GH administration patterns differently regulate epidermal growth factor signaling

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Abstract

Current GH administration protocols imply frequent s.c. injections, resulting in suboptimal compliance. Therefore, there is interest in developing delivery systems for sustained release of the hormone. However, GH has different actions depending on its continuous or pulsatile plasma concentration pattern. GH levels and circulating concentration patterns could be involved in the regulation of epidermal growth factor receptor (EGFR) expression in liver. Aberrant expression of this receptor and/or its hyperactivation has been associated with the pathogenesis of different types of carcinoma. Considering that one of the adverse effects associated with GH overexpression and chronic use of GH is the increased incidence of malignancies, the aim of this study was to analyze the effects of GH plasma concentration patterns on EGFR expression and signaling in livers of mice. For this purpose, GH was administered by s.c. daily injections to produce an intermittent plasma pattern or by osmotic pumps to provoke a continuously elevated GH concentration. Intermittent injections of GH induced upregulation of liver EGFR content, augmented the response to EGF, and the induction of proteins involved in promotion of cell proliferation in female mice. In contrast, continuous GH delivery in male mice was associated with diminished EGFR in liver and decreased EGF-induced signaling and expression of early genes. The results indicate that sustained delivery systems that allow continuous GH plasma patterns would be beneficial in terms of treatment safety with regard to the actions of GH on EGFR signaling and its promotogenic activity.

Key Words

- growth hormone
- epidermal growth factor
- intracellular signaling
- liver

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Introduction

Growth hormone (GH) is a pituitary hormone that stimulates longitudinal bone growth; it induces diverse effects on cell growth and differentiation, and on the metabolism of proteins, lipids, and carbohydrates (Herrington & Carter-Su 2001). The use of GH in endocrinological practice and for the treatment of various

clinical conditions is expanding. The first use of human GH (hGH) was as replacement therapy in children with GH deficiency (GHD). However, further indications have been gradually approved or proposed since the development of recombinant hGH (rhGH; Kemp & Frindik 2011, Laron 2011). Current protocols for GH treatment imply its

s.c. or i.m. injection once daily or three times a week. The main disadvantages of these administration protocols are the short plasma half life of the hormone and its renal toxicity. Moreover, injection results in poor patient compliance, high doses, nonspecific toxicity, and increased cost (Kwak *et al.* 2009, Park *et al.* 2013). Thus, development of sustained-release rhGH formulations could improve quality of life and decrease secondary effects (Laron 2011). To date, a once-weekly sustained release GH preparation has been shown to be effective for the treatment of several clinical conditions (Péter *et al.* 2012, Biller *et al.* 2013) and sustained delivery systems that last longer are being investigated (Wei *et al.* 2012, Park *et al.* 2013). However, GH shows different effects depending on its plasma concentration pattern in many species including mice, rats, and humans (Jansson *et al.* 1985, Jaffe *et al.* 2002, Waxman & O'Connor 2006). Therefore, the efficacy and toxicity of pharmaceutical systems that allow prolonged release of the hormone, which would produce near continuous elevation of circulating GH levels, should be assessed and compared with the effects of the treatments that involve intermittent injections and mimic a pulsatile concentration pattern.

The differential effects of intermittent and sustained plasma GH patterns have been studied in humans and animals, mostly focusing on the consequences for longitudinal growth and metabolism; however, the differential effects of plasma GH patterns on the activation of signaling pathways involved in cell proliferation and survival have not been analyzed. A relevant side effect associated with chronic use of GH and its overexpression is the increased risk of malignancies (Jenkins 2006, Siobhan & Shereen 2008, Laron 2011). Previous studies indicate that GH concentration patterns regulate epidermal growth factor receptor (EGFR) expression in rodent liver. The EGFR, also known as ERBB1, is a plasma membrane glycoprotein which belongs to the ERBB family of receptor tyrosine kinases (Burgess 2008). Upon ligand binding, ERBB proteins homo- or heterodimerize with other members of the ERBB family to activate downstream signaling pathways that regulate proliferation, growth, and differentiation (Riese & Stern 1998). Aberrant expression of the EGFR and/or hyperactivation of this receptor have been associated with the pathogenesis and progression of different types of cancers (Ito *et al.* 2001, Normanno *et al.* 2006). GH has been shown to modulate the expression of *Egfr* in the liver (Jansson *et al.* 1988, González *et al.* 2010). *Egfr* mRNA levels and EGF binding to liver were reported to be more efficiently induced

by a pulsatile pattern of plasma GH concentration (Jansson *et al.* 1988, Ekberg *et al.* 1989, Kashimata *et al.* 1989). However, results of these studies are controversial since other authors found no differences on the induction of *Egfr* mRNA or EGF binding when the hormone was administered by intermittent injections or continuous infusion to hypophysectomized rats (Johansson *et al.* 1989). Moreover, we have recently demonstrated that transgenic mice overexpressing GH, which exhibit a continuous plasma GH expression pattern (Norstedt & Palmiter 1984), show increased EGFR expression (Miquet *et al.* 2008, González *et al.* 2010).

While studies regarding GH concentration patterns and EGFR expression in the liver have been performed, the resulting effects on EGFR signaling have not been analyzed. Increased EGFR liver content does not necessarily imply the upregulation of EGF signaling. Actually, transgenic mice overexpressing GH showed elevated EGFR protein levels in the liver, but activation upon ligand stimulation was diminished for some of its signaling cascades (González *et al.* 2010, Díaz *et al.* 2012).

Therefore, considering the growing interest in the development of sustained delivery systems for GH administration, the potential oncogenic properties of this hormone and its likely dimorphic regulation of a receptor widely involved in cancer, the EGFR, the aim of this study was to analyze the effects of different GH administration protocols on EGFR expression, signaling, and induction of mitogenic mediators in the liver for normal mice.

Subjects and methods

Reagents

Highly purified porcine GH (pGH) was obtained from Zamira Life Sciences Pty Ltd (Knoxfield, VIC, Australia). Recombinant human EGF was obtained from Sigma Chemical Co. PVDF membranes, high-performance chemiluminescence film, and ECL-Plus were from Amersham Biosciences. Acrylamide, bis-acrylamide, and tetramethylethylenediamine (TEMED) were obtained from Bio-Rad Laboratories. Secondary antibodies conjugated with HRP, anti-major urinary proteins (MUPs), anti-EGFR, anti-cytokine-inducible SH2-domain-containing protein (CIS), and anti-signal transducer and activator of transcription 5 (STAT5) antibodies were purchased from Santa Cruz Biotechnology Laboratories. The anti-phospho-STAT5a/b Tyr694/699 antibody was from Millipore (Billerica, MA, USA). The anti-phospho-AKT Ser473, anti-AKT,

anti-p44/42 MAPK (anti-ERK1/2), anti-phospho-p44/42 MAPK Thr202/Tyr204 (anti-phospho-ERK1/2), anti-phospho-STAT3 Tyr705, anti-phospho-EGFR Tyr845, anti-cMyc, and anti-cFos antibodies were from Cell Signaling Technology, Inc. (Beverly, MA, USA). The bicinchoninic acid (BCA) protein assay kit was obtained from Thermo Scientific, Pierce Protein Research Products (Rockford, IL, USA). All other chemicals were of reagent grade.

Animals

Swiss-Webster female and male adult animals (3–4 months old, body weight: 26–30 g) were used. The mice were housed three to five per cage in a room with controlled light (12 h light:12 h darkness cycle) and temperature ($22 \pm 2^\circ\text{C}$). Sentinel animals were tested for all major murine pathogens and the results of the tests were uniformly negative. The animals had free access to a nutritionally balanced diet and tap water. The appropriateness of the experimental procedure, the required number of animals used, and the method of acquisition were in compliance with federal and local laws, and with institutional regulations.

Animal treatments

GH-treatment The hormone was administered to Swiss-Webster mice for 5 days under two different protocols: i) s.c. injections of pGH (0.5 $\mu\text{g/g}$ of body weight) twice a day and ii) s.c. continuous infusion with an osmotic minipump (model 1007D, Alzet) delivering the hormone at a dose 1 $\mu\text{g/g}$ of body weight per day (Jansson *et al.* 1988, Karlsson *et al.* 1999). Protocols for GH treatment were adapted from previously described techniques (Jansson *et al.* 1988, Ekberg *et al.* 1989). Control animals for each group were treated in a similar fashion but saline solution was administered instead.

Acute EGF stimulation Mice were fasted for 6 h before receiving an i.p. injection of recombinant human EGF at 2 mg/kg BW in 0.9% w/v NaCl. Animals were killed 10 min after administration of EGF (González *et al.* 2010) to study the phosphorylation of EGF signaling mediators, or after 30 min or 1 h to study the expression of early genes involved in the induction of cell cycle progression. Control mice received injections of saline solution to evaluate basal conditions. Urine was collected at the time the animals were killed and the livers were removed and stored frozen at -70°C until homogenization.

Processing of urine samples

Urine samples were processed immediately after being obtained. They were centrifuged at 8800 *g* for 3 min at 4°C . The supernatant was recovered and diluted at a ratio of 1:3 in 0.06 mol/l Tris, 0.001 mol/l EDTA (pH 7.4) buffer. An aliquot of each sample was diluted in Laemmli buffer, boiled for 5 min, and stored at -20°C until used for electrophoresis. The samples were subjected to SDS-PAGE using Bio-Rad Mini Protean apparatus (Bio-Rad Laboratories). Gels were stained with Coomassie Blue.

Preparation of liver extracts

Liver samples were homogenized at the ratio 0.1 g/ml as previously described (González *et al.* 2010, Díaz *et al.* 2012). Protein concentrations of supernatants were determined using a BCA protein assay kit and samples were prepared for immunoblotting (González *et al.* 2010, Díaz *et al.* 2012). For immunoprecipitation, aliquots of solubilized liver containing 4 mg of protein were incubated overnight at 4°C with anti-CIS antibody and subsequently processed as described previously (González *et al.* 2010).

Immunoblotting

Samples were subjected to SDS-PAGE. Electrophoretic transfer of proteins from gel to PVDF membranes, incubation with antibodies, reprobing, detection, and quantification of band intensity were carried out as described previously (González *et al.* 2010, Díaz *et al.* 2012).

RNA isolation and quantitative RT-PCR

Total RNA from 50 mg liver tissue was extracted with TRIzol reagent (Life Technologies) according to the manufacturer's instructions. The purity of RNA was assessed from the ratio of the optical densities at 260 and 280 nm, and the integrity was controlled by electrophoresis on 1% agarose gel. Total RNA (2 μg) were used for the RT reaction in a 20 μl volume using M-MLV reverse transcriptase (Promega, 200 U/ μl) and random hexamer primers (Biodynamics SRL, Buenos Aires, Argentina) (Frungieri *et al.* 2002).

For quantitative real-time PCR, cDNA was amplified using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems), using SYBR Green Master Mix Reagent (Applied Biosystems). The forward (F) and reverse (R) primers used were as follows: EGFR: F, ATAGTGGTGGTGGCCCTTGG and R, GAGGTTCCACGAGCTCTCTCT;

cMyc: F, TCACCAGCACAACACTACGCCG and R, TGCTTC-AGGACCCTGCCACT; *cFos*: F, CCGACTACGAGGCGT-CATCC and R, CTGCGCAAAAGTCCTGTGTGT; and cyclophilin A: F, GCGTCTCCTTCGAGCTGTT and R, AAGTCACCACCCTGGCAC. Cyclophilin A was chosen as the housekeeping gene. Reaction data were collected and analyzed using the complementary computer software (Sequence Detection Software, Applied Biosystems, version 1.3). For the assessment of quantitative differences in the cDNA target between samples, the mathematical model of Pfaffl (2001) was applied. An expression ratio was determined for each sample by calculating $(E_{\text{target}})^{\Delta C_t(\text{target})}/(E_{\text{cyclophilin}})^{\Delta C_t(\text{cyclophilin})}$, where E is the efficiency of the primer set and $\Delta C_t = C_{t(\text{normalization cDNA})} - C_{t(\text{experimental cDNA})}$. The amplification efficiency of each primer set was calculated from the slope of a standard amplification curve of log micro-liters of cDNA per reaction vs C_t value ($E = 10^{-(1/\text{slope})}$). Efficiencies of 2 ± 0.1 were considered optimal.

Statistical analysis

Experiments were carried out analyzing all groups of animals in parallel, n representing the number of different individuals used in each group. Results are presented as mean $\pm$ S.E.M. of the number of samples indicated. Statistical analyses were performed by ANOVA followed by the Newman-Keuls multiple comparison test using the GraphPad Prism 4 statistical program by GraphPad Software, Inc. (San Diego, CA, USA). Student's t -test was used when only two groups were analyzed. Data were considered significantly different if $P < 0.05$.

Results

Effects of different GH administration protocols on the expression of proteins regulated by plasma GH concentration patterns

Previous studies have demonstrated that intermittent injections and continuous administration of GH produce different plasma concentrations patterns of the hormone and effects; however, those studies were carried out in hypophysectomized animals (Jansson *et al.* 1988, Ekberg *et al.* 1989, Waxman *et al.* 1991). In the present study, non-hypophysectomized mice were studied. In normal rodents, as it occurs in several species, GH secretion is sexually dimorphic (Jansson *et al.* 1985, MacLeod *et al.* 1991, Veldhuis & Bowers 2003). A key difference between male and female GH profiles is the sustained interpulse

interval with little or no detectable circulating GH characteristic of adult males (Jansson *et al.* 1985, MacLeod *et al.* 1991, Jaffe *et al.* 1998). Normal female and male mice also have a different endocrine and metabolic background. To study the effects of different GH concentration patterns in normal mice, supraphysiological GH doses were exogenously administered in an intermittent or continuous fashion. Then, the expression of proteins regulated by the GH plasma concentration patterns was assessed in order to establish a relationship between the GH administration protocols and the GH plasma concentration patterns produced by the treatments. For this purpose, MUPs and CIS in the liver and concentrations of MUPs in the urine were determined in GH-treated and control mice.

The MUPs are acidic protein isoforms with molecular masses of between 19 and 21 kDa (Cavaggioni & Mucignat-Caretta 2000). These pheromone-binding proteins are synthesized in the liver and released through the kidney into the urine. The secretory pattern of GH regulates the expression *MUP* genes; induction of *MUP* mRNAs requires pulsatile occupancy of GH receptors, while continuous GH concentration pattern represses expression of *MUP* genes (Norstedt & Palmiter 1984, Johnson *et al.* 1995). To show the difference in hepatic contents of MUPs between male and female mice, samples from mice of both sexes were analyzed in parallel (Supplementary Fig. 1A, see section on supplementary data given at the end of this article). Intermittent administration of GH to female mice produced an increase in MUPs in liver (Fig. 1A); however, this GH administration protocol had no effect on expression of MUPs in male mice (Fig. 1B). In contrast, continuous administration of the hormone had no effect on expression of MUPs in the liver of female mice (Fig. 1C), but caused a decrease in the level MUPs in the liver of male mice (Fig. 1D). To confirm the effects of exogenously administered GH on production of MUPs, the concentration of these proteins in urine was determined. Consistent with the results obtained for MUPs in liver, the concentrations of MUPs in urine increased in female mice that received intermittent injections of GH (Fig. 1E) but diminished in male mice that received continuous administration of the hormone (Fig. 1F). The absence of effects on expression of MUPs expression observed in male mice that received injections of GH could be explained by the already high levels of MUPs induced by the endogenous secretion pattern of the hormone. In the case of female mice, no effects on expression of MUPs were found in animals treated with GH delivered by the osmotic pumps because expression of MUPs is already low.

Figure 1

Levels of MUPs and CIS in liver and concentrations of MUPs in urine in GH-treated and control mice. Solubilized liver samples from female and male mice treated with intermittent injections of GH (intGH) or continuous delivery of GH (contGH) and their respective controls were subjected to immunoblotting analysis to determine the hepatic levels of MUPs (21 kDa) (A, B, C and D). Reprobing with anti-actin antibody demonstrated similar protein loading for all lanes. Urine samples from female mice treated with intGH, male mice receiving contGH, and their respective controls were analyzed by

SDS-PAGE to assess concentrations of MUPs in urine (E and F). CIS (32 kDa) was immunoprecipitated from solubilized livers from female and male mice that received intGH or contGH treatment, respectively, and assessed by immunoblotting with anti-CIS (G and H). Quantification was performed by scanning densitometry and expressed as fold change vs values measured for GH-untreated normal mice. Data are the mean $\pm$ S.E.M. of the indicated number of subsets (n) of individual animals. Different letters denote significant differences ($P \leq 0.05$). Representative western blots are shown.

The liver content of the suppressor of cytokine signaling CIS also varies depending on GH plasma concentration patterns (Karlsson *et al.* 1999). CIS is induced by a continuous pattern of GH concentration (Ram & Waxman 2000). Consequently, its expression decreased in female mice that received intermittent injections of GH compared with controls (Fig. 1G), but increased in male mice administered GH in a continuous manner compared with untreated male mice (Fig. 1H). A tendency towards decreased CIS levels was also evidenced when GH was administered by intermittent injections to male mice; however, the difference was not significant (Supplementary Fig. 1B). Continuous administration of GH to female mice induced a slight and non-significant increase in hepatic levels of CIS (Supplementary Fig. 1C).

Therefore, a pulsatile pattern of GH concentration was evident in female mice exogenously treated with intermittent injections of GH, while a continuous pattern

of circulating levels of GH was demonstrated to be caused by sustained delivery of GH in male mice. For this reason, the effects of pulsatile GH plasma concentration patterns on EGFR expression and signaling were studied in female mice that received intermittent injections of GH or saline solution. On the other hand, the effects of constant elevated concentrations of the hormone were analyzed in male mice by implantation of sustained delivery systems for continuous release of the hormone or saline solution. In addition, control assays involving intermittent administration of GH to male mice and continuous delivery of the hormone to female mice were carried out.

EGFR expression, activation, and signaling vary with the pattern of plasma GH concentrations

The effects of plasma GH patterns on hepatic *Egfr* mRNA levels and EGFR protein content were determined in the

livers of female mice that received intermittent treatment with GH and in the livers of male mice that received continuous administration of the hormone (Fig. 2 A, B, C and D). *Egfr* mRNA levels increased in the livers of mice that received intermittent GH injections, but results were not significantly different from those for untreated mice (Fig. 2A). In contrast, administration of GH by osmotic pumps provoked a decline in *Egfr* mRNA levels (Fig. 2B). EGFR protein content was analyzed in solubilized livers of GH-treated and control mice that subsequently received acute stimulation with EGF. Immunoblotting analysis showed that the protein content of EGFR was significantly increased by a pulsatile plasma GH pattern (Fig. 2C), while continuous circulating levels of GH decreased EGFR protein abundance (Fig. 2D) in accordance with results for the determination of *Egfr* mRNA.

Ligand-induced EGFR dimerization leads to receptor autophosphorylation at tyrosine residues. Phosphotyrosine residues allow the recruitment of specific partners to activate different downstream pathways, including

Ras/MAPK, PI3K/Akt, and STAT pathways (Wells 1999, Henson & Gibson 2006, Normanno *et al.* 2006). EGFR is augmented in transgenic mice overexpressing GH; however, this does not result in an increased response to EGF (González *et al.* 2010). To ascertain the effects of the different GH plasma patterns on EGF-induced EGFR activation, EGFR phosphorylation on Tyr845 in the livers of GH-treated and untreated mice after acute stimulation with EGF was assessed (Fig. 2E and F). In accordance with the changes described for hepatic EGFR content, EGF-induced receptor phosphorylation levels increased in mice that received s.c. injections of GH compared with untreated mice (Fig. 2E), but decreased in mice that received continuous administration of GH with respect to their controls (Fig. 2F). The analysis of the ratio between EGFR phosphorylation levels and EGFR content of liver revealed that increased or decreased EGFR phosphorylation levels could be attributed to the augmented or reduced amount of hepatic protein respectively (Fig. 2G and H).

Figure 2

Hepatic *Egfr* mRNA expression, protein content, and phosphorylation. Liver samples from female mice treated with intermittent injections of GH (intGH), male mice receiving continuous delivery of GH (contGH), and their respective controls were analyzed by qRT-PCR to determine *Egfr* mRNA levels (A and B). Solubilized livers from GH-treated and control mice that subsequently received acute stimulation with EGF for 10 min (EGF) or not (–) were subjected to immunoblot analysis to determine liver EGFR (170 kDa) content (C and D) and EGFR Tyr845 phosphorylation levels (E and F). Membranes were reprobed to

assess actin content and to demonstrate uniform protein loading for all lanes. Immunoblots were scanned and quantification was performed by densitometry. Data resulting from quantification analyses were used to calculate the pY845 EGFR:EGFR ratio (G and H). Results were expressed as fold change vs. values measured for untreated normal mice (A and B) or EGF-stimulated GH-untreated mice (C, D, E, F, G and H). Data are the mean $\pm$ s.e.m. of the indicated number of subsets (n) of individual animals. Different letters denote significant differences ($P \leq 0.05$). Representative western blots are shown.

The MAPKs Erk1 and Erk2 have been extensively associated with the promotion of cell proliferation. To ascertain the effects of different plasma GH concentration patterns on the regulation of EGF-induced Erk1/2 activation, Erk1/2 phosphorylation in the livers of mice that received intermittent or sustained administration of GH followed by the acute stimulation with EGF or saline was determined. In accordance with the results described for

EGFR, phosphorylation levels of Erk1/2 were increased in female mice treated with GH injections compared with untreated mice (Fig. 3A), but decreased in the livers of male mice that received continuous administration of GH (Fig. 3B). No differences in the protein levels of Erk1/2 were observed for any of the treatments (Fig. 3A and B).

The activation of EGFR also results in phosphorylation of Akt at its two main and activating phosphorylation

Figure 3

Hepatic Erk1/2 and Akt phosphorylation and protein content. Solubilized liver from female mice treated with intermittent injections of GH (intGH), male mice receiving continuous infusion of GH (contGH), and their respective controls that subsequently received acute stimulation with EGF for 10 min (EGF) or not (–) were subjected to immunoblot analysis to determine Erk1/2 (44/42 kDa) phosphorylation levels and protein content (A and B); Akt (62 kDa) phosphorylation levels at Ser473 (C and D), and at

Thr308 (E and F) and its protein content (C, D, E and F). Reprobing with anti-actin antibody demonstrated equal protein loading for all lanes. Quantification was performed by scanning densitometry and expressed as fold change vs EGF-stimulated GH-untreated mice. Data are the mean $\pm$ s.e.m. of the indicated number of subsets (*n*) of individual animals. Different letters denote significant differences ($P \leq 0.05$). Representative western blots are shown.

sites, Thr308 in the kinase domain and Ser473 in the C-terminal regulatory domain (Fresno Vara *et al.* 2004, Osaki *et al.* 2004). Once activated, Akt promotes cell survival and proliferation. Akt protein content and phosphorylation of its activating residues in the livers of mice that received intermittent or sustained administration of GH (Fig. 3C, D, E and F). In accordance with the results recorded for Erk1/2, Akt activation was augmented in the livers of female mice that received intermittent administration of GH (Fig. 3C and E), but was diminished in the livers of male mice that received the hormone in a continuous fashion (Fig. 3D and F). The protein abundance of the kinase did not significantly vary after the different GH treatments (Fig. 3C, D, E and F).

EGFR activation also results in activation of STATs (Guren *et al.* 2003, Quesnelle *et al.* 2007), which has been associated with development and progression of many malignancies (Buettner *et al.* 2002, Quesnelle *et al.* 2007). EGF-induced STAT3 phosphorylation in mice that received the different GH administration protocols was compared with the results for the respective untreated control mice (Fig. 4). In accordance with the findings described for Erk1/2 and Akt activation, EGF-induced STAT3 phosphorylation increased in the presence of a pulsatile GH concentration pattern (Fig. 4A), whereas it was diminished when the GH plasma concentration pattern was continuous (Fig. 4B). However, protein content was not modified

in response to the different GH administration protocols (Fig. 4A and B). Similar results were found for STAT5 activation when EGF-induced phosphorylation and protein levels were studied in mice that received GH treatments and in their respective controls (Fig. 4C and D).

EGFR protein levels and EGF-induced activation of Erk1/2 and Akt were also determined, as a control, in female mice that received continuous administration of GH and in male mice that received intermittent doses of the hormone by injection. Continuous administration of GH to female mice had no effects on liver EGFR content, while GH injections induced a slight but non-significant increase in hepatic EGFR content (Fig. 5A and B). In accordance with the absence of effects on EGFR levels, neither continuous administration of GH to female mice nor intermittent injection of GH into normal male mice induced changes in Erk1/2 or Akt phosphorylation levels in response to EGF (Fig. 5C, D, E and F). The absence of effects of GH administered by the osmotic pumps to female mice on EGFR expression and EGF signaling correlates with the lack of effects of the treatment observed for expression of MUPs (Fig. 1C). Similarly, intermittent GH injections to male mice had no effects on hepatic levels of EGFR and EGF signaling as well as on expression of MUPs (Fig. 1B). In both cases, exogenous administration of GH does not seem to induce a net change with respect to endogenous GH concentration patterns.

Figure 4

STAT3 and STAT5 phosphorylation and protein content in the liver. Solubilized livers from female mice treated with intermittent injections of GH (intGH), male mice that received continuous delivery of GH (contGH), and their respective controls that subsequently received acute stimulation with EGF for 10 min (EGF) or not (–) were subjected to immunoblot analysis to determine phosphorylation levels of STAT3 (86–91 kDa) (A and B) and STAT5 (92 kDa)

(C and D) as well as their protein contents (A, B, C and D). Reprobing with anti-actin antibody demonstrated uniform protein loading for all lanes. Quantification was performed by scanning densitometry and expressed as fold change vs EGF-stimulated GH-untreated mice. Data are the mean $\pm$ S.E.M. of the indicated number of subsets (*n*) of individual animals. Different letters denote significant differences ($P \leq 0.05$). Representative western blots are shown.

The pattern of plasma GH concentrations affects EGF induction of proteins involved in cell cycle promotion

As a consequence of cellular stimulation with several mitogenic signals, including the EGF, *cMyc* expression and activation are induced mainly via the MAPK/ERK pathway (Curran *et al.* 1985). Considering the differential effects on EGFR expression and signaling observed in mice

treated with intermittent injections of GH or with a sustained delivery system for GH administration, *cMyc* induction upon EGF stimulation was assessed to elucidate the possible differential effects of plasma GH concentration patterns on EGF-induced proliferation in liver. For this purpose, the kinetics of *cMyc* mRNA and protein expression upon exogenous administration of EGF for different times

Figure 5

Hepatic EGFR content, Erk1/2, and Akt phosphorylation and protein detection. Solubilized livers from female mice receiving continuous delivery of GH (contGH), male mice treated with intermittent injections of GH (intGH), and their respective controls which subsequently received acute stimulation with EGF for 10 min (EGF) or not (–) were analyzed by immunoblotting to determine EGFR protein levels (A and B), Erk1/2 and Akt phosphorylation, and

protein content (C, D, E and F). Membranes were reprobated to assess actin content and demonstrate equal protein loading for all lanes. Quantification was performed by scanning densitometry and expressed as fold change vs EGF-stimulated GH-untreated mice. Data are the mean $\pm$ S.E.M. of the indicated number of subsets (*n*) of individual animals. Different letters denote significant differences ($P \leq 0.05$). Representative western blots are shown.

were first evaluated in the liver of female and male mice with no GH treatment. No sex differences were observed, both mRNA and protein expression showed maximal induction 1 h after EGF injection (Fig. 6A and B); therefore, this time period was selected for evaluation of the effects of different GH concentration patterns. In accordance with variations described for EGFR expression and signaling, EGF-induced *cMyc* mRNA and protein content were increased by the pulsatile GH concentration pattern induced in female mice by GH injections (Fig. 6C and E) whereas they were decreased by the continuous GH concentration pattern provoked by sustained delivery of the hormone in male mice (Fig. 6D and F).

EGF-induced expression of the oncogene *cFos* was also analyzed in the livers of mice that received GH by different modes of administration. The kinetics of *cFos* transcription and transduction upon EGF stimulation were first

assessed in the livers of non-GH treated female and male mice. *cFos* mRNA levels reached a maximum 30 min after EGF injection, while protein content attained higher levels 1 h after EGF administration (Fig. 7A and B) in both sexes. Considering the data from kinetic studies, *cFos* mRNA and protein content in the livers of untreated and GH-treated mice, under basal conditions and after acute stimulation with EGF, were measured. In accordance with previous results, both hepatic *cFos* mRNA and protein levels were increased by the pulsatile GH concentration pattern (Fig. 7C and E) whereas they were decreased by the continuous plasma GH pattern (Fig. 7D and F).

Discussion

Currently, there is increasing interest in developing delivery systems that allow sustained release of GH to

Figure 6

Hepatic *cMyc* expression and protein content. Liver samples from mice stimulated with EGF over different time periods or not stimulated were analyzed by qRT-PCR and immunoblotting to determine *cMyc* expression and its protein content induction respectively (A and B). *cMYC* (67 kDa) expression was analyzed by qRT-PCR in female mice treated with intermittent injections of GH (intGH), male mice receiving continuous infusion of GH (contGH) and their respective controls that received acute stimulation with EGF for 1 h (EGF 1 h) or not (-) (C and D). Protein contents of liver samples were analyzed by immunoblotting for GH-treated and

control mice that received acute stimulation with EGF for 1 h (EGF 1 h) or not (-) (E and F). Reprobing with anti-actin antibody demonstrated equal protein loading in all lanes. Immunoblots were scanned and quantification was performed by densitometry. Results were expressed as fold change vs values measured for EGF-stimulated GH-untreated mice (A, C, D, E and F). Data are the mean $\pm$ S.E.M. of the indicated number of subsets (n) of individual animals. Different letters denote significant differences ($P \leq 0.05$). Representative western blots are shown.

Figure 7

Hepatic *cFos* expression and protein content. Liver samples from mice stimulated with EGF during different time periods and their nonstimulated controls were analyzed by qRT-PCR and immunoblotting to determine *cFos* expression and its protein content induction respectively (A and B). *cFos* (62 kDa) expression was analyzed by qRT-PCR in female mice treated with intermittent injections of GH (intGH), male mice receiving continuous infusion of GH (contGH), and their respective controls that received acute stimulation with EGF for 30 min (EGF 30 min) or not (–) (C and D). Protein content of liver samples was analyzed by immunoblotting in GH-treated

and control mice that received acute stimulation with EGF for 1 h (EGF 1 h) or not (–) (E and F). Reprobing with anti-actin antibody demonstrated uniform protein loading for all lanes. Immunoblots were scanned and quantification was performed by densitometry. Results were expressed as fold change vs values measured for EGF-stimulated GH-untreated mice. Data are the mean $\pm$ s.e.m. of the indicated number of subsets (*n*) of individual animals. Different letters denote significant differences ($P \leq 0.05$). Representative western blots are shown.

prolong administration intervals of the hormone during a chronic treatment. However, plasma GH concentration patterns induce differential physiological actions in several species. For this reason, continuous administration of the hormone must be validated regarding not only its efficacy but also its safety.

In humans, only a few studies have compared the effects of continuous s.c. infusion vs daily s.c. injections of GH. The studies were mainly focused on the actions on longitudinal growth, body weight, body composition, and metabolism, and revealed some differences regarding insulin-like growth factor 1 (IGF1) and IGF-binding protein 3 (IGFBP3) plasma levels, glucose tolerance (Johansson *et al.* 1996), and lipid metabolism, but the effects on longitudinal growth and body composition were not of critical significance (Laursen *et al.* 2001). In animals,

the physiological importance of GH concentration patterns has been extensively described. Actually, GH concentration patterns have differential effects on the hepatic expression of numerous genes (Norstedt & Palmiter 1984, Waxman *et al.* 1991, Waxman & O'Connor 2006) and GH administered in a pulsatile manner induces growth and generation of IGF1 more effectively than continuous administration (Clark & Robinson 1988, Udy *et al.* 1997).

Although several studies have been carried out to establish the efficacy of continuous administration of GH, there are not enough studies to determine the security of this treatment compared with intermittent administration of GH. Among the main problems associated with long-term GH treatment is the increased tendency to develop tumors. The expression of the EGFR, broadly involved in

the pathogenesis of different types of tumors, varies depending on plasma levels of GH. EGFR plays an essential role in the development of not only epithelial cells but also of tumors of epithelial cell origin (Singh & Harris 2005). Therefore, the objective of this study was to investigate how different GH plasma patterns, induced by different administration protocols, affect EGFR expression, signaling, and induction of mitogenic mediators in the livers of normal mice.

The effects of plasma levels of GH on EGFR expression have been extensively studied. GH administration was found to induce the expression of EGFR in the liver while hypophysectomy reduced the hepatic *Egfr* mRNA concentration in rats (Ekberg *et al.* 1989). In accordance, EGFR expression in liver is increased in transgenic mice overexpressing GH but decreased in GH-receptor knockout mice (Miquet *et al.* 2008, González *et al.* 2010). However, studies concerning the effects of plasma GH pattern on EGFR expression are still controversial. In rodents, EGFR expression is higher in males than in females as evidenced by specific binding of labeled EGF to purified liver membranes, determination of EGFR mRNA, and immunoblotting from solubilized liver (Ekberg *et al.* 1989, Johansson *et al.* 1989, Miquet *et al.* 2013). Furthermore, in hypophysectomized rats of both sexes, intermittent GH treatment enhanced hepatic *Egfr* mRNA concentrations compared with levels for normal male rats, while continuous GH administration was less effective or did not have any effects at all (Ekberg *et al.* 1989, Kashimata *et al.* 1989). In contrast, other authors found no differences between intermittent and continuous administration of hGH on the induction of *Egfr* mRNA or EGF binding when administered to hypophysectomized male and female rats (Johansson *et al.* 1989). Moreover, increased GH levels with a continuous pattern in transgenic mice overexpressing GH also result from the upregulation of EGFR expression in liver (Miquet *et al.* 2008, González *et al.* 2010). These genetically modified mice present high GH levels during the entire lifespan of the animals and many physiological and endocrine alterations may be associated with GH overexpression; therefore, it is difficult to dissect the mechanisms involved in EGFR expression and signaling changes. For this reason, the influence of GH plasma patterns was studied in normal mice supplemented with the hormone for a short period. GH administered by s.c. injections increased hepatic EGFR content in livers of female mice, while continuous administration of the hormone to male mice reduced hepatic receptor mRNA levels and protein content. Therefore, these results support the

relevance of the GH concentration pattern as an important determinant of EGFR expression. In accordance, EGFR levels did not change in livers of female mice after continuous administration of GH or in male mice that received intermittent injections of the hormone. As previously discussed for expression of MUPs, EGFR was not significantly induced in male mice that received injections of GH because EGFR liver content is already high due to the endogenous secretion pattern of the hormone. In contrast, a reduction in EGFR levels was not evident in livers of female mice after continuous GH treatment, probably because the receptor content is already low compared with male mice (Miquet *et al.* 2013).

Continuous elevation of GH plasma levels reduced EGFR protein content and mRNA levels in liver. In contrast, EGFR protein levels were found to be increased in the livers of female mice that received the intermittent treatment with GH, but quantitative RT-PCR of the EGFR showed a tendency to higher levels which were not significantly different from those for control mice. The discrepancy between immunoblotting and quantitative PCR determinations of *Egfr* might depend on effects of GH on EGFR turnover. GH and prolactin, a protein related to GH, were shown to control the production of EGFR by phosphorylation on serine/threonine residues (Huang *et al.* 2003, 2004, 2006), which delay EGFR downregulation and therefore potentiate acute EGF-induced signaling (Huang *et al.* 2003, 2004, 2006, Li *et al.* 2008). Besides inducing EGFR expression, intermittently administered GH could provoke retardation of EGFR downregulation, thus increasing EGFR protein content to a greater extent than *Egfr* mRNA levels.

Results from the present study demonstrated that the effects of GH administration on hepatic EGFR content directly correlated with effects on EGF-induced signaling. Such effects were not necessarily the expected results as we had previously observed that transgenic mice overexpressing GH showed an increase in hepatic EGFR content but not in EGF signaling (González *et al.* 2010). Transgenic mice overexpressing GH display high GH levels throughout their lifespan which trigger several mechanisms to downregulate EGF signalling through the STATs and the PI3K/Akt pathways (González *et al.* 2010, Díaz *et al.* 2012). Short-term treatment with GH would not induce such compensatory mechanisms; consequently, changes in EGF signaling were directly correlated with modifications in EGFR liver content.

Considering the role of EGFR in cell proliferation and tumor development, EGF-induced cMYC and cFOS protein and mRNA content were determined. cMYC is a

pleiotropic transcription factor that promotes growth and expansion of somatic cells, its activation results in the upregulation of cyclins with concomitant downregulation of cell cycle inhibitors like p21. *cMyc* is an important proto-oncogene found to be upregulated in many types of cancers (Soucek *et al.* 2008, Lin *et al.* 2010). *cFos* is a transcription factor that belongs to the family of immediate early genes which is upregulated in response to several mitogenic signals, including EGF (Curran *et al.* 1985). Increased expression of *cFOS* has been associated with development of cancer, including hepatocarcinoma (Yuen *et al.* 2001, Liu *et al.* 2012). In accordance with the effects of plasma GH pattern on EGF signaling, induction of *cMyc* was higher in female mice that received intermittent injections of GH compared with untreated animals; and *cMyc* induction was lowered by continuous GH administration in male mice. Similar results were described when *cFos* expression and protein content were determined.

In conclusion, a pulsatile GH concentration pattern, produced by intermittent injections of the hormone, induced the upregulation of liver EGFR content, which resulted in an increased response to EGF in female mice, but non-significant effects on EGFR expression and signaling in male mice. In contrast, continuous GH concentration patterns, induced by sustained administration of the hormone, had no effects on EGFR expression or EGF-induced responses in livers of female mice, but decreased EGFR expression and activation of EGF-induced signaling mediators in male mice, resulting in diminished pro-mitogenic effects. The results indicate the beneficial effects of use of sustained delivery systems that allow continuous GH plasma patterns in terms of treatment security relating to EGFR-mediated protumorigenic adverse effects. Future studies should be carried out in GHD animal models to confirm the benefits of use of those systems for the treatment of GHD-associated states. To extend this conclusion to humans, species differences in GH secretion patterns should be considered and studies on the effects of different plasma GH patterns should be carried out in humans. Moreover, assessment of other GH-regulated proteins such as serum IGF1 and IGFBP3 levels, which have been described as predictors of hepatocarcinoma development, should also be considered in order to determine the potential adverse effects of different administration protocols on tumor promotion.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-13-0447>.

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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