Regulation of Insulin-Like Growth Factor–Binding Proteins in Rats with Insulin-Dependent Diabetes Mellitus (43944)

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Abstract. **As previously reported, activation of the adrenocorticotropic hormone (ACTH)–adrenal cortical axis in rats with insulin-dependent diabetes mellitus (IDDM) reduces their growth and circulating insulin-like growth factor–I (IGF-I) levels and induces a resistance to growth hormone (GH) and IGF-I. The studies reported herein were conducted to determine whether the pituitary and/or adrenal gland influence the changes in basal and GH-stimulated serum concentrations of IGF-binding proteins (IGFBPs) in rats with IDDM. Male rats were made diabetic by injections of streptozotocin. Intact nondiabetic (NonDb), diabetic (Db), hypophysectomized diabetic (HxDb), and adrenalectomized diabetic (AxDb) rats were injected twice daily with 50 μg porcine (p) GH or with 0.9% saline for 2 weeks following the surgeries. Changes in serum IGFBP concentrations were determined by Western ligand- or immuno-blot analysis.**

Neither IGFBP-5 nor -6 was detected in any of the treatment groups. Induction of IDDM increased serum concentrations of IGFBP-1 and -2 and reduced those of IGFBP-3 and -4. Although serum IGFBP-1 and -2 concentrations remained elevated in the HxDb rats compared with the NonDb controls, IGFBP-1 levels were reduced compared with those in the Db controls. Serum IGFBP-3 and -4 were reduced to levels below those in Db controls. Although IGFBP-3 and -4 concentrations were elevated to normal in AxDb rats, the IGFBP-2 concentration was increased above those in both NonDb and Db rats and the IGFBP-1 concentration was reduced.

Administration of pGH increased serum IGFBP-4 concentrations in all groups and IGFBP-3 concentrations in all groups except the Db. In addition, pGH reduced the concentration of IGFBP-1 in HxDb rats and nearly abolished it in AxDb rats, but had no effect on IGFBP-1 concentration in NonDb or Db rats. Administration of corticosterone (B; 25 μg/ml of 0.9% saline drinking water) to AxDb rats restored Db-like profiles of all IGFBPs.

The refractoriness of Db rats to pGH is associated with a failure of the hormone to elevate IGFBP-2 and -3 titers and to reduce those of IGFBP-1. Adrenal B production appears to be responsible for this resistance to GH. However, the elevated IGFBP-2 concentration in Db rats does not appear to be due to B or any other pituitary-controlled or -derived factors. Impaired growth was associated with substantially reduced IGFBP-3 concentrations and elevated IGFBP-1, whereas growth restoration was associated with the opposite changes. [P.S.E.B.M. 1995, Vol 210]

Reduced growth and lowered circulating insulin-like growth factor–I (IGF-I) concentrations are often associated with uncontrolled insulin-dependent diabetes mellitus (IDDM) (1–3). Administration of growth hormone (GH) to diabetic rats or humans fails to restore growth or the depressed IGF-I levels to normal (1–3). In addition, tissues from rats with IDDM are resistant to the anabolic actions of

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IGF-I *in vitro* (4). We and others have previously reported that hypophysectomy and/or adrenalectomy of rats with IDDM alleviate their metabolic symptoms and partially restore the ability of GH to augment somatic growth and circulating IGF-I levels *in vivo* (5–8). Furthermore, hypophysectomy restores cartilage responsiveness to IGF-I *in vitro* in these animals (4). Replacement of corticosterone (B) to adrenalectomized diabetic (AxDb) rats re-established GH resistance and suppressed growth in the same study (8). Therefore, it appears that activation of the adrenocorticotropic hormone (ACTH)–adrenal cortical axis contributes to the GH resistance in rats with IDDM.

Several IGF-binding proteins (IGFBPs) are thought to influence IGF-I availability and action both *in vitro* and *in vivo* (9). Therefore, changes in the circulating concentrations of IGFBPs may also contribute to diabetic growth impairment. IDDM is associated with reduced circulating concentrations of IGFBP-3 and IGFBP-4, and elevated IGFBP-1 and IGFBP-2 concentrations (10–14). Various factors influence IGFBP synthesis and serum concentrations including GH, insulin, B, glucagon, catecholamines, and glucose (15–17). In most instances, it is unclear how these factors influence the changes in circulating IGFBP levels associated with IDDM. Circulating B levels rise with IDDM (18); it is possible, therefore, that B influences IGFBP production directly as well as indirectly by maintaining GH resistance. The studies reported herein were conducted to determine whether the pituitary and/or adrenal gland influence the changes in basal and GH-stimulated serum concentrations of IGFBPs in rats with IDDM.

Material and Methods

Animals. Male Long-Evans rats weighing 120–140 g were purchased from Simonsen Laboratories, Inc. (Gilroy, CA). They were maintained in an environmentally controlled room at 23° ± 1°C, with a 12:12-hr light:dark cycle, and were fed *ad libitum*. All experiments were described in detail in a protocol that was approved by our Institutional Animal Care and Use Committee. The care and use of the rats were in strict compliance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

Treatments, Surgeries, and Experimental Design. Rats were made diabetic by two intraperitoneal (ip) injections of streptozotocin (STZ; Sigma Chemical Co., St. Louis, MO) at 85 mg/kg body wt and randomly place into their respective groups (four rats/cage and one cage/treatment group). The STZ was dissolved in sterile 0.9% NaCl, pH 4.5, immediately before use. Urine glucose and ketone bodies were checked in the morning of the 3rd day with Chemstrip urine test strips (Boehringer-Mannheim, Indianapolis, IN) and rats with <5% urine glucose and those that were ketotic were excluded in order to reduce the range of the severity of diabetes among the animals. The remaining rats were injected subcutaneously twice daily for 2 days with 15 IU recombinant human insulin (Novolin; Novo Nordisk Pharmaceuticals Inc., Princeton, NJ) to normalize their metabolic status before surgery. This therapy enables the animals to survive the procedures of hypophysectomy or adrenalectomy. On the following day, they were anesthetized by an ip injection of 100 mg/ml ketamine-HCl (Aveco, Fort Dodge, IA) and 10 mg/ml acepromazine maleate (Fermenta, Kansas City, MO) solution at a dose of 1 ml/kg body wt. They were subsequently anesthetized and either hypophysectomized (Hx) (19) or were bilaterally adrenalectomized (Ax). The latter were given 0.9% NaCl to drink. Rats were injected ip with 100 μl of either sterile 0.9% NaCl vehicle or 50 μg pGH/injection (Monsanto Co., St. Louis, MO) twice daily for 14 days. The pGH was dissolved in a 0.03 *M* $NaHCO_3$ and 0.15 *M* NaCl solution at pH 9.5 and brought up to an injection volume of 50 μg/100 μl with sterile 0.9% NaCl. The purity of the pGH (lot #v202-001) was determined to be 94% by HPLC. In a separate experiment, B (Sigma) was dissolved in 100% ethanol and then added to the 0.9% saline drinking solution of AxDb rats at a concentration of 25 μg B/ml 0.9% saline and 0.2% ethanol for a period of 14 days. The purity of B (lot #42H0855) was determined to be 99.7% by HPLC. Both experiments were subsequently repeated.

Western Ligand- and Immuno-Blot Analysis. Changes in circulating IGFBP-3 and -4 levels were analyzed by Western ligand- and immuno-blotting of serum samples from individuals (20). Several rat IGFBPs exist in the molecular mass range of 32–34 kDa. Therefore, individual IGFBPs within this range, including IGFBP-1 and IGFBP-2, cannot be reliably distinguished by ligand-blot analysis (21). Furthermore, the relatively close proximity of the glycosylated form of IGFBP-4 (28 kDa) to these IGFBPs could contribute to the confusion. Accordingly, changes in IGFBP-1 and IGFBP-2 levels were analyzed by Western immunoblotting of pooled serum samples (22). Serum samples were taken from each rat and were pooled for each treatment group. The immuno-blots shown in Figure 3 and 4 are representative of three Westerns run independently; thus differences are not due to loading efficiency.

Anti-rat IGFBP polyclonal rabbit antisera were generated against synthetic peptide fragments and were donated by Dr. Nicholas Ling of the Whittier Institute (La Jolla, CA). Although anti-IGFBP-2

through -6 have been previously characterized (22) and were found not to significantly cross-react with other IGFBPs (22), the antiserum generated against IGFBP-1 recognized a 45-kDa protein as well as the 30-kDa IGFBP-1. Serum aliquots were first subjected to SDS-PAGE under nonreducing conditions using 12.5% acrylamide separating and 4% acrylamide stacking slab gels. Separated proteins were transfered to a 0.45-μm nitrocellulose Trans-Blot transfer medium (Bio-Rad, Richmond, CA) using a Semi-Dry Transfer Cell (Bio-Rad) and a 48 m*M* Tris base and 39 m*M* glycine transfer buffer. The nitrocellulose was then blocked by an overnight incubation at 4°C, in 1% nonfat dry milk for ligand-blots or 1% bovine serum albumin (BSA) for immuno-blots, 20 m*M* Tris base, 500 m*M* NaCl, and 0.05% Tween-20 solution at pH 7.5 (TTBS-milk or -BSA). Ligand-blots were probed overnight at 4°C with 150,000 CPM ^{125}I-IGF-I/ml TTBS-BSA and exposed to a Phosphorimager SF (Molecular Dynamics, Sunnyvale, CA) cassette for 2 days and were analyzed by densitometry using the ImageQuant (Molecular Dynamics) program. Immuno-blots were incubated overnight at 4°C, in TTBS-BSA, with antisera dilutions of 1:500 for IGFBP-3 and 1:2000 for other IGFBPs, followed by a 12-hr incubation at 4°C with a peroxidase-conjugated AffiniPure goat-anti-rabbit antiserum (lot #25612) at a dilution of 1:4000 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Positive immunoreactions were detected using enhanced chemiluminescence reagents (Amersham Co., Arlington Heights, IL). Autoradiograms of the immunoblots were scanned with a UMAX UC630 scanner and analyzed by densitometry with NIH Image for the Macintosh.

Data Analysis. Statistical differences in mean optical density units of Western ligand-blots were analyzed by a one- or two-way analysis of variance in conjunction with Fisher's least significance difference test for pairwise mean comparisons. These analyses were performed using the Systat 5 computer program for the Macintosh (SYSTAT Inc., Evanston, IL). In each histogram, significant differences are indicated by different letters ($P \leq 0.001$–0.05), whereas the same letters indicate no differences.

Results

Ligand-Blot Analysis of Serum IGFBPs. A ligand-blot of serum samples pooled from each rat in a treatment group is shown in Figure 1. Circulating concentrations of IGFBP-3 (Fig. 2) and IGFBP-4 (Fig. 3) in the Db controls (saline-injected = control) were reduced by 68% ($P \leq 0.05$) and 42% ($P \leq 0.01$), respectively, compared with those in NonDb controls. In the HxDb controls, IGFBP-3 and -4 concentrations were further reduced by 92% ($P \leq 0.001$) and 60%, respectively, ($P \leq 0.001$) of NonDb control values. By contrast, circulating concentrations of both IGFBP-3 and -4 in the AxDb controls were restored to equal those in the NonDb controls.

Compared with the respective saline injections, injections of pGH significantly increased the circulating concentrations of IGFBP-3 in NonDb, HxDb, and AxDb rats by 260% ($P \leq 0.001$), 171% ($P \leq 0.01$), and 160% ($P \leq 0.001$), respectively, but were ineffective in Db rats. The concentration of IGFBP-4 was also elevated by pGH injections in these groups by 57% ($P \leq 0.001$), 72% ($P \leq 0.01$), and 25% ($P \leq 0.01$), respectively, and by 34% ($P \leq 0.05$) in the Db rats.

Concentrations of IGFBP-3 and IGFBP-4 were reduced in AxDb controls receiving B in their drinking water by 57% ($P \leq 0.01$) and 79% ($P \leq 0.001$), respectively, compared to AxDb controls drinking only saline (Fig. 4). Thus, serum concentrations of both IGFBPs were restored towards Db-like levels by B administration. The ability of pGH to augment circulating IGFBP-3 and -4 levels in AxDb rats was suppressed by administration of B.

Immuno-Blot Analysis of Serum IGFBPs. No significant binding was detected with the use of non-immune serum as a negative control for the immuno-blots. The results of immuno-blotting for IGFBP-3 and -4 are similar to the ligand-blotting results (Fig. 3a and 4a). Serum concentrations of immunoreactive IGFBP-1 and IGFBP-2 increased in the Db controls compared to those in NonDb controls (Fig. 5). Hypophysectomy of Db rats appeared to slightly reduce the elevated levels of IGFBP-1 and IGFBP-2. The concentration of IGFBP-1 was also reduced by adrenal-

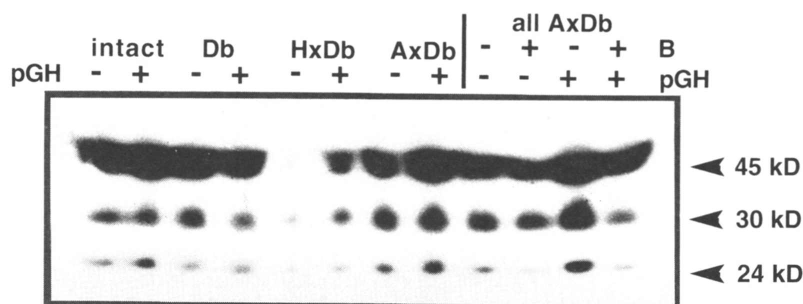
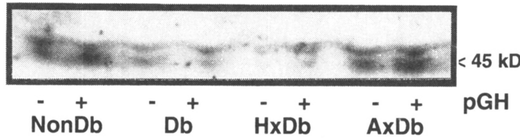


Figure 1. Western ligand-blot of serum samples pooled from six or seven rats per treatment group.

a. immuno-blot for IGFBP-3



b. ligand-blot ODUs for IGFBP-3

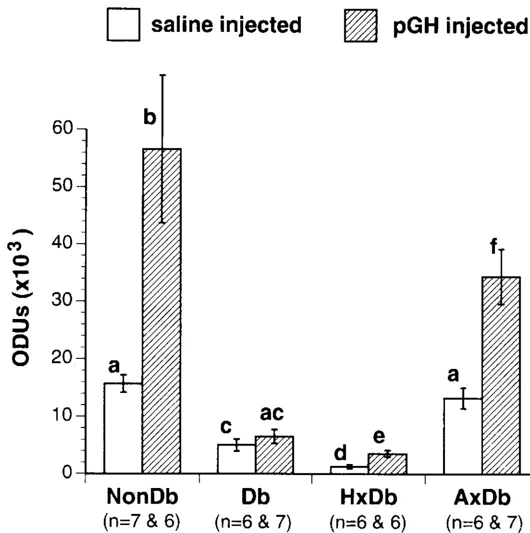
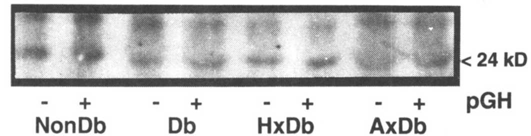


Figure 2. Serum concentrations of IGFBP-3 in intact nondiabetic (NonDb), diabetic (Db), hypophysectomized diabetic (HxDb), and adrenalectomized diabetic (AxDb) rats (± 50 μg pGH 2×/day) as determined by Western (a) immuno-blotting of pooled serum samples for each treatment group and (b) ligand-blotting of individual samples (note the difference in scale from other figures). Histogram values are mean ± SEM (*n* = saline-injected and pGH-injected). Significant differences between any two means are indicated by different letters ($P \leq 0.05$), whereas the same letters indicate no differences.

a. immuno-blot for IGFBP-4



b. ligand-blot ODUs for IGFBP-4

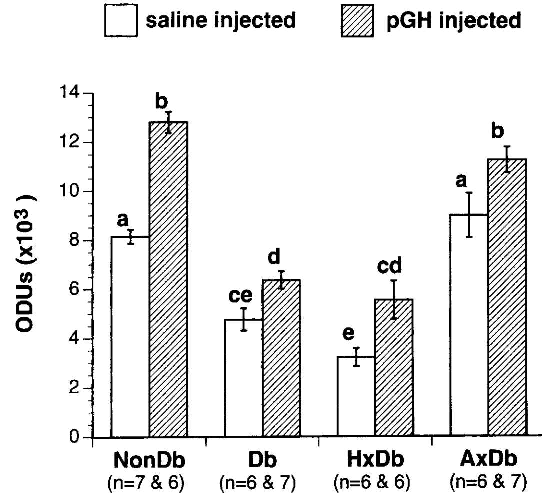


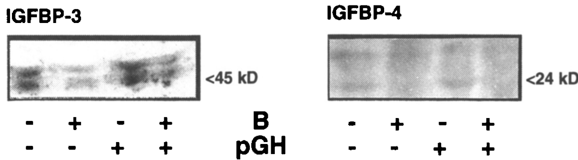
Figure 3. Serum concentrations of IGFBP-4 in intact nondiabetic (NonDb), diabetic (Db), hypophysectomized diabetic (HxDb), and adrenalectomized diabetic (AxDb) rats (± 50 μg pGH 2×/day) as determined by Western (a) immuno- and (b) ligand-blotting (note the difference in scale from other figures). Otherwise as in the legend for Figure 2.

ectomy. By contrast, the concentration of IGFBP-2 was increased above Db levels in the AxDb controls. The serum IGFBP-1 concentration was elevated and that of IGFBP-2 was reduced in AxDb controls receiving B in their drinking water compared with AxDb controls drinking only saline (Fig. 6). Thus, B administration restored Db-like serum levels of both IGFBPs.

The concentrations of immunoreactive IGFBP-1 and IGFBP-2 were unaffected by pGH injections in NonDb and Db rats. However, compared with the saline injections, pGH injections reduced the elevated IGFBP-1 concentration in the HxDb and AxDb rats. Injections of pGH failed to influence the IGFBP-2 concentration in AxDb rats, but they elevated its concentration in HxDb rats, compared with the respective saline controls. By contrast, injections of pGH had no effect on IGFBP-1 and -2 levels in AxDb rats receiving B.

Discussion

Relationships among Changes in IGFBP Concentrations and Growth Responses. The changes in circulating IGFBP-3 and -4 may be due to protease activity, although no bands were detected below the expected molecular weight range with immunoblotting techniques. However, the antisera used may not have recognize the proteolyzed IGFBPs. Thus, it is impossible to determine whether the changes are due to proteolysis, synthesis or just release of the specific proteins. The changes discussed are therefore representative of intact protein levels only. Our results, which are summarized in Figure 7, are consistent with previous studies which demonstrated that insulin deficiency reduces serum concentrations of IGFBP-3 and -4 and elevates the concentrations of IGFBP-1 and -2 (10–14). These changes are accompanied by a significant reduction in serum IGF-I concentration and growth (1–3, 7, 8). When Db rats are hypophysectomized, their growth and serum IGF-I concentration are further reduced (7, 8). Relative to the Db controls, serum IGFBP-2 and -4 concentrations decreased slightly in the HxDb rats, but those of IGFBP-1 and -3 were reduced considerably. This re-



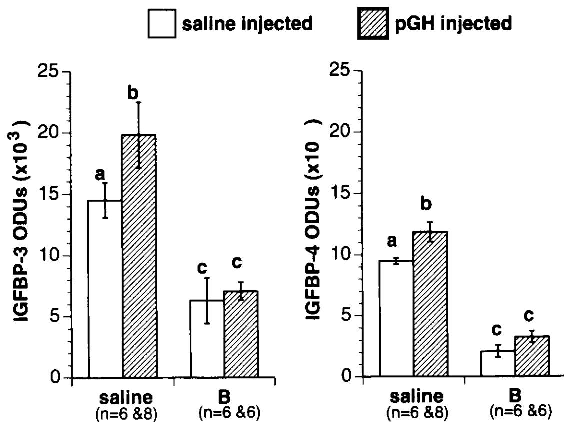
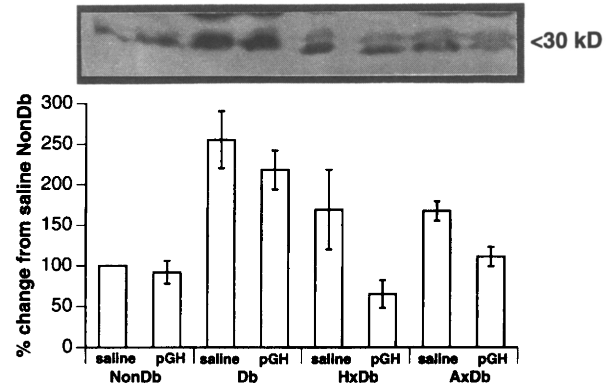


Figure 4. Serum concentrations of IGFBP-3 and -4 in AxDb rats with or without B replacement (25 μg B/ml 0.9% saline drinking water) as determined by Western (a) immuno- and (b) ligand-blotting (note the difference in scale from other figures). Otherwise as in the legend for Figure 2.

duction in IGFBP-1 brought it near the range of NonDb controls. The 75% decrease in IGFBP-3 concentration was considerably greater than the 33% decrease in IGFBP-4 concentration. Thus, the more severe growth impairment and reduction in IGF-I concentration in the HxDb rats versus the Db animals is associated primarily with a relatively large decrease in IGFBP-3 concentration.

Adrenalectomy of Db rats appeared to normalize their serum IGFBP-1, -3, and -4 concentrations, but that of IGFBP-2 was increased substantially. These results are consistent with those previously reported by Unterman *et al.* (14). We reported earlier (8) that the AxDb controls did not grow any more than the Db controls, even though their serum IGF-I concentration was normalized. Administration of B reversed these changes in serum IGFBP concentrations and reduced IGF-I levels in AxDb animals from both studies (8, 14). Furthermore, B replacement reduced body weight and tail and tibial growth in both the saline- and pGH-injected AxDb animals (8). These results suggest that although IGFBP-1, -3, and -4, and IGF-I were restored to levels of growing NonDb rats, the high serum IGFBP-2 concentration may have prevented restoration of growth. It is unknown how abnormally high circulating IGFBP-2 titers will affect growth. Free



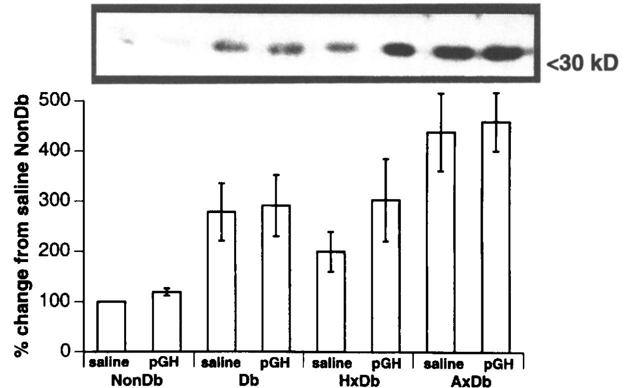
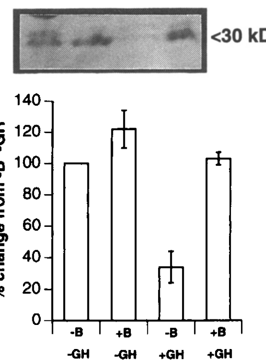


Figure 5. Serum concentrations of immunoreactive IGFBP-1 (a) and -2 (b) in intact nondiabetic (NonDb), diabetic (Db), hypophysectomized diabetic (HxDb), and adrenalectomized diabetic (AxDb) rats (±50 μg pGH 2×/day) as determined by Western immuno-blotting of pooled serum samples for each treatment group. Westerns for each IGFBP were run separately, but all lanes on each panel are from the same blot. Histogram values are % differences (mean ± SEM) in optical density units of only the lower (approximately 30 kDa) band and represent the variability between Westerns not groups.



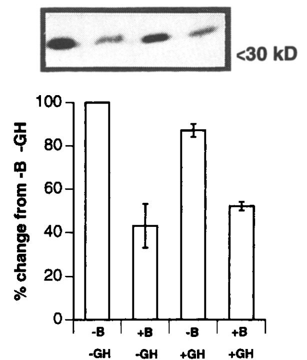
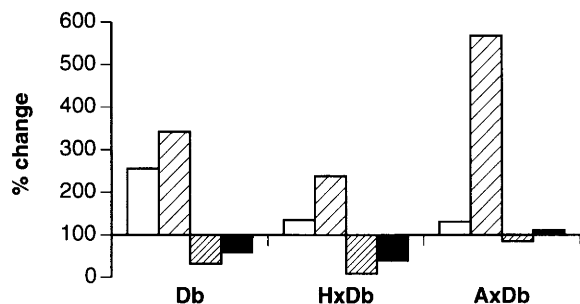


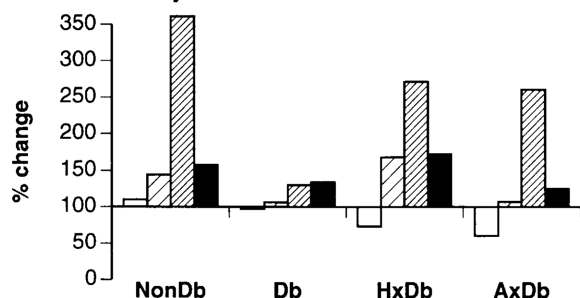
Figure 6. Serum concentrations of immunoreactive IGFBP-1 (a) and -2 (b) in AxDb rats (±50 μg pGH 2×/day) with or without B replacement (25 μg B/ml 0.9% saline drinking water) as determined by Western immuno-blotting of pooled serum samples for each treatment group. Otherwise as in Figure 5.

IGFBP-1 IGFBP-2 IGFBP-3 IGFBP-4

a. [IGFBP]s relative to NonDb controls



b. [IGFBP]s in pGH injected rats relative to saline injected controls



c. [IGFBP]s relative to saline drinking and injected AxDb rats

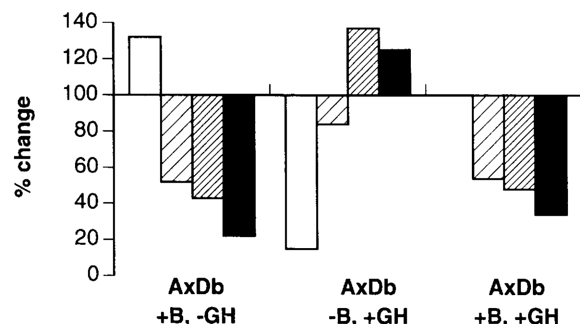


Figure 7. Summary of changes in the concentrations of IGFBPs in intact nondiabetic (NonDb), diabetic (Db), hypophysectomized diabetic (HxDb), and adrenalectomized diabetic (AxDb) rats (±50 μg pGH 2×/day) with or without B replacement (25 μg B/ml 0.9% saline drinking water) as determined by Western ligand- or immuno-blotting. (a) IGFBPs relative to NonDb controls. (b) IGFBPs in pGH-injected controls relative to saline-injected controls. (c) IGFBPs relative to saline-drinking and -injected AxDb rats.

IGF-I may have been compromised in these animals; Unfortunately, only total serum IGF-I concentrations were determined.

Injections of pGH into the NonDb rats caused substantial increases in serum concentrations of IGFBP-3 and -4, but had little or no effect on those of IGFBP-1 and -2. These changes were accompanied by an increase in serum IGF-I concentration and growth of the tibial epiphysial growth plate (8). By contrast, injections of pGH elevated IGFBP-4 concentrations in Db rats, but failed to influence those of the other IGFBPs. However, this elevation in IGFBP-4 was not accompanied by any growth responses or by an increase in serum IGF-I concentration. Thus, the refractoriness of Db rats to GH is associated with a failure of the hormone to elevate IGFBP-2 and -3 titers and to reduce those of IGFBP-1.

In HxDb rats, pGH injections further reduced the already lowered IGFBP-1 concentration to equal that found in the NonDb controls and further increased the elevated concentration of IGFBP-2. The GH treatment also significantly elevated IGFBP-3 and -4 concentrations, although their levels remained significantly lower than those of NonDb rats. These changes in the IGFBPs were accompanied by significant growth responses and elevated serum IGF-I concentration (8). Similarly, adrenalectomy caused a substantial improvement in the responsiveness of Db rats to pGH, as measured by the increase in IGFBP-3 and -4, and by the considerable decrease in IGFBP-1 concentration. Accordingly, the improved growth and normalized IGF-I concentration that result from adrenalectomy of Db rats (8, 14) is associated with a dramatic increase in IGFBP-3 concentration and a normalization of that of IGFBP-1.

Endocrine Control of Serum IGFBP Concentrations. The relative changes in IGFBP-3 concentration in all of the treatment groups are identical to the changes in serum IGF-I concentration and growth previously reported (8). Thus, the improved responses to pGH in the HxDb and AxDb rats are associated primarily with increased IGFBP-3 concentrations and a parallel rise in IGF-I, which maintains circulating IGFBP-3 levels (23).

The elevated IGFBP-2 concentration in the Db controls was reduced slightly by hypophysectomy, which is consistent with reports that pituitary ablation reduces serum IGFBP-2 concentration and gene expression in several tissues of nondiabetic animals (24–26). Although some studies have shown that GH and IGF-I increase IGFBP-2 production both *in vivo* and *in vitro* (27, 28), other studies have reported conflicting results (29, 30). Serum IGFBP-2 concentration was further elevated in the AxDb controls and were restored towards Db-like levels by administration of B to AxDb rats. The rise in circulating IGFBP-2 concentration in Db rats occurs despite lowered serum GH concentration (31–33). Thus, it is unlikely that the pituitary or adrenal factors are responsible for the elevation in serum concentrations associated with IDDM. Injections of pGH elevate IGFBP-2 only in the HxDb

rats. The failure of the pGH to have such an effect in AxDb rats, which did show other responses to pGH, may be due to the fact that the IGFBP-2 concentration was already considerably elevated.

The rise in serum concentration of immunoreactive IGFBP-1 in Db rats is likely due to several factors, including insulin deficiency, elevated circulating concentrations of B, glucagon, and catecholamines; and low intracellular glucose (14–17), but may also be due to GH resistance. Injections of pGH lowered the IGFBP-1 concentration in HxDb and AxDb rats, but not in the Db or NonDb animals. Serum glucose was elevated in the NonDb and AxDb rats by pGH, but was unaffected in the Db and HxDb rats (8). Thus, these changes in circulating IGFBP-1 are independent of changes in serum glucose. GH and IGF-I inhibit IGFBP-1 synthesis and release *in vitro* (34–36), although these effects may be due to IGF-I activation of insulin receptors (37) rather than GH *per se*. The ability of pGH to reduce IGFBP-1 levels appeared to be inversely related to circulating B concentrations, which are reduced by hypophysectomy (38–40) and virtually eliminated by adrenalectomy. Furthermore, pGH injections had no effect on IGFBP-1 concentration in AxDb rats receiving B. Therefore, the rise in circulating IGFBP-1 concentration and hepatic gene expression in catabolic states are undoubtedly influenced by the rise in serum counteregulatory hormone concentrations and may contribute to growth inhibition by sequestering IGF-I. This suggestion is supported by the ability of IGFBP-1 to inhibit the insulin-like metabolic effects of IGF-I both *in vitro* and *in vivo* (41) and the somatotropic effects of GH and IGF-I *in vivo* (42).

The changes in IGFBP-4 concentrations were generally similar to those of IGF-I as well, although injections of pGH in Db rats significantly elevated IGFBP-4 concentration but had no effect on serum IGF-I levels, as previously reported (8). Serum IGFBP-4 concentration appears to be somewhat more responsive to B administration than that of serum IGFBP-3 in AxDb rats, suggesting that B may directly affect IGFBP-4 production and/or clearance and may be partially responsible for the reduced levels in rats with IDDM. Dexamethasone has been reported to stimulate IGFBP-4 production (43). However, a recent study by Unterman *et al.* (14) is consistent with our findings: Compared with Db rats, the circulating IGFBP-4 concentration was elevated in AxDb rats and reduced by injections of B acetate.

In conclusion, growth impairment during IDDM may be due to several factors including insulin deficiency, intracellular starvation, GH and IGF-I resistance, and changes in IGFBP concentrations. Pituitary activation of adrenal B production may contribute to this impairment in several ways, which may involve alterations in circulating IGFBP-1, -3, and -4, but not IGFBP-2, concentrations. Although the concentrations of metabolites in the circulation of fasting humans and animal models are opposite to those found in IDDM, the concentrations of counterregulatory hormones are elevated in both of these catabolic conditions. The influence of these hormones on circulating IGFBP concentrations may contribute to growth impairment by altering the availability and action of IGFs when metabolite preservation and the diversion of energy away from growth processes are required.

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