Skeletal Muscle Proteolysis in Rats With Acute Streptozocin-Induced Diabetes

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Skeletal muscle proteolysis was studied in rats 1 day after induction of diabetes with 65 mg/kg streptozocin. An evisceration procedure, including functional hepatectomy-nephrectomy, was performed, and the rate of proteolysis in the remaining tissues, primarily skeletal muscles, was evaluated over 2 h. With cycloheximide to block protein synthesis, total protein breakdown was measured from the rate of rise in plasma tyrosine concentration. The rate of degradation of contractile (myofibrillar) protein was estimated from the rate of rise in plasma concentration of 3-methylhistidine released from the breakdown of actomyosin. Compared with nondiabetic control preparations, the total protein degradation rate was increased 30% by diabetes ($P < .001$), and myofibrillar catabolism was accelerated by 60% ($P < .005$). In diabetes, the increase in proteolysis was accompanied by reductions in circulating insulin to 25–50% of normal level, whereas food intake did not differ from control. Treatment of diabetic rats with exogenous insulin, including acute infusions postoperatively, completely reversed the proteolytic effects of diabetes. The findings demonstrate that the hypoinsulinemia of acute diabetes increases the catabolism of skeletal muscle protein and that the inhibitory effect of normal levels of insulin includes a specific action to restrain myofibrillar proteolysis. *Diabetes* 38:1117–1122, 1989

One of the most debilitating consequences of untreated diabetes mellitus is a decline in the strength and mass of the skeletal muscles. Depletion of muscle protein is seen as diabetes progresses, which has led to the idea that diabetes causes muscle protein to break down at an accelerated rate. In insulin-deficient diabetic humans, whole-body amino acid tracer studies suggest that there is an increased turnover of body protein (1–3) that may reflect, at least in part, increased skeletal muscle proteolysis (4). This concept is consistent with other human studies suggesting that insulin may act to inhibit proteolysis in the whole body (5,6) and skeletal muscles (7).

Unfortunately, when protein degradation in skeletal muscles from animals with experimental diabetes is examined in detail by either in vivo or in vitro techniques, the results are conflicting. Some discrepancies could be due to the fact that experimental diabetes may cause an initial acceleration that is transformed in chronic diabetes to a decrease or no change in skeletal muscle proteolysis (8–14). However, because of technical limitations, even the acute proteolytic effect of experimental diabetes has not been a universal finding (15,16). Attempts to test effects of diabetes on degradation of myofibrils have also yielded conflicting results. Although early studies with in vitro hind limb preparations suggested that degradation of myofibrils was increased by diabetes (17,18), recent work led to the conclusion that insulin was not involved in the regulation of myofibrillar proteolysis (16). Consequently, there is no proof that diabetes actually has any catabolic effect on skeletal muscle protein.

To assess muscle protein degradation under more controlled physiological conditions, we developed an eviscerated rat preparation using the rate of tyrosine release into plasma during protein synthesis blockade as a measure of muscle proteolysis (19). The comparatively intact skeletal muscles of this preparation are in a much less catabolic state than muscles isolated and studied in other in vitro systems (20). With eviscerated rats, we were able to show that total protein degradation in peripheral tissues was enhanced when insulin concentration was low during fasting and that fasting-induced proteolysis was reversed by insulin treat-

Corticosterone 1 nM = 0.035 ng/ml Insulin 1 pM = 0.139 μU/ml

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ment (21). These results *1*) suggested that the lower plasma insulin concentrations in fasted rats play a role in the stimulation of proteolysis and *2*) raised the question of whether insulin levels similarly lowered by experimental diabetes would have a similar effect.

These experiments were undertaken to determine whether muscle protein degradation rates are accelerated during the first 24 h after induction of mild diabetes with streptozocin (STZ-D). In addition to total proteolysis, myofibrillar proteolysis was estimated from the release of 3-methylhistidine (3-MH) into the plasma after evisceration. In vivo urinary 3-MH excretion (22) and in vitro 3-MH release into the perfusate of isolated muscle preparations (23) have been used to measure the rate of degradation of actomyosin. In these experiments, an increased concentration of 3-MH in the plasma should also reflect the rate of myofibrillar proteolysis in skeletal muscles of eviscerated rats. Finally, we examined the effect of insulin replacement on muscle proteolysis in diabetic rats.

RESEARCH DESIGN AND METHODS

Animal studies were performed in adherence with guidelines established by the National Institutes of Health (24). Male Sprague-Dawley rats (Camm Research, Wayne, NJ) weighing 250–300 g were individually caged and given water and laboratory chow (Ralston-Purina, St. Louis, MO) ad libitum. Environmental temperature was controlled to 25°C, and relative humidity was kept >30%. Lighting was automatically controlled for a 12-h light-dark cycle, starting at 0700.

Acute diabetes was produced by injection of 65 mg/kg i.p. STZ buffered to pH 4.5 at 1500 the day before the experiment. The nondiabetic control animals received buffer alone. The next morning (~20 h after STZ injection), food intake and change in body weight were recorded. Urine was tested for glucose and ketone bodies with Keto-Diastix (Ames, Elkhart, IN), and the plasma of the rats with glucosuria was sampled for analysis of glucose concentration. Rats with hyperglycemia >20 mM were selected for the diabetic groups, but those that ate poorly or appeared ill or whose ketonuria exceeded 1+ were excluded. In one additional series of diabetic rats, STZ was given 48 h before evisceration.

Control and diabetic rats were then anesthetized with pentobarbital sodium (50 mg/kg i.p.) and functionally hepatectomized-nephrectomized according to an evisceration procedure described earlier (20). Briefly, the blood supply to the viscera is ligated, and the entire gastrointestinal tract is removed. The liver and kidneys are left in situ but remain completely isolated from the circulation. The care and selection of the eviscerated rat preparations were similar to our earlier experiments (20). The animals were placed in a warm box and heat lamps used to maintain a mean (±SE) rectal temperature of 37 ± 1°C. Their condition was carefully monitored, and at the end of the experiment, they were killed by thoracotomy. Because of the adverse effects of tissue anoxia per se on proteolysis, only data from healthy animals without hemorrhage or respiratory or circulatory distress were included. Postoperatively, saline and glucose must be given intravenously to maintain fluid balance and prevent hypoglycemia after removal of the glucogenic organs (liver and kidneys).

Fluids were administered through a heparinized polyethylene cannula in the right femoral vein. Both the control and diabetic rats received ~2 ml 5% glucose in saline/h by an infusion pump (Harvard Bioscience, South Natick, MA). As a result of this treatment, final plasma glucose concentrations for the total protein degradation studies and the myofibrillar degradation series, respectively, were 8.7 ± 0.6 and 8.8 ± 1.2 mM for control rats and 10.0 ± 1.0 and 13.5 ± 0.5 mM for diabetic rats. A subset of diabetic rats received exogenous insulin to determine the specific role of lack of insulin on muscle proteolysis in STZ-D rats. Some diabetic rats received insulin subcutaneously (5 U/kg) 1–2 h before evisceration, whereas others were given insulin intravenously as a bolus (100 mU/kg) at evisceration. All diabetic rats treated with insulin were given extra glucose to offset the effects of insulin on plasma glucose levels and therefore prevent hypoglycemia. Final glucose concentrations in the subcutaneously and intravenously insulin-treated rats were, respectively, 7.2 ± 0.7 and 12.5 ± 1.9 mM in the total protein degradation studies and 11.2 ± 2.7 and 12.5 ± 2.2 mM in the myofibrillar protein studies.

Immediately before evisceration and 1 and 2 h afterward, blood samples were taken for analysis of plasma concentrations of glucose and tyrosine or 3-MH. The initial and 1-h samples were obtained from the cut tip of the tail, and 2-h samples were taken from the vena cava. The final sample was also analyzed for insulin concentration. Heparin was used as the anticoagulant, and the plasma was separated promptly in a microfuge (Beckman, Fullerton, CA). A portion of the plasma was tested immediately for glucose concentration and the rest stored at −20°C until analyzed for the other constituents.

Total protein degradation. As in experiments described earlier (20), the accumulation of tyrosine in the plasma in the presence of cycloheximide was used to measure the rate of total protein degradation in the peripheral tissues after evisceration and functional hepatectomy-nephrectomy (primarily skeletal muscles). To block protein synthesis, 5 mg/kg of a freshly prepared 0.1% solution (in sterile saline) of cycloheximide was injected intravenously immediately after evisceration. At this dose, cycloheximide had a maximal effect on plasma tyrosine accumulation and was effective within 30 min (20), which agrees with reports that cycloheximide blocks protein synthesis in tissues, including skeletal muscles, by 96% in 15 min (25). The change in plasma tyrosine concentration was determined for 2 h after evisceration while the rate of increase in plasma tyrosine concentration was linear (20). Thereafter, at least in isolated skeletal muscles in vitro, cycloheximide may inhibit not only protein synthesis but also degradation (26).

Myofibrillar protein degradation. To test the effect of acute diabetes on the degradation rate of actomyosin, the increase in plasma 3-MH concentration was measured in another series of nondiabetic and diabetic eviscerated rats. Because 3-MH is an amino acid from actin and myosin that is neither degraded nor reutilized (27), its rate of accumulation in the plasma, in the absence of cycloheximide, should reflect its rate of release from the degradation of the myofibrils. 3-MH was measured over 2 h during which the increase in plasma 3-MH concentration was linear (Fig. 1).

To further validate this measurement, we preliminarily ex-

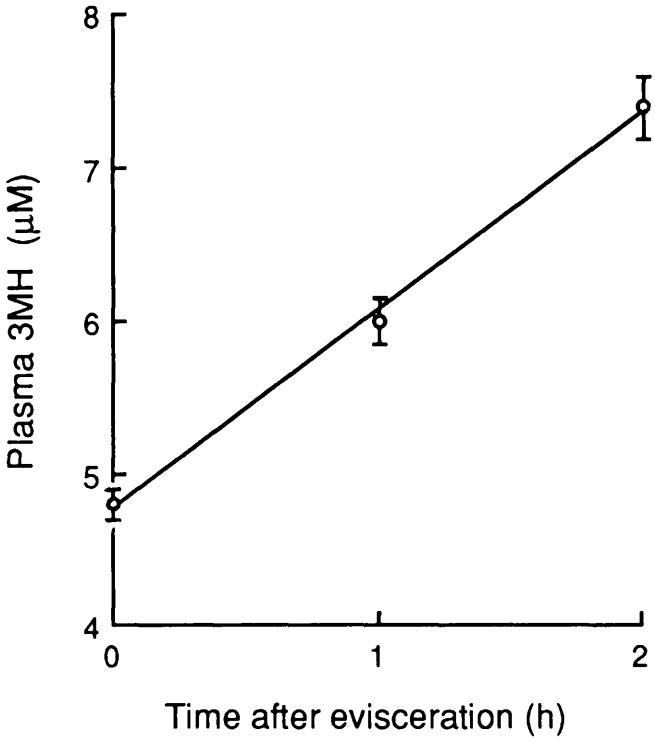

FIG. 1. Rise in concentration of 3-methylhistidine (3MH) in plasma of normal eviscerated rats (n = 4).

amined whether the increase in plasma 3-MH after evisceration reflects a similar change in skeletal muscle free 3-MH. Portions of left gastrocnemius muscles were frozen with tongs cooled in a mixture of acetone and dry ice and were then excised, weighed, and placed in storage at −20°C until analyzed for 3-MH. We compared muscle and plasma samples of 3-MH from nondiabetic, noneviscerated rats (5.53 ± 0.48 nmol/g wet wt and 4.36 ± 0.15 μM, respectively) with those obtained 2 h after evisceration (7.74 ± 0.28 nmol/g wet wt [$P < .005$ vs. intact rat] and 7.14 ± 0.30 μM [$P < .001$ vs. intact rat], respectively). After evisceration, muscle 3-MH concentrations increased in parallel with the plasma concentrations from the same preparations. Therefore, the increases in plasma concentrations could not be attributed to release of preformed intracellular 3-MH but more likely arose directly from degradation of myofibrillar protein.

An additional group of control and STZ-D rats were decapitated without anesthesia to obtain plasma to be frozen and stored for later analysis of corticosterone and insulin concentrations.

Analyses. Plasma glucose was determined by the glucose oxidase method with a glucose analyzer (Beckman). Tyrosine was analyzed fluorometrically by the method of Waalkes and Udenfriend (28) and 3-MH by high-performance liquid chromatography according to Wassner and Li (29). Kits for radioimmunoassay of plasma samples were obtained from Ciba Corning Diagnostics (Medfield, MA) for insulin and Radioassay Systems Laboratories (Carson, CA) for corticosterone.

Statistical evaluations were made with Student's two-tailed t test for unpaired data and analysis of variance for repeated treatments. Statistical significance was set at $P < .05$.

TABLE 1
Characteristics of rats with 20 h of streptozocin-induced diabetes compared to control rats before evisceration

	n	Food intake (g)	Body weight (g)		Plasma glucose (mM)
			Initial	Change	
Control	22	25 ± 1	269 ± 7	+11 ± 1	8.2 ± 0.2
Diabetic	43	24 ± 1	266 ± 4	−8 ± 1*	25.2 ± 0.4*

Values are means ± SE.
*$P < .001$ vs. control rats.

RESULTS

The preoperative condition of all rats used in the evisceration experiments is summarized in Table 1. As might be expected, when compared to control rats given buffer alone, the group given STZ developed hyperglycemia and lost some body weight. However, note that these acutely diabetic rats were in good condition, had minimal ketonuria, and consumed normal amounts of food. In addition, we measured the plasma hormone levels of similar groups of normal and diabetic rats that had been decapitated without anesthesia (Table 2). STZ-D reduced plasma insulin to half of control concentrations and increased corticosterone 2.5 times.

Total skeletal muscle protein degradation. Table 3 shows the concentration increase of plasma tyrosine and final plasma insulin concentrations in each experimental group of eviscerated cycloheximide-exposed rats. In normal preparations, the rate of accumulation of tyrosine over 2 h resembled the rate we had observed earlier (21). However, in the animals with STZ-D of 20 h duration, this indicator of proteolysis was very significantly enhanced. Diabetes caused only a minor increase in initial tyrosine concentration, but in the final concentration, there was a marked elevation so that the increment in plasma tyrosine was 33% greater in the diabetic group. This change corresponded to a 50% reduction in mean final plasma insulin concentration in the diabetic group. Longer experiments showed that the acceleration in protein breakdown persisted for at least 48 h after STZ exposure (n = 8). In fact, in the rats with STZ-D of 48 h, mean 2-h accumulation of plasma tyrosine (265 ± 4 μM), plasma glucose (24.2 ± 0.6 mM), and final insulin level (17.5 ± 2.8 μU/ml) were all virtually identical to those of rats diabetic for 20 h (Table 3).

Conversely, if the diabetic rats received various types of insulin treatment to raise plasma insulin levels, the diabetes-induced augmentation of plasma tyrosine accumulation was completely reversed (Table 3). Whether the diabetic rats received insulin subcutaneously during the hours before

TABLE 2
Effect of diabetes on plasma concentrations of glucose, insulin, and corticosterone in intact, unanesthetized rats

	n	Glucose (mM)	Insulin (μU/ml)	Corticosterone (ng/ml)
Control	10	8.4 ± 0.2	69.3 ± 17.0	76.1 ± 11.2
Diabetic	8	26.3 ± 0.8*	34.8 ± 2.5*	186.1 ± 50.4†

Values are means ± SE.
*$P < .001$, †$P < .05$, vs. control rats.

TABLE 3
Effect of diabetes and insulin treatment on total protein degradation in eviscerated rats

		Plasma tyrosine (μM)			Final plasma insulin (μU/ml)
	n	Initial	Final	2-h increase	
Control	13	72 ± 2	286 ± 6	214 ± 5	34.2 ± 6.1
Diabetic	7	86 ± 6*	365 ± 14†	279 ± 10†	15.4 ± 1.4*
Diabetic given insulin					
Subcutaneous, 1–2 h preevisceration	4	63 ± 3‡	259 ± 7§	196 ± 8§	107 (*n* = 3)
Intravenous, at evisceration	8	72 ± 2‡	287 ± 10§	215 ± 9§	78.9 ± 10.1§

Values are means ± SE.
*$P < .05$, †$P < .001$, vs. control rats.
‡$P < .05$, §$P < .001$, vs. untreated diabetic rats.

evisceration or intravenously at evisceration, tyrosine concentrations rose less postoperatively than those of untreated diabetic rats, so that the increase in plasma tyrosine level was again in the normal range (Table 3). Administration of insulin at evisceration elevated final plasma insulin concentration to 78.9 ± 10.1 μU/ml compared to 15.4 ± 1.4 μU/ml in untreated diabetic rats.

Myofibrillar proteolysis. To determine whether diabetes specifically accelerates myofibrillar proteolysis, another group of STZ-D rats was eviscerated along with control rats, and the accumulation rate of 3-MH in the plasma was measured (Table 4).

The initial concentration of 3-MH in the plasma of normal rats was 4.10 ± 0.39 μM, which was similar to values previously reported (30) and not significantly affected by diabetes. However, when preparations from eviscerated STZ-D rats were compared with normal preparations over 2 h, the rate of accumulation of 3-MH in the plasma was greatly enhanced. In these diabetic rats, plasma insulin concentrations were reduced to 25% of normal fed levels (Table 4). Restoration of insulin concentrations to more normal levels by treatment with exogenous insulin completely abolished the diabetes-induced excess rise in 3-MH concentration (Table 4). As was the case with plasma tyrosine, insulin treatment given intravenously at evisceration was just as effective as pretreatment 1–2 h beforehand.

DISCUSSION

It would be useful to know whether diabetes in humans enhances and insulin inhibits the breakdown of protein in the skeletal muscles. Indirect evidence from studies of protein turnover suggests that this might be so (1–7). However, animal experiments are needed to reveal how changes in insulin concentration actually influence the rate of proteolysis in the muscles, and the results from such in vitro skeletal muscle preparations have been controversial. It is well known that muscles removed from the body and perfused with artificial mediums are in negative nitrogen balance in the basal state (31) and that in vitro inhibition of proteolysis in both nondiabetic (32) and diabetic (11) muscles requires large doses of insulin. Consequently, the physiological significance of findings obtained from in vitro muscles during diabetes and insulin treatment is still in doubt.

We demonstrated with eviscerated rat preparations that, in the peripheral tissues, acutely lower insulin levels during fasting were associated with an accelerated rate of total proteolysis, measured as tyrosine release. The extra proteolysis in fasted preparations responded to insulin treatment in a dose-dependent fashion (21), suggesting that in vivo endogenous insulin normally acts to inhibit the breakdown of skeletal muscle protein. The improved sensitivity to insulin of proteolysis in this preparation is probably related to the fact that the skeletal muscles are maintained in a more physiologically intact condition. As described in detail earlier (20,21), such preparations maintain normal circulation to the tissues during the experimental period if care is taken to prevent hypothermia and dehydration (20) and prevent hypoglycemia after removal of the liver and kidneys from the circulation (21). Consequently, the high basal rate of protein breakdown usually associated with in vitro muscle preparations is avoided (20).

In this study, we examined the effects of 1 day of STZ-D in the eviscerated rat model. Mild acute diabetes was used to minimize the secondary consequences of chronic insulin deficiency, and protein degradation was measured (20). Because of the uncertain status of myofibrillar proteolysis in

TABLE 4
Effect of diabetes and insulin treatment on myofibrillar protein degradation in eviscerated rats

		Plasma 3-methylhistidine (μM)			Final plasma insulin (μU/ml)
	n	Initial	Final	2-h increase	
Control	9	4.10 ± 0.39	6.24 ± 0.44	2.14 ± 0.16	40.8 ± 5.8
Diabetic	11	4.43 ± 0.20	7.80 ± 0.39*	3.37 ± 0.28†	10.0 ± 1.6‡
Diabetic given insulin					
Subcutaneous, 1–2 h preevisceration	8	4.23 ± 0.33	6.42 ± 0.39§	2.19 ± 0.38§	73.2 ± 15.1‖
Intravenous, at evisceration	6	5.02 ± 0.41	6.60 ± 0.59	1.58 ± 0.29‖	47.7 ± 12.5‖

Values are means ± SE.
*$P < .02$, †$P < .005$, ‡$P < .001$, vs. control rats.
‖$P < .001$, §$P < .02$, vs. untreated diabetic rats.

diabetes, the rate of degradation of contractile protein was also estimated from the rate that 3-MH accumulated in plasma. Finally, in view of the apparent resistance of other isolated muscle preparations to the antiproteolytic effects of insulin, we sought to determine whether proteolysis in the undisturbed skeletal muscles of diabetic eviscerated rats would decrease in response to more physiological increments in plasma insulin concentration.

Twenty hours after STZ, before evisceration and cycloheximide, the intact diabetic rats showed small but significant increases in plasma tyrosine concentrations. Plasma tyrosine concentrations are usually unaffected by diabetes in the rat (33), but such an increase could be caused by either excess tissue protein breakdown or lower rates of tissue protein synthesis. After evisceration and cycloheximide treatment of the rats with diabetes, it was possible to demonstrate more specifically that total proteolysis in the peripheral tissues was enhanced by ~30% compared with normal control preparations. The possibility that STZ administration had transient toxic side effects at 20 h unrelated to insulin deficiency is unlikely, given the very similar results obtained when animals were studied 48 h after STZ. Despite the differences in the preparations used, our results are remarkably similar to those obtained recently from soleus muscles in vitro, where tyrosine release was elevated 29% by 2 days of STZ-D (11).

Insulin deficiency after STZ administration was also found to accelerate myofibrillar proteolysis by 60% in these preparations (Table 4). Although the effect on myofibrillar degradation appeared to be more pronounced than that seen for total proteolysis (30%), precise quantitative comparisons between total and myofibrillar proteolysis are difficult in view of the use of different animals and experimental approaches. However, the weakness and loss of skeletal muscle mass that occurs in untreated diabetes may well be related to excessive rates of degradation of the contractile proteins of the myofibrils.

Our results contrast with those reported by Goodman (16), where 3 days of STZ-D affected neither tyrosine nor 3-MH release from the tissues into the perfusate of rat hindquarters. Only in very severely diabetic rats was there evidence of excess proteolysis, but it was linked to failure of the sick animals to eat rather than the diabetes. This was not unexpected, because in normal rats, fasting per se enhances total and myofibrillar proteolysis in the hemicorpus preparation (23) and perfused hindquarters (34) and also increases total protein breakdown in skeletal muscles of eviscerated preparations (21). However, we found that diabetes enhanced proteolysis even when poor food intake was not a factor (Table 1).

That lack of insulin causes the proteolysis induced by diabetes is supported by data from our experiments where insulin was given. In the eviscerated diabetic rats described here, as in fasted preparations reported earlier (21), treatment that raised plasma insulin concentration to the high normal (fed) range (74.2 ± 10.0 μU/ml, n = 12) effectively reversed the accelerated total protein degradation caused by experimental diabetes. Although earlier work had shown that the excess total protein degradation in the diabetic soleus muscle responded to insulin, only pharmacological doses were used (11).

Restoration of normal insulin concentrations (64.1 ± 10.2 μU/ml, n = 14) also completely prevented the more rapid increase in 3-MH that otherwise occurred in untreated diabetic eviscerated rats. These findings agree with two earlier reports in which insulin suppressed the release of 3-MH from the perfused hind limbs of diabetic rats (17,18), although recently, on the basis of similar experiments with the skinned perfused hindquarters, it has been concluded that insulin does not influence myofibrillar proteolysis (16). Our results suggest that in the comparatively intact, undisturbed skeletal muscles of eviscerated rats not only total protein but also myofibrillar degradation is sensitive to physiological amounts of insulin.

Of the tissues remaining after evisceration, the skin also contains 3-MH. The amounts are small (total bound in skeletal muscles is 16 times greater than in skin), but skin protein appears to turn over rapidly, based on the high rates of protein synthesis (35). Whether this translates into high rates of degradation is questionable, because large amounts of the protein products of skin are constantly lost to the environment as hair and sloughed dead cells. Because it is impossible to keep a skinned, eviscerated animal in good physiological condition, especially with regard to body temperature and fluid balance (factors that also affect body protein turnover), the contribution of skin to our results could not be directly investigated. However, the likelihood that skin rather than muscle accounts for our findings seems remote.

Although the final insulin levels in the treated rats were in the normal physiological range (averaging 67 μU/ml), we recognize the possibility that earlier, immediately after the intravenous insulin load, a brief period of supraphysiological hyperinsulinemia probably occurred. However, we showed previously that total protein degradation in eviscerated fasted rats responded equally well to constant infusions of insulin designed to maintain the concentration within physiological limits (21). Although it is still unclear which enzymes are responsible for muscle proteolysis in vivo (36), further support for the view that insulin affects myofibrillar protein degradation can be found in some studies of the enzyme activities in skeletal muscles, where experimental diabetes activates and insulin inhibits neutral serine proteinases that degrade myofibrillar proteins (37–40)

The rate of degradation of myofibrillar and other proteins in skeletal muscle is undoubtedly influenced by numerous factors and catabolic hormones that change during acute diabetes; most notable among these is the increased secretion of adrenal corticosteroids (Table 2). The possible contributions of these regulators are yet to be fully examined in diabetic skeletal muscle. Nevertheless, our results strongly suggest that the excess catabolism of skeletal muscle protein that occurs in diabetes results, at least in part, from the absence of the direct restraining influence on proteolysis of normal amounts of insulin.

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