Pancreatic hormones differentially regulate insulin-like growth factor (IGF)-I and IGF-binding protein production by primary rat hepatocytes

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Abstract
We investigated the influence of and interactions among pancreatic hormones on the secretion of insulin-like growth factor-I (IGF-I) and IGF-binding proteins (IGFBPs) by treating primary hepatocytes from young male Long-Evans rats with insulin or glucagon in combination with rat GH (rGH). The concentration of IGF-I secreted into the medium was estimated by radioimmunoassay after formic acid-acetone cryoextraction, and secreted IGFBPs were analysed by Western ligand blot and immunoblot; accumulation of IGF-I mRNA was analysed by Northern blot. Both insulin (0–100 nmol/l) and rGH (0–5, 5 and 50 pmol/l) produced a dose-dependent stimulation of IGF-I secretion over a 24-h incubation period. In contrast, glucagon (0–1–100 nmol/l) inhibited IGF-I production in a dose-related manner. Glucagon (10 nmol/l) also inhibited IGF-I secretion stimulated by rGH (5 pmol/l) and insulin (10 nmol/l). Northern blot analysis of total RNA isolated from rat hepatocytes revealed that rGH (5 pmol/l) elevated IGF-I mRNA levels, glucagon (10 nmol/l) alone had no effect on this parameter, but glucagon significantly reduced IGF-I transcript accumulation in response to rGH. IGFBPs secreted by rat hepatocytes run in two molecular weight ranges on SDS-PAGE: ~25 kDa (IGFBP-4) and ~29–31 kDa (IGFBP-1 and -2); the predominant hormonally regulated IGFBP was identified as IGFBP-1. Insulin produced a dose-dependent inhibition of production of IGFBP-1, while glucagon was stimulatory; when given together at an equivalent concentration (1 nmol/l), the effects of insulin were dominant to glucagon on IGFBP-1. These observations provide support for significant opposite roles for the pancreatic hormones, insulin and glucagon, in the regulation of liver IGF-I and IGFBP-1 production. As the production of pancreatic hormones is influenced by nutritional status, these polypeptides may mediate the effects of changing nutritional state on the hormonal control of protein anabolism and glucose homeostasis by directly influencing the circulating level of liver-derived IGF-I and its binding proteins.

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Introduction
The vascular link between the pancreas and the liver presents the potential for direct regulation of liver insulin-like growth factor (IGF) secretion by polypeptide hormones produced by the islets of Langerhans. While pituitary growth hormone (GH) is thought to be the primary hormonal regulator of liver IGF production (Mathews et al. 1986, see Sara & Hall 1990), insulin has also been shown to influence circulating levels of IGF-I and IGF-binding proteins (IGFBPs) and to act directly on hepatocytes to alter the levels of their mRNA and peptide (Scott et al. 1985, Johnson et al. 1989, Salamon et al. 1989, Boni-Schnetzler et al. 1990, 1991, Sara & Hall 1990, Lewitt & Baxter 1991, Phillips et al. 1991). Work from our laboratory has demonstrated a physiological role for insulin in the control of IGF-I (Griffen et al. 1987).

While much attention has focused on insulin as an important regulator of liver IGF-I and IGFBPs, little is known about the roles of the other major pancreatic hormones, somatostatin (SRIF) and glucagon. Several studies, both in vivo and in vitro, using the superactive analogue of SRIF, octreotide, suggest a role for SRIF at the level of the liver cell in IGF-I (inhibitory) and IGFBP-1 (stimulatory) regulation (Ezzat et al. 1991, 1992, Flyvbjerg et al. 1991, Ren et al. 1992, Serri et al. 1992). Contradictory evidence for a role for glucagon in hepatic IGF-I and IGFBP regulation has been reported. While Kachra et al. (1991) reported stimulatory effects of glucagon on IGF-I, Arany et al. (1993) found glucagon to be inhibitory to IGF-I; these groups also obtained contradictory effects of glucagon on IGFBP production.

Given the important actions of glucagon on glucose metabolism, and the recently recognized roles for IGF-I
and IGFBP-1 in glucose homeostasis (reviewed by Lewitt & Baxter 1991), regulatory interactions among these hormonal peptides might be expected. We investigated the possibility that physiological concentrations of glucagon regulate the production of IGF-I and IGFBP by primary rat hepatocytes, and that glucagon interacts with insulin and pituitary rat GH (rGH).

Materials and Methods

Hormones and reagents

Powdered culture medium and antibiotics were obtained from Gibco (Grand Island, NY, USA). Collagenase (type IV), DNase, bacitracin and bovine insulin were from Sigma (St Louis, MO, USA). Bovine glucagon was obtained from two sources: Sigma and Calbiochem (La Jolla, CA, USA). rGH was supplied by the National Hormone and Pituitary Program of the NIDDK. Recombinant human IGF-I (rhIGF-I) was from Amgen (San Diego, CA, USA), and recombinant human IGFBP-3 (non-glycosylated) was from Celtrix Pharmaceuticals, Inc. (Santa Clara, CA, USA). The rat IGF-I cDNA probe was kindly supplied by Dr L Murphy (Department of Internal Medicine, University of Manitoba, Winnipeg, Canada; see Murphy et al. 1987). 32P-α-dCTP was from Amersham (Arlington Heights, IL, USA) and the random primed labelling kit was from Boehringer Mannheim (Indianapolis, IN, USA).

Hepatocyte isolation and culture

Primary rat hepatocytes were isolated from juvenile Long-Evans rats (38–40 days old; 140–160 g body weight) by in situ collagenase perfusion (0–1 mg/ml=0·17 units/ml) using the two-step procedure with recirculating buffer described by Seglen (1976) with modifications described by Bissell & Guzelian (1980) and Aiken et al. (1990). The preperfusion solution was a Ca2+-free Krebs–Henseleit buffer (Sigma) with 24 mmol NaHCO3/l, 10 mmol Hepes/l, 0·5 mol EGTA/l added and the enzyme buffer was Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (50:50) with 15 mmol Hepes/l, 29 mmol NaHCO3/l. Dispersed cells liberated from the liver capsule were incubated in DNase (1 mg/ml=580 Kunitz units/ml) for 5 min at 37 °C to inhibit cell clumping caused by DNA leaked from damaged cells. The hepatocytes were then separated from liver non-parenchymal cells by gravity sedimentation (15–20 min, 4 °C); this method gives 98% or greater parenchymal cells (see Seglen 1976). Cell viability as assessed by trypan blue exclusion ranged from 75 to 95%.

Hepatocytes were cultured in Williams medium E (WME; Williams & Gunn 1974; with 2000 mg glucose/l as supplied by Gibco) which was formulated specifically for the culture of adult rat hepatocytes. Ichihara et al. (1980) found this medium to be the most suitable for obtaining high levels of protein synthesis and urea formation in cultured hepatocytes. The cell pellet was resuspended in plating medium (WME, 5% calf serum, 10 µg insulin/ml (1·7 µmol/l), 1 µmol dexamethasone (DEX)/l) and the cells were plated in six-well Primaria culture plates (Falcon, Becton–Dickinson) or rat-tail collagen (prepared as described by Richards et al. 1983)–coated six-well plates at a density of 150 000 cells/cm2 in a volume of 2 ml. For RNA isolation, cells were plated in 100 mm Primaria dishes at a density of 150 000 cells/cm2 in a volume of 8 ml. Cell densities of 100 000/cm2 and above favour the expression of liver-specific functions but suppress growth (Ichihara 1991). Also, higher cell densities result in greater enzyme induction (Ichihara et al. 1980). Cells were cultured under a humidified atmosphere of 5% CO2, 95% air. The initial incubation in WME with serum (plating period) was continued for 3 h, after which time the medium was changed to serum-free WME with 10 nmol insulin/l, 100 nmol DEX/l to enhance attachment and cell viability (see Ichihara et al. 1980, 1982) and the incubation was continued for 18–20 h (preincubation). We found that the presence of DEX in the culture medium was required during this period to maintain cell attachment. Many investigators include higher concentrations of insulin during this preincubation period (i.e. 1 µmol/l to 100 nmol/l); for examples see Scott et al. 1985, Norstedt & Moller 1987, Johnson et al. 1989, 1991, Tollet et al. 1990, Boni–Schnetzer et al. 1991, Harp et al. 1991); however, we were concerned that such high doses of insulin would alter subsequent cellular responses to the hormone (e.g. see data of Houston & O’Neill 1991) with chicken hepatocytes. We found that a lower dose of insulin (10 nmol/l) was sufficient to maintain cell viability and this dose did not alter the basal output of IGF-I or cellular responsiveness to insulin or rGH during the 24-h test period (data not shown).

The hepatocytes became attached to the surface of the Primaria culture dishes during the 3-h plating period and cells spread and flattened during the next 18–20 h to form a monolayer. There was no significant difference in plating efficiency or hormone responsiveness of cells plated on Primaria plates or plates coated with rat-tail collagen (t-test).

After the preincubation, cells were washed with Earle's balanced salt solution and 2 ml WME containing 0·01% bacitracin (a peptidase inhibitor) were added to the wells. Ten microlitres of test substances were added directly to the wells and incubations were continued for 24 h. At the termination of the cultures, the medium was collected, cells were rinsed with phosphate buffered saline (PBS; phosphate, 0·02 mol/l; saline, 0·9%; pH 7·4), harvested in 1 ml PBS and sonicated for 20 s with a Branson Sonifier. For RNA analyses, cells were rinsed with PBS, suspended in RNA extraction buffer (4 mol guanidinium
thiocyanate/1, 25 mmol sodium citrate/1, pH 7, 10% sarcosyl, 0.72% 2-mercaptoethanol) and frozen at −80 °C until extraction. Medium samples were frozen at −80 °C before assay.

**IGF-I extraction techniques**

Since the presence of IGFBPs in biological fluids can influence radioimmunoassay (RIA) estimates of IGF-I concentration, the IGFBPs were removed from hepatocyte-conditioned medium (HCM) before IGF-I RIA. The first method was used routinely because of greater time efficiency and better recoveries while the second method was used to corroborate the results obtained with the first (see Results).

**Acid-acetone cryoextraction** We used the technique described by Bowsher et al. (1991) for the extraction of IGFs from rat biological fluids with some modifications. Acetone was used at 80% instead of 70% since we found that this concentration precipitated most of the binding activity without precipitating the IGF-I (as assessed by tests with 125I-labelled IGF-I prepared by the iodogen procedure (Pierce, Rockford, IL, USA)). We also added bovine γ-globulins (Sigma) as carrier protein to form a larger pellet and conducted the precipitation overnight at −20 °C (cryoprecipitation: see Breier et al. 1991). The basic technique was as follows: 50 μl of a 1% bovine γ-globulins solution were added to 400 μl HCM, the medium was acidified with 250 μl 8.3 formic acid containing 0.1% Tween 20 and kept at room temperature for 30 min. Binding proteins were then precipitated with 3 ml acetone at −20 °C for 18-20 h. The mixture was then centrifuged at 3000 g for 30 min and a 2 ml aliquot of the supernatant was removed and dried in a vacuum oven at 50 °C. The extracts were allowed to redissolve overnight at 4 °C in a volume of RIA buffer (phosphate, 0.05 mol/l; protamine sulphate, 0.02%; Tween 20, 0.05%; EDTA, 0.01 mol/l; sodium azide, 0.02%; pH 7.5) equivalent to the amount of HCM in the extract, and dilutions of the extracts were analysed in the RIA (see below). Recovery of added 125I-labelled IGF-I and unlabelled IGF-I averaged 88% and 97% respectively. Residual IGF-I-binding activity in the extracts as assessed by charcoal-binding assay (see below) averaged less than 8% of that in the unextracted medium.

**Acid-gel filtration** Samples of HCM were subjected to acid-gel filtration chromatography as described by Daughaday et al. (1987) and Phillips et al. (1991) with minor modifications. The column buffer used was 0.25 mol formic acid/l, 0.025 mol NaCl/l, pH 2.0 and we used 20 ml disposable chromatography columns (Bio-Rad, Hercules, CA, USA) packed with Biogel P-30 (Bio-Rad) with a bed volume of 18 ml. Columns were calibrated with 125I-labelled IGF-I added to a pool of HCM; 1 ml fractions were collected and counted in a gamma counter or dried in a centrifugal concentrator and tested for binding activity by solution binding assay (see below). Binding activity eluted in the first 11 ml and the free IGF-I in the next 8 ml. Medium samples (200 μl) were prepared by adding 1000 c.p.m. 125I-labelled IGF-I (repurified just before use to remove free I), incubating for 30 min at room temperature, then acidifying with 10 μl 88% formic acid and incubating for an additional 30 min at room temperature before loading onto the columns. Aliquots (1 ml) of the 8 ml pooled IGF-I fractions were dried in a centrifugal concentrator and resuspended in 250 μl RIA buffer; this concentration of salt in the reconstituted extracts did not interfere in the RIA. Recoveries of added 125I-labelled IGF-I and unlabelled IGF-I averaged 80% and 77% respectively. Residual IGF-I binding activity in the extracts averaged less than 5% of that of the unextracted medium.

**Charcoal binding assay**

Residual binding in the extracts was assessed by solution binding assay as described by Hintz et al. (1981).

**Western ligand and immunoblotting**

Samples of HCM were analysed by Western ligand blotting as described by Hossenlopp et al. (1986). Aliquots of HCM were mixed with 3 × non-reducing SDS sample buffer (Tris base, 0.125 mol/l; SDS, 4%; glycerol, 20%; 2-mercaptoethanol, 10%; bromophenol blue, 0.1%; pH 6.8), heated to 65 °C, and electrophoresed in 12-5% SDS-polyacrylamide gels. The separated proteins were transferred to Transblot transfer medium (Bio-Rad) using a methanol-free transfer buffer (50 mmol Tris base/l, 40 mmol glycine/l; pH 8.4) and probed with 125I-labelled IGF-I (150 000 c.p.m./ml). Autoradiography was carried out at −80 °C for 7-12 days.

Transferred proteins were immunodetected using antirat IGFBPs (Liu et al. 1993). The antisera were raised against synthetic peptide fragments of IGFBP-1, -2, -4, -5 and -6, and the IGFBP-3 antiserum was raised against native rat IGFBP-3; there is no significant cross-reaction of the antisera among the different binding proteins (Liu et al. 1993). Blots were incubated with the antiserum overnight at 4 °C at dilutions ranging from 1:500 to 1:2000. Positive immunoreactions were detected using enhanced chemiluminescence reagents supplied by Amersham.

**IGF-I RIA techniques**

Concentrations of IGF-I in HCM extracts were determined by a standard double-antibody, disequilibrium human IGF-I RIA (Furlanetto & Marino 1987) with minor modifications. The IGF-I antiserum (polyclonal human SmC antiserum; UB3–189; supplied by Drs L
Underwood and J J Van Wyk, Division of Pediatric Endocrinology, University of North Carolina at Chapel Hill, and distributed by the National Hormone and Pituitary Program of the NIDDK) was used at a final dilution of between 1:15 000 and 1:20 000 depending on the quality of the $^{125}$I-labelled IGF-I tracer. The RIA was terminated and free IGF-I was separated from bound by the addition of cold 5% polyethylene glycol (molecular weight 8000) in deionized H$_2$O containing goat anti-rabbit serum and normal rabbit serum as carrier. The precipitation was carried out for 30 min at 4°C followed by centrifugation at 2000 g for 25 min. Intra- and inter-assay coefficients of variation for the IGF-I RIA were 2-8% and 12-6% respectively.

**Northern blotting**

For Northern blot analysis, total RNA was isolated from cells by the method of Chomczynski & Sacchi (1987). RNA concentrations were determined spectrophotometrically and 20 µg denatured total RNA per sample was fractionated in a 1:2% agarose, 2:2 mol formaldehyde gel/1 (Sambrook et al. 1989) containing 1 µg ethidium bromide (EtBr)/ml. After visualization and photography of the EtBr-stained ribosomal RNA bands under u.v. light (for normalization of RNA loading), the RNA was transferred to nylon membrane (Nytran; Schleicher and Schuell, Inc. Keene, NH, USA) by capillary transfer and the membrane baked at 80°C for 2 h. The Northern blot was performed essentially as described by Murphy et al. (1987). The membrane was prehybridized for 4 h at 42°C in 50% formamide, 20 mmol NaH$_2$PO$_4$/1, 4 × Denhardt’s solution (1 × =0·02% each of bovine serum albumin, ficoll and polyvinylpyrrolidone), 100 µg sonicated denatured herring sperm DNA/ml, 4 × SSC, 0·1% SDS. Hybridization was carried out in the same solution at 42°C for 24 h with 2 × 10$^8$ c.p.m. of denatured probe. The rat IGF-I cDNA probe was labelled to high specific activity with $^{32}$P-α-dCTP by random primed labelling. After hybridization, the membrane was washed briefly with 2 × SSC, 0·1% SDS at room temperature and then for 1 h with 0·1 × SSC, 0·1% SDS at 65°C. The membrane was exposed to X-ray film for 16–72 h at −80°C. Subsequently, the membrane was stripped (see Sambrook et al. 1989) and rehybridized with random primed-labelled human β-actin probe (Clontech Laboratories, Palo Alto, CA, USA) as a control for RNA loading. Appropriately exposed autoradiograms were analysed by densitometry using a Macintosh-linked image scanner and the NIH image program (version 3·0).

**Data analysis**

Three to six replicate wells per hormone treatment were included in each experiment and experiments were replicated three or four times. The absolute amount of hormone produced by the cells differed slightly between experiments due to variability in the primary cell preparations; however, the profiles of changes in IGF-I among replicate experiments were virtually identical. Data were analysed by analysis of variance (ANOVA) and linear regression using the SOLO computer program (BMDP statistical software, Los Angeles, CA, USA) with $P<0.05$ considered significant. Medium concentrations of IGF-I are expressed as ng/1·5 million cells added to the wells.

**Results**

**Extraction and RIA of IGF-I from rat hepatocyte-conditioned medium**

Dilutions of acid-acetone cryoextracts produced displacement curves in the RIA that were parallel to the rhIGF-I standard, while dilutions of unextracted HCM were non-parallel (Fig. 1a). We determined the possible effects of residual binding protein in the HCM extracts on the RIA estimation of medium concentrations of IGF-I by adding recombinant human IGFBP-3 (7·5 ng/ml) or the concentrated IGFBP fraction of HCM from acid-gel filtration to the RIA (see Fig. 1b). These tests showed that the main effects of the presence of IGFBP on the RIA were to increase the apparent amount of IGF-I in the sample and produce dilution curves that were non-parallel to the IGF-I standard. However, an amount of the IGBP fraction collected from the acid-gel filtration column corresponding to 10 µl of the unextracted HCM gave less than 5% displacement in the RIA (see Fig. 1b). Since we included 10 µl or less of the pooled acid-acetone cryo-extraction fractions in our RIA, which, by solution binding assay contained less than 8% of the binding activity of the unextracted medium, we conclude that the effect of any residual binding activity in the acid-acetone cryoextracts would account for less than 1% of the displacement of $^{125}$I-labelled IGF-I from the antibody in the RIA. Thus, since the acid-acetone cryoextraction gave better recoveries and similar results and was much more time-efficient compared with the acid-gel filtration method, the former technique was used in all subsequent tests.

**Time-course of IGF-I production by rat hepatocytes**

Freshly isolated liver cells exhibit very low levels of protein synthesis and hormonal responsiveness, both of which increase during several days in culture (see Ichihara et al. 1980). Figure 2 illustrates the time-course of IGF-I peptide secretion by primary rat hepatocytes in monolayer culture; medium was collected and replaced with fresh medium and hormones at 6-, 12-, 24- and 48-h intervals and assayed for IGF-I. IGF-I secretion increased over the 72-h incubation period and was significantly (P<0·001) greater in cells exposed to insulin (10 nmol/l=60 µg/l)
Glucagon inhibits IGF-I peptide but stimulates IGFBP production by rat hepatocytes

Glucagon inhibited basal IGF-I secretion with a maximal inhibitory dose of 1 nmol/l (Fig. 5). In addition, glucagon (10 nmol/l) and rGH (5 pmol/l), both significantly inhibited insulin (10 nmol/l) and rGH (5 pmol/l)-stimulated IGF-I secretion by rat hepatocytes. (P<0.02 to P<0.001 (ANOVA); see Fig. 6). In contrast to this inhibitory action on IGF-I, glucagon produced a dose-dependent stimulation of the production of the 29–31 kDa IGFBP (Fig. 7; maximal stimulation with 1 nmol glucagon/l). When cells were treated with both insulin and glucagon at a dose of 1 nmol/l, the effects of insulin on IGFBP production were dominant to those of glucagon.
Effects of hormones on accumulation of IGF-I transcripts in primary rat hepatocytes

Levels of hepatocyte IGF-I mRNA were elevated by rGH (5 pmol/l; 2.5-fold). When glucagon was given alone at a dose of 10 nmol/l, no change in IGF-I mRNA levels was observed (see Fig. 9); however, when glucagon and rGH were given together, glucagon reduced the stimulation of IGF-I mRNA levels induced by rGH (~66%). Analysis of ethidium bromide-stained ribosomal RNA bands was used routinely as a control for RNA loading; this showed that RNA of approximately equal amount and quality was loaded in each lane (data not shown). The β-actin probe was not useful for the normalization of RNA loading since these mRNA levels were also altered by the hormone treatments (elevated by rGH; data not shown); the inappropriateness of this probe as a control for RNA loading in the isolated rat hepatocyte model has been found by other investigators (see Johnson et al. 1989, 1991).

Discussion

We have shown that glucagon at concentrations within the physiological range inhibits IGF-I production (both peptide and mRNA accumulation) while stimulating the secretion of IGFBP-1 by primary rat hepatocytes. The inhibitory effects of glucagon on IGF-I were dominant to the stimulatory actions of rGH and insulin at the doses tested in this study; however, insulin was dominant over glucagon in inhibiting IGFBP-1 when tested at 1 nmol/l. Our results are in agreement with those of Lewitt & Baxter (1989) and Kachra et al. (1991) who showed a stimulatory action of glucagon on production of IGFBP-1 by liver cells. Our results showing an inhibitory action of glucagon on IGF-I production agree with those of Arany et al. (1993) but are in conflict with those of Kachra et al. (1991) who claimed that bGH and glucagon synergized to elevate IGF-I mRNA, but such a synergy was not evident when they measured IGF-I peptide. A possible explanation for the discrepancies between our results and those of Kachra et al. (1991) is that their culture conditions differed from ours and those of Arany et al. (1993) in several respects. (1) They cultured the hepatocytes for 72 h before testing with hormones. We chose 24 h after isolation to begin our tests since this earlier time-point presumably more closely reflects normal liver function (see Bissell & Guzelian 1980, Ichihara 1980). (2) The basal medium that they used (see Barash et al. 1988 for a formulation) contained a high concentration of glucagon (500 ng/ml). Matsumoto et al. (1991) suggested that glucagon may exert a stimulatory action on IGF-I production but only at doses greater than 10 nmol/l. Thus, the effects of high doses of glucagon may represent a pharmacological effect of the hormone.

We tested preparations of glucagon from two different sources (Sigma and Calbiochem) and obtained similar
results with both. We also addressed the possibility that residual binding protein in our extracts may alter our RIA estimates, as glucagon stimulated the production of IGFBP-1 and insulin reduced it. Our results show that this extraction technique removes most of the binding activity in the samples, and any residual binding activity had negligible effects on the RIA. Indeed, any effect of binding protein on this RIA would be in the direction of overestimation of IGF-I concentrations rather than underestimation.

Growth hormone is a well-known stimulator of liver IGF-I production (see Mathews et al. 1986, Sara & Hall 1990). We showed that while rGH elevated IGFBP-1 transcripts in rat hepatocytes, glucagon inhibited this stimulation; glucagon also inhibits IGF-I peptide production (see Figs 5 and 9). We found, as have others (Scott et al. 1985, Johnson et al. 1989, 1991, Boni-Schnetzler et al. 1991, Phillips et al. 1991), that insulin elevates IGF-I peptide production and mRNA by liver cells (see Fig. 3 and data not shown). By using homologous anti-rat IGFBP antibodies we have shown that while both IGFBP-1 and IGFBP-2 are produced by rat hepatocytes, only IGFBP-1 exhibits regulation by pancreatic hormones (see Fig. 8). Also, changes in binding activity, as assessed by ligand blot, and the changes in protein accumulation, as assessed by immunoblot, paralleled one another. This suggests that changes in binding activity reflect alterations in the concentration of the protein rather than an alteration in its affinity or saturation. Results of attempts to identify the IGFBP-1 produced by rat hepatocytes using heterologous human antibodies differ from ours. Arany et al. (1993) showed that the human IGFBP-1 antiserum recognized two bands, one 29–30 kDa and the other 32 kDa; in the present study, the rat antibodies differentiated between these two bands, identifying one as IGFBP-1 and the other as IGFBP-2. Thus, it seems likely that the human IGFBP-1 antibody cross-reacted with rat IGFBP-2.

Suppression of rat IGFBP-1 mRNA levels and putative peptide (as determined by Western ligand blotting and immunoblotting with anti-human IGFBP-1 antiserum) by insulin has been demonstrated by a number of investigators (Villafuerte et al. 1991, 1992, reviewed by Lewitt & Baxter 1991). Stimulation of IGFBP-1 by glucagon has been reported (Lewitt & Baxter 1989, Kachra et al. 1991), but others found no change in IGFBP-1 following glucagon treatment (Villafuerte et al. 1992, Arany et al. 1993); this discrepancy may be related to variation in culture conditions or possibly the loss of activity of glucagon in the culture medium. IGFBP-1 changes rapidly with the prevailing metabolic and hormonal state of the animal and
it has been suggested that this IGFBP may function as a glucocounter-regulator (see Lewitt & Baxter 1991). Regulation of this IGFBP by insulin and glucagon, which themselves change rapidly in response to changing nutritional state, may be a mechanism for controlling minute to minute changes in levels of IGFBP-1. This IGFBP may then regulate levels of free IGF-I in the blood and thus maintain in check the hypoglycaemic potential of IGF-I (see Lewitt & Baxter 1991).

Whether rat hepatocytes produce IGFBP-2 has been controversial. Several investigators have reported the presence of IGFBP-2 mRNA in hepatocytes (Margot et al.

**Figure 5.** Dose-related inhibition of IGF-I peptide production by bovine glucagon in primary rat hepatocytes. Cells were incubated for 24 h in the presence (●) or absence (solid bar) of varying doses of glucagon. The concentration of IGF-I secreted into the medium was estimated by radioimmunoassay after acid-acetone cryoextraction. This experiment was done with glucagon from two sources, Sigma and Calbiochem, with virtually identical results. Each point represents the average of six replicate wells; vertical lines represent the S.E.M.

**Figure 6.** Inhibition of insulin (Ins; 10 nmol/l)- and rat GH (rGH; 5 pmol/l)-stimulated (a) IGF-I secretion (b) by treatment with glucagon (10 nmol/l) in primary rat hepatocytes. Cells were incubated for 24 h in the presence or absence of hormones. The concentration of IGF-I secreted into the medium was estimated by radioimmunoassay after acid-acetone cryoextraction. Bars represent the means (n=3 wells/treatment) and vertical lines are the S.E.M. Within (a) or (b), different letters indicate significant differences (P<0.05, Duncan's multiple range test) between treatment groups. The levels of IGF-I secreted by cells exposed to glucagon are all significantly lower compared with the respective treatment in the absence of glucagon (t-test); control: P=0.02; insulin: P<0.001; rGH: P<0.001; rGH+insulin: P=0.004.

**Figure 7.** Dose-related stimulation of IGF-binding protein (IGFBP) production by glucagon in primary rat hepatocytes. Cells were incubated for 24 h with varying doses of glucagon (Glu; each dose represented by duplicate lanes). Two lanes with conditioned medium from insulin (Ins)-treated cells and two from insulin+glucagon-treated cells (both at a dose of 1 nmol/l) were included for comparison. Fifty microlitre aliquots of conditioned medium were fractionated on 12.5% SDS-PAGE and analysed by Western ligand blotting for IGFBPs (see Materials and Methods). Positions of molecular weight markers are shown on the left.

Figure 8. Identification by immunoblotting of IGF-binding proteins (IGFBPs) secreted by rat hepatocytes in culture. Cells were incubated for 24 h with insulin (Ins), glucagon (Glu) or both (at 1 nmol/l). Proteins in 50 µl aliquots of conditioned medium were fractionated on 12.5% SDS-PAGE and transferred to Transblot transfer medium. Blots were probed with (a) anti-rat IGFBP-1 or (b) anti-rat IGFBP-2. Positive immunoreactions were identified by electrochemiluminescence. Positions of molecular weight markers are shown on the left.
Figure 9. Effects of glucagon (Glu; 10 nmol/l) with or without rat GH (rGH; 5 pmol/l) on IGF-I transcript accumulation in primary rat hepatocytes. Total RNA extracted from rat hepatocytes was analysed by Northern blot using an IGF-I cDNA probe. Cells (10 × 10^6 cells per dish) were incubated for 24 h in the absence or presence of hormones. Total RNA (20 µg) was fractionated in a 1.2% formaldehyde gel, transferred to a nylon membrane and probed with 32P-α-dCTP-labelled IGF-I cDNA (see Methods). Positions of size markers are shown on the left. The bottom figure shows the average densitometric values for the data shown in the Northern blot (top).
1989, Boni-Schnetzler et al. 1990), but others did not find this IGFBP either by Northern blot or heterologous (anti-human antibodies) immunoblot (Villafuerte et al. 1992, Arany et al. 1993). We have shown, using a homologous rat anti-IGFBP-2 antiserum that hepatocytes from juvenile male rats secrete detectable levels of IGFBP-2. Boni-Schnetzler et al. (1990) found that insulin reduced IGFBP-2 mRNA levels; however, we did not observe any change in IGFBP-2 peptide levels produced by rat hepatocytes after insulin treatment. Although the liver is a major site of IGFBP-3 mRNA, IGFBP-3 peptide is not produced by rat hepatocytes in short-term culture (see Figs 4 and 7). Takenaka et al. (1991) found that the mRNA for IGFBP-3 is not present in hepatocytes but is confined to the non-parenchymal cells of the liver. We have found (R J Denver, unpublished results) that co-cultures of rat hepatocytes and non-parenchymal cells treated with IGF-I produce IGFBP-3 peptide (by Western ligand blot and immunoblot), but only after at least 5 days in culture; similar findings have been reported by Villafuerte & Phillips (1992).

Further evidence for a physiological role for glucagon in controlling IGF-I and IGFBP production comes from our preliminary in vivo studies using osmotic minipump infusions of glucagon into the hepatic portal vein. Infusion of glucagon at 4 or 1 U/kg per day for 6 days significantly reduced body weight and IGF-I concentrations, but increased circulating IGFBP-1 (Denver 1993). Taken together, our in vitro and in vivo results support a physiological role for glucagon in the regulation of IGF-I and IGFBP-1 production that is opposite to that of insulin. Since IGFBP-1 is rapidly regulated in vivo and changes with the prevailing metabolic and hormonal state (see Lewitt & Baxter 1991), the reciprocal regulation of this binding protein may be one mechanism in which short-term regulation of both the anabolic and hypoglycaemic actions of IGF-I is achieved.

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