Tissue-specific response of IGF-I mRNA expression to obesity-associated GH decline in the male Zucker fatty rat

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Abstract

Diminished GH secretion is a well known association of obesity. As in obese humans, Zucker fatty rats develop a progressive GH deficiency, present at 6 weeks of age and maximal at 10 to 12 weeks. The aim of this study was to investigate the GH dependence of IGF-I gene expression in liver and extrahepatic tissues of the obese Zucker rat as a model of progressive GH reduction during adult life. Six- and 11-week-old obese Zucker rats and their lean littermates were used to compare body weight, glycemia, insulinemia, serum GH and IGF-I levels and IGF-I mRNA expression in liver, heart, aorta, kidney and skeletal muscle. In comparison with lean controls, obese Zucker rats showed at both ages comparable glycemia, severe hyperinsulinemia (mU/ml, mean ± S.E.M.; 6 weeks 138 ± 10 vs 45 ± 6 $P<0{\cdot}001$; 11 weeks 147 ± 14 vs 46 ± 3, $P<0{\cdot}001$) and lower GH (ng/ml; 6 weeks 1·7 ± 0·9 vs 2·7 ± 1·1; 11 weeks 1·5 ± 0·9 vs 4·2 ± 1·2) in the presence of similar circulating IGF-I levels (ng/ml; 6 weeks 774 ± 26 vs 694 ± 28; 11 weeks 1439 ± 182 vs 1516 ± 121). Hepatic IGF-I mRNA expression was already reduced at 6 weeks of age due to a significant decrease in the IGF-Ib transcript compared with lean controls (relative units; IGF-Ia: 99 ± 2% vs 100 ± 5%; IGF-Ib: 69 ± 10% vs 100 ± 2%, $P<0{\cdot}05$) and this reduction was more marked in 11-week-old animals when both IGF-I transcripts were significantly diminished (relative units; IGF-Ia: 80 ± 6% vs 100 ± 1%, $P<0{\cdot}05$; IGF-Ib: 65 ± 5% vs 100 ± 2%, $P<0{\cdot}01$). Extrahepatic tissues expressed almost exclusively the IGF-Ia transcript, the amount of which relative to controls was: (1) similar at 6 weeks and decreased at 11 weeks in kidney and skeletal muscle extracts (relative units; kidney: 6 weeks 88 ± 10% vs 100 ± 2%; 11 weeks 76 ± 3% vs 100 ± 4%, $P<0{\cdot}05$; vastus lateralis: 6 weeks 95 ± 7% vs 100 ± 10%; 11 weeks 59 ± 4% vs 100 ± 2%, $P<0{\cdot}001$); (2) similar at both ages in thoracic aorta (relative units; 6 weeks 121 ± 6% vs 105 ± 5%; 11 weeks: 91 ± 14% vs 100 ± 4%); and (3) increased at both ages in left ventricle extracts (relative units; 6 weeks 114 ± 2% vs 99 ± 9%, $P<0{\cdot}05$; 11 weeks 119 ± 7% vs 95 ± 3%, $P<0{\cdot}05$).

These data support the existence of tissue-specific dependence of IGF-I mRNA on GH levels during adulthood, reflected by the different behavior of IGF-I expression for each tissue in conditions of progressive decrease of GH levels.

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Introduction

Insulin-like growth factor I (IGF-I) is involved in the promotion of growth and differentiation of a variety of vertebrate tissues (Daughaday & Rotwein 1989). The liver is considered the primary source of endocrine IGF-I production in mammals, growth hormone (GH) being the major regulator of hepatic IGF-I synthesis and secretion (Schwander *et al.* 1983). In addition to liver, many other tissues synthesize IGF-I with essentially autocrine/paracrine functions but its regulation is less well known (D'Ercole *et al.* 1984, Moller *et al.* 1991). GH seems to regulate extrahepatic IGF-I production in conditions of severe deficit suggesting there is a minimal threshold level of this hormone below which local IGF-I production fails. Thus, IGF-I mRNA is decreased in variable degrees in most tissues of hypophysectomized (hx) rats and exogenous GH efficiently reverses this decrease (Murphy *et al.* 1987, Roberts *et al.* 1987). Two animal models of dwarfism have been used to study *in vivo* the GH dependence of IGF-I synthesis in extrahepatic tissues comparing it with regulation in the liver: the lit/lit mouse in which there are markedly reduced serum GH levels as a result of a recessive inactivating mutation (Mathews *et al.* 1986) and the sex-linked dwarf chicken lacking GH receptor protein because of an inappropriate splicing of the GH receptor gene transcript secondary to a mutation (Tanaka *et al.* 1996). In the first model, 13-week-old homozygous males showed a significant decrease in liver IGF-I mRNA level in the presence of a slight and GH-dependent decrease in kidney and brain, whereas expression in heart was increased but unchanged in testis and lung. In the second model, 4-week-old chickens with the mutant GH receptor showed no IGF-I mRNA expression in liver, but there

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were similar levels in extrahepatic tissues such as heart, muscle, testis or brain compared with normal controls. Aorta and kidney were not analyzed in this study.

The role of GH in extrahepatic IGF-I production in conditions of more physiological and progressive GH deficiency, such as associated with aging or obesity, is less well established and has been the recent focus of our laboratory research (Melián *et al.* 1997, Velasco *et al.* 1998). In this line of work the purpose of the present study was to characterize the IGF-I system in genetically obese Zucker rats as a model of obesity-associated relative GH deficiency. Using age-matched lean rats as controls, we examined serum IGF-I levels and IGF-I mRNA expression in liver, heart, aorta, kidney and skeletal muscle from 6-week-old rats (when the GH defect is already present) and 11-week-old rats (when it is maximal). To confirm dependency of GH in conditions of total endogenous lack of this hormone we also measured IGF-I mRNA in tissues from hx obese rats before and after one i.p. dose of GH.

Materials and Methods

Animals and experimental design

Male lean (Fa/−) and genetically obese (fa/fa) rats were obtained from Criffa (Barcelona, Spain). Animals were freely fed a standard chow (RMN Labsure, Biosure, Barcelona, Spain) and water, and were housed at constant temperature (23 °C) with a fixed (12 h:12 h) light: darkness cycle. After adaptation, animals were anesthetized and killed by decapitation at 0900 h. Blood was collected from the cervical vessels and serum was stored at −20 °C until assayed for glycemia, insulinemia, rat GH and IGF-I. Liver, heart, aorta, kidneys and vastus lateralis muscle were removed rapidly, snap-frozen in dry ice, and stored at −80 °C. At the time of the experiments animals were 6 or 11 weeks old.

Hx male obese rats were delivered to our animal facility 7 days after hypophysectomy (Criffa, Lyon, France). The adequacy of hypophysectomy was assessed at the time of killing by visual inspection of sella turcica. Rats were housed for 5 days under controlled conditions, freely feeding and with water with 5% glucose and 0·9% NaCl. After adaptation, rats received one i.p. dose of 1·5 μg/g body weight of recombinant human GH (rhGH) and were decapitated 4 or 8 h later. At the time of the experiments animals were 11 weeks old. Tissues were collected and stored at −80 °C.

RIAs and biochemical parameters

IGF-I was measured by a commercial RIA (Nichols Institute, San Juan Capistrano, CA, USA) after acid–ethanol extraction. Rat GH was determined using the National Pituitary Hormone Distribution Program rat hormone kit (National Hormone and Pituitary Program of the National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, MD, USA), with a sensitivity limit of 0·8 μg/l. Serum insulin was measured using a commercial kit (Coat-a-Count Insulin, Diagnostic Products Corp., Los Angeles, CA, USA) and serum glucose by the glucose oxidase method (ITC Diagnostics).

All the parameters were measured in serum from individual rats and the samples of groups to be compared were analyzed in the same assay, to avoid interassay variations.

RNA probes

IGF-I One construct of 376 bp from the rat IGF-I cDNA, containing part of the A domain, the entire D and E domains and part of the 3′-untranslated region was generated in order to simultaneously quantify IGF-Ia and IGF-Ib mRNAs, yielding protected fragments of 224 and 376 bp respectively. This is due to a 52 bp insert present in the E domain resulting in two different transcripts depending on whether exon 4 is spliced to exon 5 (Eb region: IGF-Ib) or exon 6 (Ea region: IGF-Ia). For the protection assay this template was linearized with HindIII and transcribed with T7 RNA polymerase (Lowe *et al.* 1988).

Cyclophilin The rat cyclophilin cDNA was a 132 bp fragment linearized with ApaI and transcribed in order to generate the antisense probe with SP6 polymerase following previously described methods (Danielson *et al.* 1988).

Ribonuclease protection assay (RPA)

RNA was extracted using the Chomczynski and Sacchi (1987) method. In the RPA, total RNA of each individual rat was hybridized overnight with approximately 600 000 c.p.m. labeled antisense rat IGF-I riboprobe at 45 °C. The hybridization solution contained 75% (vol/vol) formamide, 80 mM Tris–HCl pH 7·6, 4 mM EDTA, 1·6 M NaCl and 0·4% SDS. After hybridization, samples were digested using RNAse A (40 mg/ml) and RNAse T1 (2 mg/ml) for 1 h at 30 °C. Protected hybrids were isolated by ethanol precipitation after phenol–chloroform extraction and separated according to size on an 8% polyacrylamide/8 M urea denaturing gel. Gels were exposed to X-ray film (Kodak, Cambridge, UK) at −80 °C for 24–96 h. Quantitation of the intensities of the autoradiography bands corresponding to protected hybrids was done by densitometric scanning using Adobe-Photoshop 2·0 and NIH-Image 1·47 programs for the Apple Macintosh (Wpertino, CA, USA). All samples were hybridized at the same time with cyclophilin in order to correct for the differences in gel loading.

Table 1 Body weight and serum parameters in 6- and 11-week-old lean and obese Zucker rats. Values are means ± S.E.M. ($n=5$)

	Body weight (g)	Glucose (mg/dl)	Insulin (mU/ml)	I/Glu	Rat GH (ng/ml)	IGF-I (ng/ml)
6 weeks						
Lean	162 ± 3·3	164 ± 2·7	44·7 ± 6·5	0·24 ± 0·04	2·7 ± 1·1	694·4 ± 28
Obese	200 ± 3·5***	155 ± 5·0	138 ± 10·0***	0·92 ± 0·08***	1·7 ± 0·9	774·6 ± 26
11 weeks						
Lean	257 ± 3·7	142 ± 5·2	46·3 ± 3·0	0·32 ± 0·02	4·3 ± 1·2	1516 ± 121
Obese	381 ± 6·7***	148 ± 5·0	147 ± 14·0***	1·0 ± 1·0***	2·4 ± 0·9	1439 ± 182

***$P<0·001$ vs lean animals at the same age.

Statistical analysis

All values are means ± S.E.M. The statistical significance of differences between values was calculated by unpaired Student's *t*-test and/or variance analysis. The differences were considered statistically significant when P values were below 0·05.

Results

Six-week-old lean and obese Zucker rats

As shown in Table 1, at 6 weeks obese rats were already significantly heavier that lean controls. Serum IGF-I levels were comparable between phenotypes, in the presence of reduced GH levels in obese rats. Statistics for GH were avoided because some rats showed GH levels below the limit of detection of the RIA and were arbitrarily assigned the minimum value of sensitivity of the assay. As already described, obese rats showed no significant differences in glucose levels compared with lean, but were severely hyperinsulinemic and had increased basal insulin/glucose (I/Glu) ratio as an expression of their insulin resistance (Bray 1979).

IGF-I mRNA levels in tissues of 6-week-old lean and obese Zucker rats and its relative expression are shown in Figs 1 and 2 respectively. Both IGF-I transcripts were present in the liver, the IGF-Ib transcript being significantly decreased in obese rats vs lean (relative units; 69 ± 10% vs 100 ± 2%, $P<0·05$) and the IGF-Ia transcript being similar (99 ± 2% vs 100 ± 5%). In contrast IGF-Ib was barely detectable in extrahepatic tissues (unmeasurable in kidney and absent in heart, aorta or skeletal muscle) whereas IGF-Ia was clearly detectable. At this age we found no significant differences in IGF-I mRNA expression between obese animals and their lean littermates for aorta, kidney or skeletal muscle while there was an increased amount in the heart – left ventricle – of obese rats (119 ± 6·6% vs 95 ± 2·8%, $P<0·05$).

Eleven-week-old lean and obese Zucker rats

At 11 weeks lean and obese rats showed an increase of weight associated with the progression of age, the difference in body weight of obese rats vs lean controls being more evident than at 6 weeks. Serum IGF-I levels were comparable between phenotypes, in the presence of reduced GH levels in obese rats. As was the case at 6 weeks, obese rats showed similar glucose levels compared with the lean ones, severe hyperinsulinemia and an increased I/Glu ratio.

IGF-I mRNA levels in tissues of 11-week-old lean and obese Zucker rats and their relative expression are shown

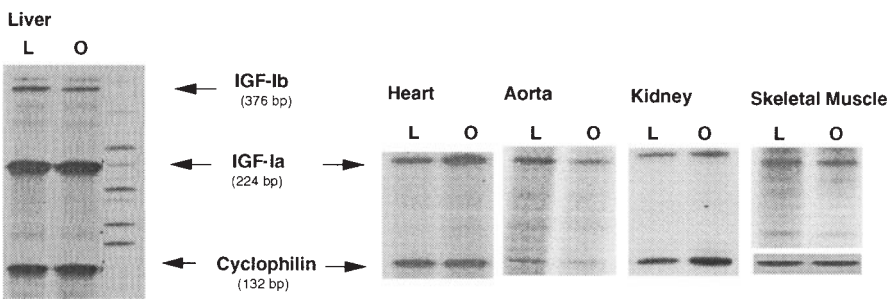

Figure 1 RPA of IGF-I mRNA levels in liver, heart, aorta, kidney and skeletal muscle from 6-week-old lean (L) and obese (O) Zucker rats. Ten to twenty micrograms total RNA from each tissue were subjected to solution hybridization/RPA using the antisense probes described in Materials and Methods. The position of each protected fragment is indicated by an arrow.

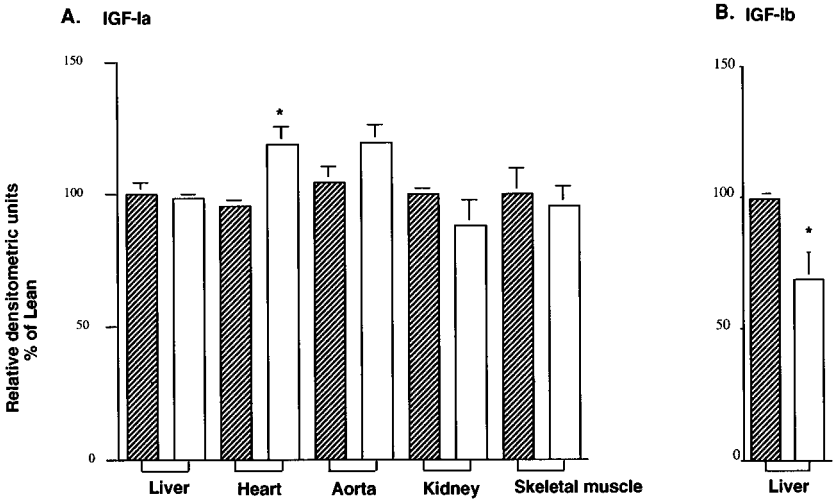

Figure 2 Relative levels of IGF-I mRNA expression in tissues of lean (hatched columns) and obese (open columns) Zucker rats at 6 weeks of age. After correction of both transcripts for cyclophilin levels, relative densitometric units were adjusted so that the ratio obtained from tissues of intact lean rats equaled 100. Results are means ± S.E.M. (n=3–5). *P<0·05 vs lean rats.

in Figs 3 and 4 respectively. At this age both hepatic IGF-I mRNA transcripts were decreased in the obese group, this decrease being more evident for the IGF-Ib transcript (relative units; IGF-Ia: 80 ± 6% vs 100 ± 1%, P<0·05; IGF-Ib: 65 ± 5% vs 100 ± 2%, P<0·01). IGF-I mRNA expression compared with controls was: (1) similar for aorta extracts; (2) decreased for kidney and skeletal muscle extracts (kidney: 76 ± 3% vs 100 ± 4%, P<0·05; muscle: 59 ± 4% vs 100 ± 2%, P<0·001); and (3) increased for left-ventricle (114 ± 2% vs 100 ± 9%, P<0·05).

To confirm that extrahepatic tissues from obese Zucker rats showed GH dependence in conditions of absolute deprivation, we measured IGF-I mRNA amounts in liver and extrahepatic tissues from 11-week-old hx obese rats before and 4 and 8 h after acute rhGH administration (skeletal muscle not done). Figure 5 shows the quantitation of IGF-Ia and IGF-Ib transcripts in this group of rats. In the liver, rhGH administration increased 10 times the IGF-Ib and 2·5 times the IGF-Ia transcripts over basal values. Additionally in all the extrahepatic tissues studied, exogenous rhGH was able to induce IGF-I gene expression. A remarkable response was observed in aorta and heart where basal lGF-I amount was very low in the absence of GH.

Discussion

At the two ages studied obese and GH-deficient Zucker rats had serum levels of IGF-I similar to those of lean littermates in the presence of decreased hepatic mRNA expression, which was more marked at 11 weeks. Extrahepatic IGF-I mRNA showed different patterns of expression with decreased levels in skeletal muscle and kidney at 11 weeks, normal levels in aorta at both ages and increased levels in heart at both ages. At present the factors that regulate gene expression of IGF-I in adult extrahepatic tissues and the molecular basis for the tissue differences in GH responsiveness are unknown. Obese Zucker rats develop progressive and partial GH deficiency through adult life (Gelato & Berelowitz 1994) and provide a useful model to evaluate GH dependence in conditions more physiological than GH deprivation by hypophysectomy or transgenic models. Conscious of the limitations derived from the analysis of only one sample of serum GH, we chose for our purpose animals of two ages where the degree of GH deficiency in obese rats is well established. Thus, it has been demonstrated that plasma levels of GH, pulsatile GH release, pituitary GH concentration, and mRNA expression are reduced in obese male rats when compared with lean littermates, and that this defect is maximal at 10–12 weeks of age (Finkelstein *et al.* 1986, Ahmad *et al.* 1990, Tannenbaum *et al.* 1990, Leidy *et al.* 1993).

IGF-I of endocrine action is primarily derived from liver and GH has a crucial role in its synthesis and secretion (Schwander *et al.* 1983). Hepatic IGF-I mRNA expression in GH-deficient models such as aging rats or dwarfism is diminished in direct correlation to the low GH (Mathews *et al.* 1986, Tanaka *et al.* 1996, Velasco *et al.* 1998). In agreement with these reports, Zucker rats showed a decrease in hepatic IGF-I mRNA levels compared with lean which was more marked in 11-week-old rats. These results are in concordance with previous studies

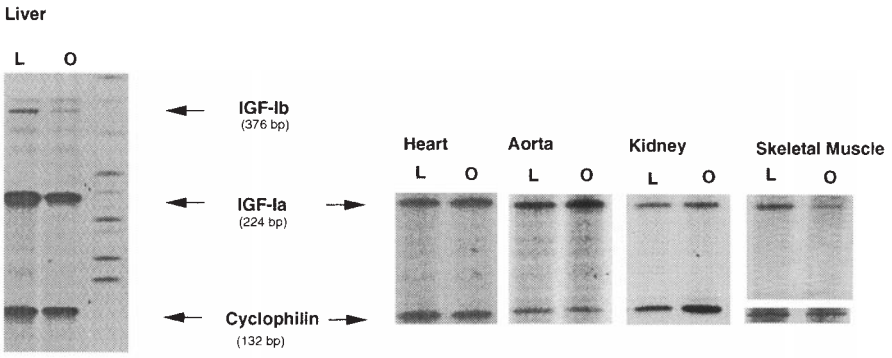

Figure 3 RPA of IGF-I mRNA levels in liver, heart, aorta, kidney and skeletal muscle from 11-week-old lean (L) and obese (O) Zucker rats. Ten to twenty micrograms total RNA from each tissue were subjected to solution hybridization/RPA using the antisense probes described in Materials and Methods. The position of each protected fragment is indicated by an arrow.

performed in our laboratory where we found that hepatic IGF-I and insulin-like growth factor-binding protein-3 (IGFBP-3) mRNA expression in 11-week-old obese Zucker rats under different GH conditions tightly correlated with GH levels (Melián *et al.* 1997). However, obese rats had serum IGF-I levels comparable to lean at both ages, in spite of GH differences. In fact, as occurs in children with idiopathic obesity, obese Zucker rats have normal or increased rates of linear growth accompanied by normal or increased circulating IGF-I levels (Vignolo *et al.* 1988, Nguyen-Yamamoto *et al.* 1994). Moreover, obese rats, like sheep or humans, show a greater response to exogenous GH in serum IGF-I compared with lean controls (Nguyen-Yamamoto *et al.* 1994, McCann *et al.* 1997). It has been suggested that obesity-associated hyperinsulinemia could be responsible for this finding by directly increasing hepatic mRNA transcription, as apparently occurs in primary cultures of hepatocytes, even in the absence of GH (Boni-Schnetzler *et al.* 1991). Our data do not support this hypothesis since severely hyperinsulinemic obese rats showed a progressive decrease in liver IGF-I mRNA levels compared with lean as GH deficiency progressed. Moreover, hyperinsulinemia was not associated with an increase in the hepatic IGF-I amount under

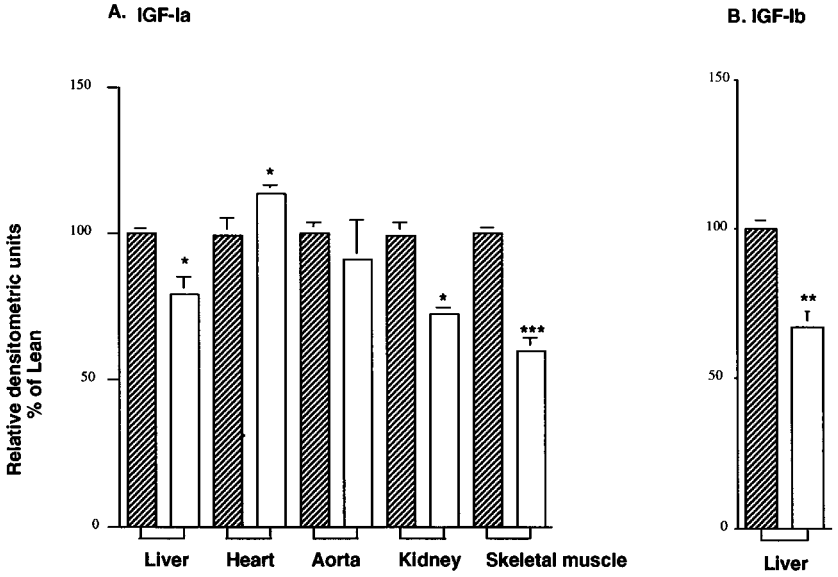

Figure 4 Relative levels of IGF-I mRNA expression in tissues of lean (hatched columns) and obese (open columns) Zucker rats at 11 weeks of age. After correction of both transcripts for cyclophilin levels, relative densitometric units were adjusted so that the ratio obtained from tissues of intact lean rats equaled 100. Results are means ± S.E.M. (n=3–5). *P<0·05, **P<0·01 and ***P<0·001 vs lean rats.

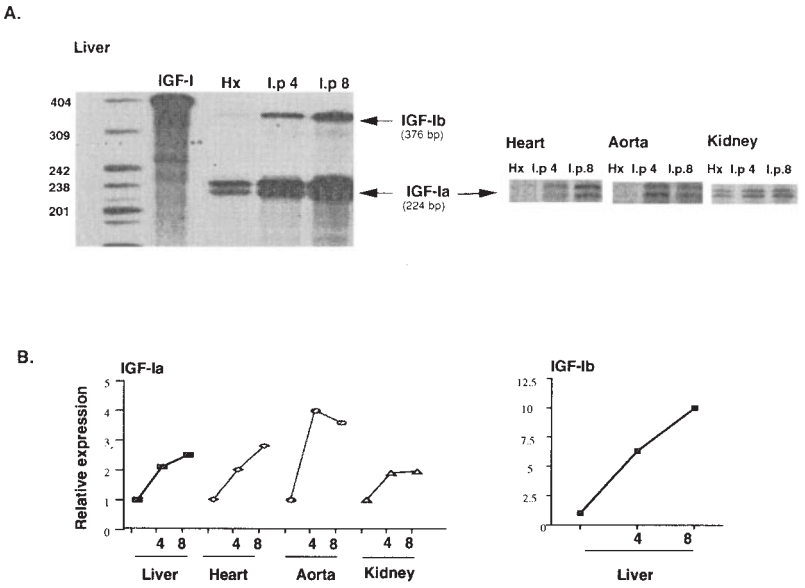

Figure 5 (A) Representative RPA of IGF-I mRNA levels in liver, heart, aorta and kidney from hx 11-week-old obese Zucker rats before and 4 or 8 h after acute rhGH treatment. (B) Relative levels of IGF-I mRNA expression. For this purpose the mean IGF-I band abundance in hx rats was assigned a value of 1, since cyclophilin was also partially inducible by acute GH treatment.

comparable GH levels (Melián *et al.* 1997). Taken together, these data suggest that obesity-associated imbalance between low serum GH/normal serum IGF-I levels is primarily due to translational or post-translational events distal to the GH-dependent decrease of IGF-I expression in the liver. Changes in the final amount of free IGF-I secondary to modulation of IGFBPs by insulin and/or other nutritional factors could have a relevant role in this sense (Thissen *et al.* 1994). In fact obese Zucker rats have, compared with lean, low hepatic mRNA amounts but similar serum levels of IGFBP-3 (Nguyen-Yamamoto *et al.* 1994, Melián *et al.* 1997) suggesting that post-transcriptional events regulate serum levels of this binding protein (Albinston & Herington 1992). On the other hand these rats show a 67% suppression of IGFBP-1 serum levels (Lewitt *et al.* 1994). This decrease could account for an increase in free IGF-I without alteration in total IGF-I levels as has been described in human obesity, showing direct correlation with hyperinsulinemia and inverse correlation with IGFBP-I levels (Frystyk *et al.* 1995).

The hepatic IGF-Ib transcript appears to be more tightly regulated by GH than IGF-Ia. Its decrease was already manifest at a younger age and was more marked in hx animals. Indeed several data support the view that the rat hepatic IGF-Ib mRNA variant of the IGF-I gene may be more sensitive to GH status (Mathews *et al.* 1986, Lowe *et al.* 1988, 1989, Tanaka *et al.* 1996, Melián *et al.* 1997, Velasco *et al.* 1998). A specific role for the Eb mRNA in directing hepatic IGF-I to the circulation on the basis of its post-natal presence, mainly in the liver, and its higher responsiveness to GH has been suggested, but there is little evidence to support this at present (Gilmour 1994).

Extrahepatic tissues express predominantly the IGF-Ia transcript and it is clear that, at least in rats, most tissues show ability to respond to acute GH treatment when severely deprived of GH by hx (Hynes *et al.* 1987, Roberts *et al.* 1987). Therefore the fact that extrahepatic tissues from hx Zucker rats elicit a remarkable response of local IGF-I mRNA expression after one i.p. rhGH dose is not surprising. However, the physiological role of GH on extrahepatic IGF-I gene expression should be evaluated in conditions of progressive and relative GH decline, when significant GH levels are still present. In our model we found a variable behavior in extrahepatic tissues of obese rats when compared with lean littermates. Skeletal muscle and kidney showed similar IGF-I mRNA expression at 6 weeks and decreased expression at 11 weeks with a more relevant reduction for skeletal muscle. In contrast, the level of aorta IGF-I mRNA was similar and that in left ventricle was higher than in controls at the two ages studied, suggesting in both cases its GH independence. Considering that aorta and heart showed almost undetectable levels of IGF-I mRNA in hx rats we suggest there might exist a differential threshold level of GH for each extrahepatic tissue which may condition full expression of the GH-dependent component of IGF-I gene transcription. This threshold level would be higher for skeletal muscle, intermediate for kidney and lower for aorta and heart. This

contention, consistent with data from other models of moderate GH deficits (Mathews *et al.* 1986, Lemmey *et al.* 1997, B Velasco, unpublished observations) could be due to tissue-specific affinities of the GH receptor for its ligand (Wells *et al.* 1993).

It could be argued that hyperinsulinemia associated with obesity in Zucker rats from 3 to 5 weeks of age could be attenuating a GH-dependent decrease of IGF-I mRNA expression in extrahepatic tissues, since insulin has been found to slightly increase IGF-I mRNA expression in the rat aorta *in vivo* (Murphy *et al.* 1990). Moreover, chronic insulin administration in pituitary-intact rats has been associated with an increase of IGF-I mRNA in kidney and heart from Sprague–Dawley rats, although not statistically supported (Salamon *et al.* 1989). Our data cannot exclude hyperinsulinemia as a cause of no decrease in IGF-I mRNA expression in heart and aorta from obese rats at the two ages. However, three arguments can be invoked against this hypothesis: (1) the consistency of our findings with those observed in the non-hyperinsulinemic GH-deficient lit/lit mouse (Mathews *et al.* 1986); (2) the fact that factitious hyperinsulinemia is not associated with overexpression of IGF-I in aorta, heart or skeletal muscle of normoglycemic diabetic rats (Bornfeld *et al.* 1992); and (3) the absence of increased IGF-I mRNA levels compared with lean in hearts of intact 11-week-old hyperinsulinemic obese rats killed 6 h after one dose of rhGH (1·5 μg/g body weight) (data not shown).

Finally, the existence of differences between species in the steady-state level of IGF-I mRNA and its sensitivity to GH has been described (Moller *et al.* 1991). This fact has been more solidly established in skeletal muscle because of its easier access. IGF-I gene expression in muscle appears to be less sensitive to GH in humans, pigs or chickens (Weller *et al.* 1994, Hamilton *et al.* 1995, Tanaka *et al.* 1996) and more in sheep (Oldham *et al.* 1996). In rats, skeletal IGF-I mRNA expression seems to be highly sensitive to GH in hx and non-hx models (Murphy *et al.* 1987, Isgaard *et al.* 1989, Moller *et al.* 1991, Gosteli-Peter *et al.* 1994). Whether the severe local IGF-I decrease in skeletal muscle of 11-week-old obese rats has some relevance in the alteration of the skeletal structure and functionality of these animals is something not settled at the moment (King & Betts 1994, He *et al.* 1995).

In summary, our data suggest the existence of considerable differences between adult tissues with regard to GH dependence of IGF-I gene expression. Models of partial and progressive GH deficit, as obesity or aging, can provide a useful system to elucidate GH-dependent and -independent mechanisms in the regulation of IGF-I in physiological situations during adulthood.

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