An Insulinlike Growth Factor I–Resistant State in Cartilage of Diabetic Rats Is Ameliorated by Hypophysectomy

Possible Role of Metabolism

KEVIN M. KELLEY, SHARON M. RUSSELL, MARK L. MATTEUCCI, AND CHARLES S. NICOLL

This study investigated the effect of IDDM on cartilage anabolic activity in rats. Rats were injected with STZ to induce IDDM, were hypophysectomized, or were injected with STZ and hypophysectomized. After 14 days, control (intact and sham-Hx) and Hx rats were normoglycemic, whereas the rats with IDDM exhibited hyperglycemia and glycosuria. The HxDb rats, however, had normal blood glucose levels and no glycosuria. Body growth, serum levels of IGF-I, and basal cartilage $^{35}SO_4$ incorporation measured in vitro were decreased in the Hx, IDDM, and HxDb groups. IGF-I added in vitro significantly stimulated $^{35}SO_4$ incorporation by cartilage explants from control and Hx animals, whereas explants from the animals with IDDM were unresponsive. Explants from the HxDb rats, however, were stimulated by IGF-I in a dose-related manner. Because Hx corrected the glycemic status of the IDDM rats and restored cartilage responsiveness to IGF-I, a second set of experiments was undertaken to further investigate the relationship between cellular metabolism and anabolic activity in cartilage. Cartilage explants from rats fasted for 48 h showed significantly decreased basal $^{35}SO_4$ incorporation, which was as low as that in explants from rats with severe IDDM. Whereas explants from the IDDM rats were completely unresponsive, those from the fasted rats (and fed rats) were significantly stimulated by the added IGF-I. However, incubation in the presence of 2-D-G, which causes intracellular glucopenia, or in the absence of glucose, completely blocked the anabolic response to IGF-I in otherwise responsive tissues. In conclusion, an important component of diabetic growth inhibition appears to be tissue resistance to the anabolic action of IGF-I, a condition that is correctable by Hx and that may be a result of metabolic impairment at the tissue level. *Diabetes* 42:463–69, 1993

Growth processes and intermediary metabolism are interrelated at several levels. At the hepatic level, GH-stimulated production of IGF-I is not maintained during uncontrolled IDDM or malnutrition (1–3). The hepatic resistance to GH action during IDDM or malnutrition is apparently caused by post-GH receptor defects (4) and to reduced GH receptor number and gene expression (3,5–8). This deficit leads to decreased circulating IGF-I and inhibited growth (4–11). IGF-I gene expression in many extrahepatic tissues is also reduced during IDDM, although in some tissues (e.g. kidney) IGF-I peptide concentration (12,13), as well as expression of the gene for its specific receptor (IGF type-I receptor; 14,15), may be increased.

Studies of rats with IDDM have raised questions regarding the relationship between metabolic control and growth inhibition. Scheiwiller et al. (10) demonstrated that treatment of diabetic rats with either IGF-I or a low dose of insulin can stimulate growth without normalizing their glycemic status. As endogenous IGF-I levels were increased in the insulin-treated rats, these investigators proposed that insulin promoted growth independently of its metabolic effects by increasing IGF-I. A study by Griffen et al. (11) is consistent with this proposal. Griffen et al. found that infusion of a small dose of insulin directly into the liver of IDDM rats via the hepatic portal vein increased circulating IGF-I levels and growth but did not normalize their glycemic status.

An alternative explanation, proposed by Robinson et al. (16) and Carlsson et al. (17), suggests that growth arrest during IDDM is caused primarily by intracellular

From the Endocrinology and Cancer Research Laboratory, Department of Integrative Biology, University of California, Berkeley, California.

Address correspondence and reprint requests to Professor Charles S. Nicoll, Department of Integrative Biology, LSA 281, University of California, Berkeley, CA 94720.

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IGF-I, insulinlike growth factor I; rbIGF-I, recombinant bovine IGF-I; IDDM, insulin-dependent diabetes mellitus; STZ, streptozocin; Hx, hypophysectomized or hypophysectomy; HxDb, STZ treated and Hx; 2-D-G, 2-deoxy-D-glucose; GH, growth hormone; MEM, minimal essential medium; CV, coefficient of variation; ANOVA, analysis of variance IGF-BP, IGF binding protein.

starvation of tissues in the absence of insulin, which would block anabolic responses in growing tissues and inhibit pulsatile release of GH by the pituitary (see also 18). Although these authors (16,17) suggested that any insulinlike substance would correct the intracellular imbalances that caused the growth inhibition, their treatment of diabetic rats with IGF-I (26.6 ng/day in animals weighing 120–150 g) did not restore growth, pulsatile GH release, or normoglycemia (17). The dose of IGF-I that stimulated growth of diabetic rats in the study by Scheiwiller et al. (10), on the other hand, was considerably higher (300 μg/day in animals weighing 120–130 g) and thus may have exerted insulinlike effects, restoring intracellular metabolism and growth.

The relationship between growth processes, such as cartilage-matrix synthesis, and intracellular metabolism has received little attention (19,20); in particular, little is known about possible changes occurring in IGF-I–mediated processes during IDDM. This study identifies an IGF-I–resistant state in cartilage of growth-arrested IDDM rats, as measured by $^{35}SO_4$ incorporation in vitro. Experiments were conducted to investigate a possible relationship between intracellular metabolism and anabolic responses of tissues to IGF-I.

RESEARCH DESIGN AND METHODS

Male rats (150–200 g; Simonsen Laboratories, Gilroy, CA) of the Long-Evans or Fisher-344 strains were maintained in a climate-controlled room (22–23°C; 12-h light-dark photoperiod). Water and Purina rat chow (Ralston-Purina, St. Louis, MO) were available ad libitum. Rats were killed by injection of an overdose (≥0.2 ml/100 g body wt) of anesthetic mixture composed of 10 ml ketamine, 5 ml xylazine, 1.5 ml acepromazine, and 1.85 ml distilled H_2O (21). All procedures were done in strict compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by our Institutional Animal Care and Use Committee.

Costal cartilage $^{35}SO_4$ incorporation assay. Costal cartilage was obtained as described previously (22) and cut into pieces ≥1 mm^3. The explants were placed in MEM plus Hanks' salts (Gibco, Grand Island, NY) supplemented with 400 U/ml each of penicillin and streptomycin (Sigma, St. Louis, MO) for a 30-min preincubation. Live and heat-killed (boiled for 5 min to determine nonspecific $^{35}SO_4$ uptake) explants were then cultured in MEM supplemented with 2 μCi/ml $^{35}SO_4$ (Amersham, Arlington Heights, IL) and 100 U/ml each of penicillin and streptomycin, and maintained at 37.5°C for 48 h in air. Phenol red in the medium indicated that pH was stable during incubation. rbIGF-I (Monsanto, St. Louis, MO) was dissolved in 10 mM HCl, neutralized by the same volume of 10 mM NaOH, and added to the culture medium in final concentrations of 1.32, 13.2, and 132 nM (10, 100, and 1000 ng/ml). After incubation, tissues were soaked for 6 h in saturated Na_2SO_4 solution and then flushed continuously with tap water overnight. Individual dry weights (to the nearest microgram) were measured before dissolving tissues in 99% formic acid. Samples were counted on a Beckman 5000 LSC counter and expressed as disintegrations per minute per microgram. In our serum-free system, levels of $^{35}SO_4$ incorporation were generally between 1 and 8 dpm/μg, which is in the range of 0.5–4 nmol, or about 15–120 ng, of $^{35}SO_4$ incorporated per gram dry weight. A lesser magnitude of $^{35}SO_4$ incorporation in our system compared with data reported in other studies (23,24) is probably the result of a smaller ratio of $^{35}SO_4$ to ambient SO_4 in our culture medium (MEM plus Hanks' salts) and to our serum-free conditions.

Determinations of levels of IGF-I and glucose. Blood was collected by cardiac puncture and serum stored at −80°C. Serum IGF-I levels were measured by radioimmunoassay after separating IGF-I from its binding proteins by an acid-acetone extraction method (25). An anti-human IGF-I antibody (obtained from the National Institute of Diabetes and Digestive and Kidney Diseases, lot UB3–189) and ^{125}I-labeled rhIGF-I (provided by Dr. Robert J. Denver, University of California at Berkeley) were used. All samples were assayed at the same time and had an intra-assay CV of 2.8% (interassay, 12.6%). Serum glucose was measured with a colorimetric assay (Trinder reagent, Sigma). Urine glucose and ketones were assessed using Chemstrips (Boehringer Mannheim, Indianapolis, IN).

Experimental groups. In the first experiment, Long-Evans rats were subjected to three growth-inhibitory treatments that differ in their metabolic condition, following the protocol developed by English (26). The rats were Hx by a transauricular approach (27), were sham-Hx, or were made moderately diabetic by intraperitoneal injection of STZ (Sigma) at 85 mg/kg on 2 consecutive days. Half of the STZ-injected animals that exhibited glycosuria were injected with 2 $U \cdot kg^{-1} \cdot day^{-1}$ purified bovine insulin (Sigma) in 0.9% NaCl with 0.02% NaN_3 for 2 days (to ameliorate diabetic symptoms) and then were Hx (HxDb group). For all surgical procedures, the rats were anesthetized by injection of 0.1 ml/100 g of the anesthetic mixture described above. All animals (intact, $n = 7$; sham-Hx, $n = 8$; Hx, $n = 7$; IDDM, $n = 8$; and HxDb, $n = 8$) were maintained without further treatment for 2 wk before death. Body weight and tail length changes were measured every 48 h during the 2-wk period. Serum was collected for determination of glucose and IGF-I levels as described above.

The anabolic activity of costal cartilage (basal and in response to added rbIGF-I) in the rats was assessed by measurement of $^{35}SO_4$ incorporation in vitro. Sixteen cartilage explants per animal were collected for distribution among in vitro treatments ($n = 4$/well; wells contained 0, 1.32, 13.2, or 132 nM rbIGF-I), and the total number of animals per group was $n = 9$ (intact), $n = 8$ (sham-Hx), $n = 8$ (Hx), $n = 11$ (IDDM), and $n = 10$ (HxDb).

In the second experiment, Fisher-344 rats were either fed ad libitum ($n = 4$), fasted for 48 h ($n = 4$), or injected intraperitoneally with 150 mg/kg STZ to induce severe IDDM ($n = 4$). Animals with urine glucose ≥5% were killed 4 days after STZ treatment. Cartilage explants from each of the animals were tested for their response to

TABLE 1
Percentage of body-weight change and percentage of tail-length change after 14 days of in vivo treatment

	Rat groups				
	Intact	Sham-Hx	Hx	IDDM	HxDb
Body-weight change (%)	35.6 ± 2.1	35.4 ± 3.6	−6.2 ± 2.0*[a]	20.2 ± 1.9*[b]	−7.85 ± 3.1*[a]
Tail-length change (%)	14.9 ± 1.2	11.4 ± 1.8	−0.23 ± 0.60*[a]	6.6 ± 1.4*[b]	1.1 ± 1.0*[a]
n (in each group)	8	8	7	8	8

Data are means ± SE.
*Values are significantly different from intact and sham-Hx control values; *[a] values and *[b] values are significantly different from each other ($P < 0.05$).

rbIGF-I (at doses of 0, 1.32, 13.2, and 132 nM) in different culture media as follows. A Hanks'-Ringer solution (based on formula of Gibco) was made and supplemented with 2 ml 50X MEM amino acids (Gibco)/98 ml Ringer; glucose was either excluded or added at 1 g/L. Glucose concentration was measured to be 10.6 mM in the medium with glucose. Explants (n = 4/well) from fed rats were cultured in complete medium with or without 2-D-G (Sigma; at a concentration equal to that of glucose); induction of intracellular glucopenia by treatment with this nonmetabolizable glucose analogue has been described by several studies (18,28,29). Explants (n = 4/well) from fasted rats were cultured in medium with or without glucose, and explants (n = 4/well) from rats with IDDM were cultured in complete medium.

Statistical analyses. Data are means ± SE and were subjected to ANOVA and Bonferroni multiple comparisons tests using CRISP version 3.05A software (Crunch, San Francisco, CA) on an IBM-PC/2.

RESULTS

First experiment. The data in Table 1 show that intact and sham-Hx rats gained 35% in body weight and 15 and 11%, respectively, in tail length over 14 days. Hx and HxDb rats lost body weight ($P < 0.05$), and their tails did not grow ($P < 0.001$), whereas rats with moderate IDDM exhibited a 40% reduced body weight gain ($P < 0.05$) and inhibited tail growth ($P < 0.01$) compared with controls.

Mean serum glucose levels (Fig. 1) were between 8 and 11 mM in intact, sham-Hx, and Hx rats, whereas animals with IDDM had significant hyperglycemia of (~23 mM, $P < 0.001$). By contrast, the HxDb rats were normoglycemic. The presence of glucose in the urine was detectable only in the IDDM rats (≥5% concentration). None of the rats had detectable urine ketones.

Serum IGF-I levels (Fig. 2) were high in intact rats and were reduced by ~30% in sham-Hx animals ($P < 0.05$). The rats with IDDM had a mean serum IGF-I concentration ~33% of that of intact rats, but the Hx and the HxDb rats had levels that were reduced by ~95%. Basal $^{35}SO_4$ incorporation by cartilage explants (Fig. 3*A*) showed a pattern generally similar to that of the serum IGF-I levels in all but the HxDb group. Cartilage from this latter group showed basal incorporation at a rate significantly lower than that measured in the Hx group ($P < 0.01$), in contrast to the results with serum IGF-I levels.

The cartilage explants from the intact control group had the highest basal activity and showed a tendency toward increased $^{35}SO_4$ incorporation in response to higher doses of IGF-I (Fig. 3*A*); when normalized to change in disintegrations per minute per microgram (relative to untreated controls; Fig. 3*B*), the effect was statistically significant ($P < 0.05$) at the 132 nM IGF-I dose. The sham-Hx controls with the lower serum IGF-I

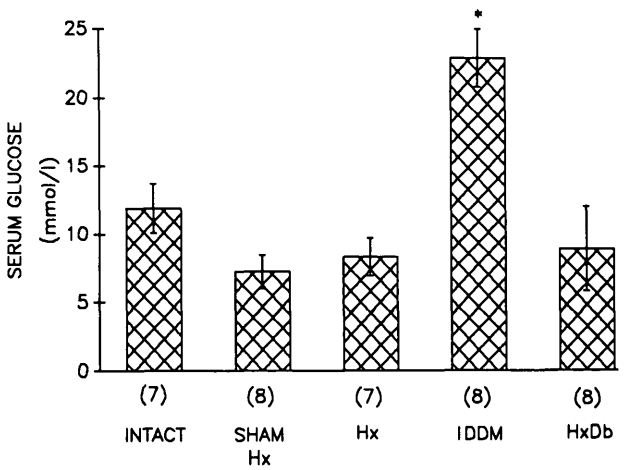

FIG. 1. Serum glucose levels (mean ± SE) of intact, Hx, sham-Hx, IDDM, and HxDb rats. Number of animals in each group is shown in parentheses. *$P < 0.001$ compared with other serum glucose values.

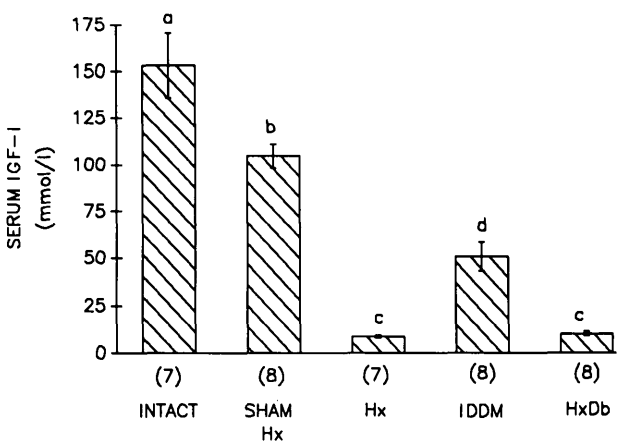

FIG. 2. Serum levels of IGF-I (means ± SE) as measured by radioimmunoassay in intact, Hx, sham-Hx, IDDM, and HxDb rats. Number of animals in each group is shown in parentheses. a,b,c,d—Values are significantly different from each other ($P < 0.05$).

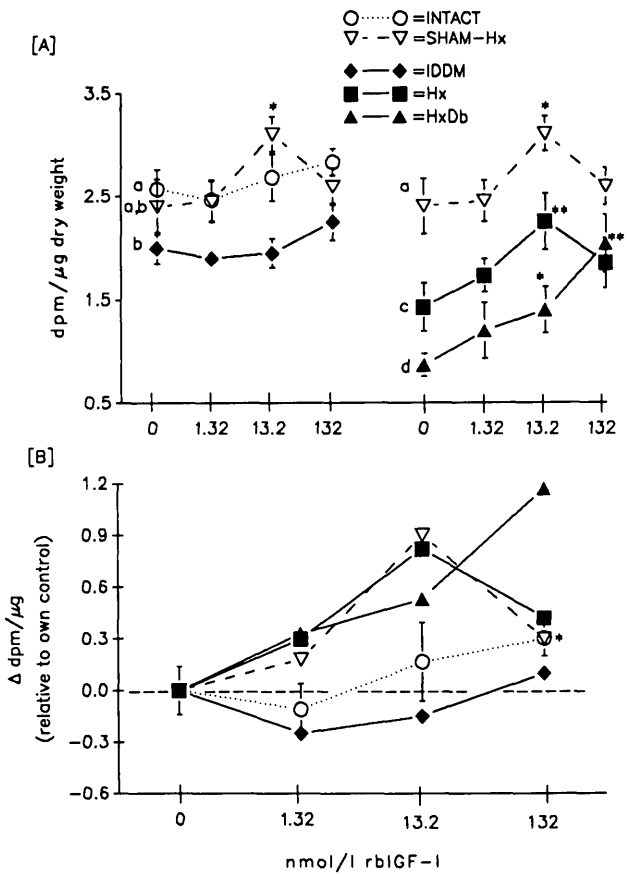

FIG. 3. In vitro effect of rbIGF-I at 1.32, 13.2, and 132 nM (10, 100, and 1000 ng/ml, respectively) on $^{35}SO_4$ incorporation by cartilage (means ± SE) expressed in disintegrations per minute per microgram (*A*) or as normalized to absolute change in disintegrations per minute per microgram in response to rbIGF-I (relative to own untreated control) (*B*). Cartilage $^{35}SO_4$ incorporation at each rbIGF-I dose was measured in quadruplicate for each animal. Number of animals that contributed cartilage for each group was $S = 9$, intact; $n = 8$, sham-Hx; $n = 8$, Hx; $n = 11$, IDDM; $n = 10$, HxDb. $^*P < 0.05$, $^{**}P < 0.01$ compared with own control. a,b,c,d—values are significantly ($P < 0.01$) different from each other.

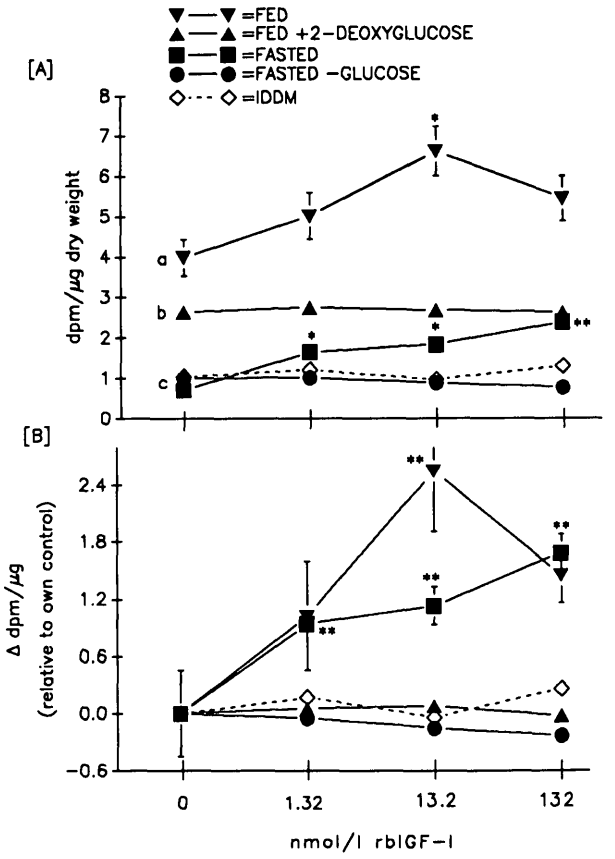

FIG. 4. In vitro effect of rbIGF-I at 1.32, 13.2, and 132 nM (10, 100, and 1000 ng/ml) on $^{35}SO_4$ incorporation by cartilage (means ± SE) expressed in disintegrations per minute per microgram (*A*) or as normalized to absolute change in disintegrations per minute per microgram in response to rbIGF-I (relative to own untreated control) (*B*). Cartilage from fed rats was tested in complete medium with or without 2-D-G. Fasted rat cartilage was tested in medium with or without glucose. Explants from rats with IDDM were tested in complete medium. Number of animals represented by each point is 4 ($^{35}SO_4$ incorporation at each rbIGF-I dose was measured in quadruplicate for each animal). $^*P < 0.05$, $^{**}P < 0.01$ compared with own control. a,b,c—Values are significantly ($P < 0.01$) different from each other.

levels, however, showed a significant stimulation of cartilage $^{35}SO_4$ incorporation at the intermediate dose (13.2 nM) of IGF-I (Fig. 3*A* and 3*B*).

By comparison with the control groups, explants from the Hx group showed a reduction of ~50% in basal synthetic activity. IGF-I significantly increased incorporation in these explants at the 13.2 nM dose; however, the stimulated level reached only 70% of that observed in the sham-Hx group at the same IGF-I dose (Fig. 3*A*). Explants from the IDDM group, although exhibiting a 35% reduction in basal activity relative to the controls ($P < 0.05$), did not significantly respond to any of the doses of IGF-I tested. Although not significant relative to its own control, incorporation at the high IGF-I dose tended to be increased in the IDDM group and was not significantly below the basal levels in the two control groups. Explants from the HxDb group had the lowest basal level of $^{35}SO_4$ incorporation and showed a highly significant ($P < 0.001$) dose-dependent stimulation by IGF-I. As in the Hx group, however, the IGF-I did not restore anabolic activity to control levels.

When the data in Fig. 3*A* were normalized to absolute change in disintegrations per minute per microgram (Fig. 3*B*), the sham-Hx, Hx, and HxDb groups clearly exhibited the greatest magnitude of responses to IGF-I (between 0.8 and 1.2 dpm/μg), whereas the IDDM group did not respond.

Second experiment. Because metabolic imbalance was associated with IGF-I resistance of cartilage from the rats with IDDM (i.e., Hx corrected their glycemic status and restored the responsiveness of their cartilage), the second experiment was conducted to investigate the effects of metabolic impairment on cartilage anabolic activity. Figure 4 shows the relationship between cartilage metabolic status and its anabolic activity and responsiveness to IGF-I in vitro. Explants from rats fed ad libitum had the highest basal synthetic activity during 48 h of incubation, which was further stimulated by IGF-I at the 13.2 nM dose ($P < 0.01$, Fig. 4*A*). The presence of 2-D-G in the medium inhibited basal $^{35}SO_4$ incorporation by >30% ($P < 0.01$) and completely suppressed the anabolic response to IGF-I. Cartilage explants from the fasted rats and from

those with IDDM had basal levels of anabolic activity that were reduced by ~80%, and the explants from the former group showed a significant ($P < 0.001$), dose-dependent response to IGF-I. However, the explants from the rats with IDDM, and those from the fasted animals that were incubated in the absence of glucose, were completely unresponsive to any of the doses of IGF-I (Fig. 4*A* and 4*B*).

DISCUSSION

Our two experiments show that cartilage from rats with IDDM is refractory to the anabolic action of IGF-I in vitro. This unresponsiveness was evident regardless of whether the tissue showed slightly (Fig. 3) or severely (Fig.4) depressed basal anabolic activity. Such IGF-I resistance was not found in cartilage from the Hx, HxDb, or fasted animals that showed similar or greater degrees of growth inhibition than the animals with IDDM. In addition, Hx, which normalized the glycemic status of the rats with IDDM (HxDb group), restored responsiveness to IGF-I. That the resistance was specific to the hyperglycemic IDDM group suggests that the ability of cartilage to respond anabolically to IGF-I is dependent on a normal metabolic state. This hypothesis is supported by the data indicating that responsive tissue from fed or fasted rats was rendered resistant to IGF-I by induction of intracellular glucopenia with 2-D-G treatment or by incubation without glucose (Fig. 4).

At the doses used (which are within the physiological range), IGF-I was apparently not capable of correcting (via its insulinlike activity?) the impairments that rendered the diabetic cartilage unresponsive, even in the moderately diabetic group in which the basal anabolic activity of the cartilage was not severely depressed (first experiment). This finding is inconsistent with the proposal (16,17) that any insulinlike substance would be effective (at least within physiological circumstances). In an earlier study by Phillips and Young (23), normal rat serum added in vitro was found to stimulate $^{35}SO_4$ incorporation by cartilage of STZ-induced IDDM rats. This effect, however, may have been the result of the presence of insulin (in addition to IGF-I) in the rat serum. In agreement with our study, Phillips and Young (23) found that cartilage from the rats with IDDM was less sensitive to low concentrations of serum than was cartilage from Hx rats. Our results also appear to be at odds with the results of Scheiwiller et al. (10), who observed anabolic responses after injection of IGF-I in rats with IDDM. However, the dose used (300 μg/day in rats weighing 120–130 g) was probably supraphysiological and thus possibly exerted both insulinlike and IGF-I effects to stimulate growth. The tendency to show increased $^{35}SO_4$ incorporation at the highest IGF-I dose in cartilage from the moderately diabetic rats (Fig. 3) may support this latter hypothesis.

In the first experiment, Hx was found to reduce basal synthetic activity of cartilage by nearly 50%, whereas the activity was further depressed in the HxDb group (Fig. 3*A*). By contrast, serum IGF-I levels were equally low in both groups (Fig. 2). The higher activity of the cartilage from the Hx rats suggests that additional factors, possibly insulin, may have maintained a higher degree of growth activity in that group. The response to IGF-I in the two groups also showed differences. Cartilage from the HxDb animals, which had very low basal anabolic activity, showed a linear dose-response relationship, with the highest IGF-I dose having the greatest effect (Fig. 3). By contrast, the Hx group showed a higher basal activity and a dose-response curve with maximal response at the intermediate IGF-I dose, and a smaller response at the highest dose (Fig. 3). The maximal IGF-I–stimulated level of $^{35}SO_4$ incorporation reached in both groups, however, was similar (~2 dpm/μg; Fig. 3*A*). That this maximum was reached at a lower IGF-I dose in the Hx group with the higher basal activity suggests that, because a larger proportion of the chondrocytes was already activated, less additional stimulation in vitro was required to reach the maximal level. Under such circumstances, the highest IGF-I dose appears to have exerted a negative influence. A possible cause of the upper limit of anabolic activity in these groups, which was significantly below that of the control groups, may be the 14-day duration of Hx. GH stimulates the differentiation of cartilage progenitor cells into cells that express type I IGF receptors and thereby become IGF-I responsive (30–32). Thus, without GH in the Hx animals, the number of IGF-I–responsive chondrocytes would be decreased.

A nonlinear response curve like that in the Hx group (i.e., maximal response at the intermediate dose) was also observed in the sham-Hx control group, but it occurred at a higher level of anabolic activity in this pituitary-intact group (Fig. 3*A*). The intact control group, by contrast, showed only a slight response to IGF-I. Because the animals in this latter group had the greatest growth rates (Table 1) and very high serum IGF-I levels (Fig. 2), it seems likely that most of the chondrocytes present in the explants were already at a stimulated level of activity, precluding much further stimulation by IGF-I in vitro. The more responsive sham-Hx group, on the other hand, had significantly lower serum IGF-I levels. The IDDM group, however, had reduced body growth, serum IGF-I, and basal $^{35}SO_4$ incorporation, and still showed no cartilage response to IGF-I.

In the second experiment, an overall higher level of cartilage anabolic activity was observed that, because most conditions were constant between the two experiments, may be attributable to differences in the rat strains used, feeding status, stress, or other unknown factors. Despite this discrepancy, dose-response characteristics similar to those observed in the first experiment were observed in this experiment, as the fasted group showed very low basal activity and a linear response, whereas the fed group showed high basal activity and a nonlinear response. In the fasted group, a considerable depression of anabolic activity was observed that could not be fully restored by the addition of IGF-I in vitro (Fig. 4*A*). Possibly, this lack of full restoration is the result of metabolic impairments preexisting in the tissue, because the in vivo metabolic changes that occur during fasting are likely to be similar to those occurring during IDDM.

A striking aspect of our study was the finding that the cartilage from HxDb rats showed restored responsive-

ness to IGF-I. In addition to correcting the refractoriness of cartilage of the insulin-deficient rats, Hx resulted in ameliorated diabetic symptoms (Fig. 1). Houssay et al. (33,34) first reported the metabolic normalization of diabetic rats by Hx, and they implicated GH and glucocorticoids as the diabetogenic agents. Indeed, GH is known to be hyperglycemic (35,36) and has been shown to antagonize insulin-regulated glucose metabolism (37,38) and insulin-sensitive [^{3}H]2-D-G transport (39). Glucocorticoids, on the other hand, increase serum levels of somatomedin inhibitors, proteins that are elevated in animals with IDDM and that antagonize both IGF and insulin action (40–42). Such inhibitors could contribute to the resistance of cartilage from rats with IDDM to stimulation by IGF-I observed in our study. Possibly, the counterregulatory pancreatic hormones, glucagon and somatostatin, may have influenced levels of IGF-BPs or inhibitors in the rats with IDDM, as these hormones have been demonstrated to affect IGF-BP production by hepatocytes (43,44).

In conclusion, STZ-induced IDDM resulted in a loss of cartilage anabolic response to IGF-I in vitro. This IGF-I resistance may be caused, at least in part, by metabolic impairment in the cartilage, as experimental changes affecting intracellular metabolism altered the cartilage response to IGF-I. Most notably, Hx of the rats with IDDM, which normalized the glycemic status of the animals, restored cartilage responsiveness to IGF-I.

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