injected with either octreotide (25 µg/kg bd wt) or an equivalent volume of physiological saline every 12 h for a total of 12 injections. Genomic expression levels were determined using a RT² Profiler PCR Array for the rat *Jak/Stat* signaling pathway. $n = 3$

Function	Gene symbol	Fold change	P value
Immune response	<i>Jak2</i>	2.35↑	<0.05
	<i>Stat3</i>	2.01↑	<0.05
	<i>Stat5a</i>	2.05↑	<0.05
	<i>Stat5b</i>	2.52↑	<0.004
	<i>A2m</i>	4.33↑	<0.05
	<i>Csf1r</i>	4.68↑	<0.004
	<i>Csf2rb</i>	8.39↑	<0.003
	<i>Fcgr1a</i>	2.20↑	<0.02
	<i>Il10ra</i>	7.78↑	<0.04
	<i>Irf1</i>	3.85↑	<0.003
Cell differentiation/ growth/maintenance	<i>Ccnd1</i>	2.18↓	<0.05
	<i>Cdkn1a</i>	8.93↑	<0.05
	<i>Egfr</i>	2.64↓	<0.04
	<i>JunB</i>	3.88↑	<0.02
	<i>Spi1</i>	5.29↑	<0.04
Negative regulators <i>Jak/Stat</i> pathway	<i>Ptpn1</i>	2.38↑	<0.05
	<i>Ptprc</i>	3.61↑	<0.004
	<i>Sirpa</i>	2.72↑	<0.05
	<i>Socs2</i>	2.47↑	<0.05
	<i>Socs3</i>	3.43↑	<0.03

By contrast, some members of a group of octreotide upregulated genes, i.e. the negative regulators of the JAK/STAT pathway (Table 2), have been implicated in the regulation of episodic GH activation of the JAK2/STAT5B pathway (Choi & Waxman 2000) and may explain, in part, the actions of octreotide on CYP expression to be presented in Discussion section.

Discussion

The experimental results raise two questions. 1) Why didn't the octreotide treatment suppress circulating GH levels? 2) How then did the octreotide treatment suppress the expression of GH-dependent CYP isoforms without the expected concomitant decline in GH secretion?

It could be argued that the octreotide treatment did not suppress GH secretion because we used a less than effective dose of the analog. However, our administered dose (25 µg b.i.d./kg bd wt) was considerably greater than the most commonly effective human dose (100 µg t.i.d./patient) used to treat acromegaly (Physician's Desk Reference 2012. <http://www.pdr.net/>, Accessed May 28, 2012). Moreover, just a single 50 µg injection of octreotide to acromegaly patients caused a rapid fall in plasma GH that took almost 9 h to completely recover (Marbach *et al.* 1985). The possibility that the rat is insensitive to the GH inhibitory effects of the somatostatin analog also seems unlikely as a dose of octreotide as small as 2 µg (Pérez-Romero *et al.* 1999) or 15 µg/kg bd wt (Pless *et al.* 1986) has been reported to substantially (~50%) reduce GH secretion in rats.

In general, animal studies investigating the effects of octreotide on GH secretion tend to examine the inhibitory effects of just a single *in vivo* dose of the analog (Marbach *et al.* 1985, Pless *et al.* 1986, Turner & Tannenbaum 1995) making it difficult to judge the relevance of these findings to our long-term study. There is, however, some evidence indicating that microgram doses of octreotide administered i.v. to adult male rats results in an enhanced rebound secretion of pulse GH when the effectiveness of the analog begins to wane and the pituitary is exposed to the next pulse release of GH-releasing hormone (GHRH; Turner & Tannenbaum 1995). Accordingly, our observation of moderately elevated pulse amplitudes, prolonged pulse durations, and greater amounts of GH per pulse following 11 doses of octreotide may reflect an over responsiveness of the pituitary somatotrophs to the undisturbed periodic secretion of hypothalamic GHRH. Although multiple octreotide infusions did not produce the expected decline in plasma GH reported in acromegaly patients (Lamberts

et al. 1993, Yang & Keating 2010), it clearly had an effect on hepatic function.

The sexually dimorphic expression levels of hepatic CYP isoforms in the rat, and likely in all other species examined, are solely regulated by the sex differences in GH secretory patterns (Shapiro *et al.* 1995, Waxman & O'Connor 2006). CYP2C11 is the predominant isoform in the male rat accounting for >50% of the total content of hepatic CYPs (Ryan & Levin 1993). Masculine expression levels of both male-specific CYP2C11 and CYP3A2 are solely dependent on exposure to the episodic GH secretory profile characteristic of the male, whereas the feminine continuous GH profile completely suppresses expression of the isoforms. Although female-predominant CYP2C7 is expressed at much lower concentrations in male liver, these masculine levels are still dependent on the normal episodic GH profile of the male rat as disruption in the masculine GH profile will suppress CYP2C7 expression. Whereas the sexually dimorphic concentrations of female-predominant hepatic CYP2C6 are determined by the sex differences in the GH secretory profiles, considerable levels of the isoform are expressed in the absence of GH indicating a high degree of GH independence (Pampori & Shapiro 1996, Agrawal & Shapiro 2000, 2001). CYP2B1 and CYP2B2 are drug-inducible (e.g. phenobarbital) isoforms, nominally, at best, expressed constitutively, and moderately responsive to the sexually dimorphic GH secretory profiles (Agrawal & Shapiro 1995). Lastly, although not a CYP, GH regulation of IGF1 expression has been extensively reported (Martinelli *et al.* 2008).

How then did octreotide alter the expression of these GH-dependent proteins without any notable disruptions in the masculine GH secretory profile? To answer this seeming contradiction, we focused our attention on CYP2C11, a paradigm for studies examining the molecular mechanism(s) activated by episodic GH secretion (Waxman & O'Connor 2006, Thangavel & Shapiro 2007). While it is correct that the masculine levels of the sex-dependent CYP isoforms are dependent on exposure to the episodic GH profile, the profile is composed of various 'signaling elements' to which each isoform is independently responsive. These signals may be recognized by the hepatocyte in the frequencies, concentrations, and/or durations of the pulse and interpulse periods. Alternatively, the hepatocyte can monitor the mean plasma concentration of the hormone (Pampori & Shapiro 1996, Agrawal & Shapiro 2000). The signal in the masculine GH profile regulating CYP2C11, and likely CYP3A2, is a minimum GH-devoid interpulse period.

The normal pulse amplitudes can be reduced by 90–95% or elevated fourfold, and the pulse frequencies can be reduced to just four a day, and still CYP2C11 expression will be normal (Waxman *et al.* 1991, Agrawal & Shapiro 2000, 2001). However, as the duration of the physiological GH-devoid interpulse becomes shorter, the hepatic CYP2C11 discriminator recognizes the profile as feminine (i.e. constant), suppressing expression of the isoforms (Agrawal & Shapiro 2001). In this regard, octreotide appeared to have reduced the duration of the GH interpulse, which may have been a critical element in the resulting suppression of CYP2C11 as well as CYP3A2. In support of our conclusion, there are some reports indicating that the somatostatin analog can decrease drug metabolism in patients (Physician's Desk Reference 2012. <http://www.pdr.net/>, Accessed May 28, 2012), and data from octreotide-infused young men suggest shortened interpulses in the monitored GH profile (Dimaraki *et al.* 2003).

It has also been reported that a toxin-induced acute inflammatory response can reduce hepatic *Cyp2C11* mRNA to 5–25% of normal in 24 h (Wright & Morgan 1990). This rapid response likely precludes any involvement of GH. However, the response may involve the synthesis of 'positive acute-phase proteins' like α_2 -macroglobulin (A2m) (Kushner 1982). Twice-daily observations of our animals indicated no acute or even moderate inflammation. In fact, normal body weight gain of ~5 g/day during octreotide infusion indicates healthy animals. However, we did observe a greater than fourfold increase in hepatic expression levels of A2m in the octreotide-treated rats. As A2m is a GH-binding protein (Kratzsch *et al.* 1995) and an upregulation in A2m has been correlated with a downregulation of several CYPs (Beigel *et al.* 2008), the octreotide-induced expression of A2m may have contributed to the general CYP suppression in our experimental animals.

A final explanation for our results may involve GH-independent mechanisms. Somatostatins have numerous extra-pituitary effects including the activation of the immune response, inhibition of cellular proliferation, and more specifically blocking the secretion of serotonin, gastrin, vasoactive intestinal peptide, secretin, motilin, pancreatin polypeptide, glucagons, and insulin explaining their therapeutic use in treating carcinoid tumors and vasoactive intestinal peptide tumors (Marbach *et al.* 1985, Lamberts *et al.* 1993, Yang & Keating 2010). We have seen the multiple extra-pituitary effects of octreotide by the number of genes involved in the immune response, cell differentiation, growth, and maintenance affected by the

analog (Table 2). Although studies examining the extra-pituitary actions of octreotide are limited, there are reports demonstrating octreotide inhibition of cyclin D1 (*Ccnd1*; Ferrante *et al.* 2006) and epidermal growth factor receptor (*Egfr*) expression (Lai *et al.* 2003, Shen *et al.* 2011) as we too report.

It seems improbable that expression of the 20 hepatic genes we found to be regulated by octreotide is involved in CYP expression. Rather, it more likely indicates that somatostatin and its analog normally regulate a myriad of functions as broadly classified earlier. However, a few of the genes upregulated by octreotide are known to be involved in CYP2C11 and IGF1 expression. GH signaling in liver by the episodic profile (in contrast to the continuous profile) is initiated by hormone binding and the resulting activation of GH receptors on the surface of the target cells. This allows for the recruitment and/or activation of two molecules of JAK2, which then cross-phosphorylate each other as well as phosphorylating the receptor on key tyrosine residues. STAT5B, a latent transcription factor, binds to these phosphorylated receptor docking sites, is in turn phosphorylated, homodimerizes, and translocates to the nucleus where it binds to promoter sites initiating transcription of CYP2C11 (Choi & Waxman 2000, Thangavel & Shapiro 2007) and IGF1 (Davey *et al.* 2001). We found that octreotide induced a greater than twofold increase in *Jak2* mRNA and *Stat5b* mRNA, with the latter being increased at the protein level as well. These findings would seem to be in direct contradiction with the accompanying ~90% decline in CYP2C11 mRNA and protein. However, the episodic GH/JAK2/STAT5B signal transduction pathway is negatively regulated by tyrosine phosphatases (Frank 2001, Waxman & O'Connor 2006) and suppressors of cytokine signaling, i.e. SOCS/CIS (Choi & Waxman 2000, Thangavel & Shapiro 2007).

In agreement with earlier studies reporting octreotide-induced activation of tyrosine phosphatases (Held-Feindt *et al.* 2001, Murray *et al.* 2004) and then subsequent inhibition of STAT5B activation and nuclear translocation (Murray *et al.* 2004), we observed an octreotide upregulation of several tyrosine phosphatases as well as *Socs* genes. Accordingly, we propose that the somatostatin analog induces the overexpression of hepatic negative regulators of the JAK2/STAT5B pathway preventing episodic GH induction of CYP2C11 expression. The upregulation of *Jak2* and *Stat5b* may reflect a reflex overexpression in response to inhibition of the JAK2/STAT5B signaling pathway.

In summary, we have found that circulating GH profiles in male rats infused with octreotide every

12 h for 6 days were characterized by larger than normal pulses in otherwise male-like episodic secretory profiles. In apparent disaccord, several episodic GH-dependent CYP isoforms as well as *Igf1* were concurrently downregulated by the analog. We explain this contradiction by identifying octreotide-induced subtle abnormalities in 'signals' in the masculine GH profile normally required for male-like expression levels of CYPs and IGF1, induction of immune response factors postulated to suppress CYP expression, and/or by octreotide-induced overexpression of negative regulators of the JAK2/STAT5B pathway normally mediating episodic GH inductions of a least CYP2C11 and IGF1.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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