Muscle Wasting in Insulinopenic Rats Results from Activation of the ATP-dependent, Ubiquitin–Proteasome Proteolytic Pathway by a Mechanism Including Gene Transcription

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Abstract

In normal subjects and diabetic patients, insulin suppresses whole body proteolysis suggesting that the loss of lean body mass and muscle wasting in insulinopenia is related to increased muscle protein degradation. To document how insulinopenia affects organ weights and to identify the pathway for accelerated proteolysis in muscle, streptozotocin-treated and vehicle-injected, pair-fed control rats were studied. The weights of liver, adipose tissue, and muscle were decreased while muscle protein degradation was increased 75% by insulinopenia. This proteolytic response was not eliminated by blocking lysosomal function and calcium-dependent proteases at 7 or 3 d after streptozotocin. When ATP synthesis in muscle was inhibited, the rates of proteolysis were reduced to the same level in insulinopenic and control rats suggesting that the ATP-dependent, ubiquitin–proteasome pathway is activated. Additional evidence for activation of this pathway in muscle includes: (*a*) an inhibitor of proteasome activity eliminated the increased protein degradation; (*b*) mRNAs encoding ubiquitin and proteasome subunits were increased two- to threefold; and (*c*) there was increased transcription of the ubiquitin gene. We conclude that the mechanism for muscle protein wasting in insulinopenia includes activation of the ubiquitin–proteasome pathway with increased expression of the ubiquitin gene. (*J. Clin. Invest.* 1996. 98:1703–1708.) Key words: protein degradation • insulin • diabetes • gene expression • skeletal muscle

Introduction

The loss of lean body mass is a devastating complication of diabetes. The mechanisms causing this problem are unknown but measurement of whole body protein turnover in insulin-dependent, diabetic patients indicates that protein degradation and leucine oxidation are increased (1, 2). In poorly controlled diabetic patients or rats, there is excessive urinary excretion of 3-methylhistidine, a nonmetabolizable amino acid found exclusively in contractile proteins (3, 4), and insulinopenia increases protein degradation in the perfused rat hindquarter (5). These reports indicate that muscle protein degradation is activated by insulinopenia.

Does insulin directly suppress muscle protein degradation? Addition of insulin to cultured L8 myotubes or other types of cells decreases proteolysis (6, 7). Infusion of insulin into the forearm of healthy subjects suppressed the rate of appearance of phenylalanine compatible with decreased protein degradation (8, 9). In rats with chronic renal failure (CRF),[1] insulin suppresses protein degradation in incubated muscle and the perfused hindquarter (10, 11). Of interest, the extent of the suppression of protein breakdown after insulin was comparable in muscles of CRF and control rats (11).

An unanswered question is, Which proteolytic pathway is stimulated by insulinopenia? In muscle, there are four pathways for protein degradation: lysosomal proteases including the cathepsins; calcium-dependent proteases; cytosolic ATP-dependent; and cytosolic ATP-independent proteolytic pathways. In hepatocytes, insulin suppresses the formation of autophagic vacuoles and protein degradation in lysosomes, suggesting that lysosomes could be activated when the level of insulin is low (12). The ATP-dependent ubiquitin–proteasome pathway is activated in several catabolic states (13–19). Proteins degraded in this pathway are first conjugated to ubiquitin in an ATP-dependent reaction. The ubiquitin–protein conjugates are then degraded by the 26S proteasome in an ATP-dependent reaction which unfolds the protein, releases ubiquitin, and degrades the protein to small peptides and amino acids (20–22). The proteasome is composed of a 20S core containing multiple subunits arranged in four rings of seven subunits each and 19S "cap" complexes of multiple protein subunits, some of which exhibit ATPase activity (23). The present studies were designed to assess the impact of insulinopenia on organ weights including muscle mass. In addition, we sought to determine which proteolytic pathway(s) is activated in muscle of these rats.

Methods

Materials. Streptozotocin (STZ) was purchased from Pfanstiehl Laboratories, Inc. (Waukegan, IL), ZetaProbe GT membranes from Bio-

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1. *Abbreviations used in this paper:* CRF, chronic renal failure; EDL, extensor digitorus longus; STZ, streptozotocin.

Rad Laboratories (Hercules, CA), [^{32}P]dCTP and [^{32}P]CTP from Amersham (Arlington Heights, IL), and Multistix 10 SG reagent strips from Miles Inc. (Elkhart, IN). All other chemicals or reagents were purchased from Sigma Chemical Co. (St. Louis, MO). The proteasome inhibitor MG132 was generously provided by ProScript, Inc. (Cambridge, MA).

Rat model. After ether anesthesia, rats weighing ~ 150 grams were given a single tail vein injection of STZ (125 mg/kg prepared fresh in 0.1 M citrate buffer, pH 4.0) at 7 a.m. These rats were fed 23% protein chow ad libitum for the first day and the quantity of food eaten by the STZ-treated rats was measured. Vehicle-injected, control rats were pair-fed the same amount of food. Urine was collected to measure glucosuria and ketonuria using Multistix 10 SG reagent strips. At 6 p.m. the night before an experiment, food was removed from both groups of rats to eliminate the variability related to differences in food absorption. After muscles were removed, arterial blood was collected to measure blood glucose.

Measurement of muscle protein degradation. The mixed-fiber epitrochlearis muscles were dissected from the forelimbs of insulinopenic and pair-fed, vehicle-injected control rats and preincubated at resting length for 30 min at 37°C in 95% O_2/5% CO_2 in Krebs-Ringer bicarbonate (KRB) media, pH 7.4, containing 10 mM glucose and 0.5 mM cycloheximide (13, 14). The muscles were then placed in fresh media, regassed, and incubated for 2 h at 37°C. At the end of the incubation period, TCA (final concentration, 10%) was added to precipitate proteins; tyrosine in the media was measured to calculate the rate of protein degradation. In another experiment, we measured the intracellular pool of free tyrosine in muscle to assess its impact on measured protein degradation (14).

The influence of insulinopenia on different proteolytic pathways was evaluated by measuring the rates of protein degradation in an epitrochlearis muscle incubated with inhibitors of different pathways while the contralateral muscle was incubated without these inhibitors. Results were compared with those from muscles of the control rat incubated in the same fashion. Lysosomal and calcium-activated proteases were blocked by fixing the muscle at resting length and adding 1 mU/ml insulin, branched-chain amino acids (200 μM valine, 170 μM leucine, 100 μM isoleucine) and 10 mM methylamine (an inhibitor of lysosomal function), by deleting calcium from the KRB and by adding 50 μM trans-epoxysuccinyl-L-leucylamido-(4-guanidino butane) (E-64), a potent inhibitor of the calpains and lysosomal proteases cathepsins B, D, H, and L (24, 25). ATP in muscles was depleted by replacing glucose with 5 mM 2-deoxyglucose and adding 0.5 mM DNP to the media (13, 14). In other experiments, the proteasome was inhibited by adding 30 μM MG132 (14, 26–28).

Northern blot hybridizations. RNA was isolated from the mixed-fiber, gastrocnemius muscles using TriReagent (Molecular Research Center, Cincinnati, OH) and separated in a formaldehyde/agarose gel by electrophoresis before transfer to a ZetaProbe GT membrane (Bio-Rad). The blots were sequentially hybridized with cDNA probes for human ubiquitin and rat proteasome C3, C5, and C9 subunits which were labeled with [^{32}P]dCTP using the random-primer method (13, 29, 30). After overnight hybridization, the membranes were washed once with 2× SSC/0.5% SDS at 42°C followed by two washes with 0.5× SSC/0.5% SDS at 65°C; the washed membranes were exposed to x-ray film at −80°C with intensifier screens. Autoradiographic bands were quantified using a scanning densitometer (model 1680; Bio-Rad).

Nuclear run-on assays. Nuclei were isolated from 12–13 grams of hindquarter muscle of insulinopenic and pair-fed, control rats and run-on assays were performed as described (14). After removal of unincorporated [α-^{32}P]CTP, equivalent dpm of ^{32}P-labeled transcripts from each reaction were hybridized with immobilized cDNAs for human ubiquitin, rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and human γ-actin for 3 d; washes were performed as described (14).

Statistical analysis. Values are presented as means±SEM. Results were analyzed using Student's *t* test when results from two experimental groups were compared or using ANOVA when data from three groups were studied; for data analyzed by ANOVA, pair-wise comparisons were made by the Student-Newman-Keuls test.

Results

Initially, control rats maintained their weight, but insulinopenic rats lost weight (Fig. 1) in part because of a decrease in food intake. Food intake was 12.7±0.7 grams/d for day 1 and rose to 24.7±0.6 grams/d by day 6. In spite of the increase in food, insulinopenic rats still did not gain weight. Rats injected with STZ developed persistent glucosuria by day 1. On day 7, the blood glucose in insulinopenic and control rats was 269±17 and 105±7 mg/dl ($P < 0.001$), respectively. Insulinopenic rats also developed ketonuria by day 1 but it disappeared by day 7 and on this day, the serum bicarbonate concentration was 26.6±1.1 mM in insulinopenic rats and 24.7±0.7 mM in control rats.

The muscles of insulinopenic rats were smaller than those of the vehicle-injected, pair-fed, control rats (Table I). The loss of muscle mass was not specific for fiber type because the white-fiber extensor digitorus longus (EDL), the red-fiber soleus, and the mixed-fiber gastrocnemius muscles were all smaller. Moreover, the muscle protein content (normalized for DNA content) was lower in the EDL and soleus muscles of insulinopenic rats compared with values from control rats. Note that these differences were not due to a failure to grow because muscles of ad libitum fed rats with comparable body weights to those of rats before STZ injection were larger (soleus = 65.1±3.3 mg; EDL = 52.9±1.6 mg; gastrocnemius = 811.3±5.5 mg) than those of rats after 7 d of insulinopenia. Thus, muscle weight declined after STZ injection. The liver of insulinopenic rats was also smaller than that of the pair-fed,

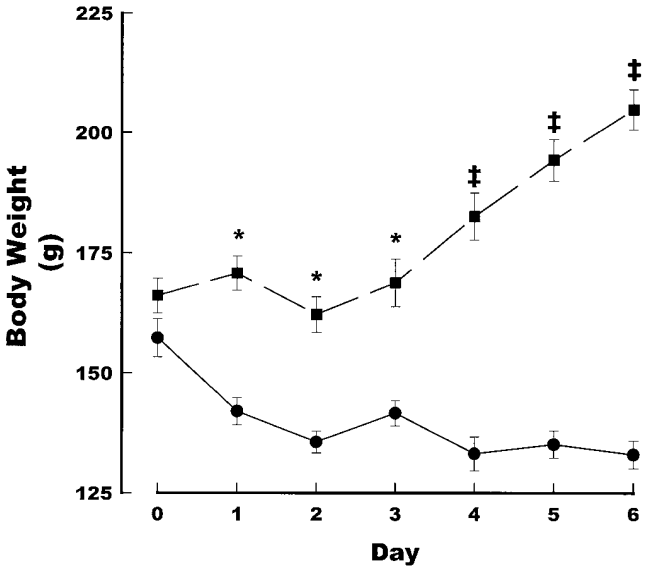

Figure 1. Daily weights of insulinopenic and sham-injected, pair-fed control rats. Mean±SE values of the body weights of nine pairs of insulinopenic (*filled circles*) and pair-fed control (*filled squares*) rats are presented. Rats were weighed at 6 a.m. the morning of injection with STZ or vehicle and at 9 a.m. on subsequent days. Body weights on the day when muscles were studied are not reported since the rats were fasted the night before. *$P < 0.001$; ‡$P < 0.0001$.

Table I. Organ Weights and Protein Content of Muscles from Insulinopenic and Pair-fed, Control Rats

	Group	Soleus weight	Soleus protein content	EDL weight	EDL protein content	Gastrocnemius weight	Liver weight	Epididymal fat weight
		mg	*μg protein/μg DNA*	*mg*	*μg protein/μg DNA*	*grams*	*grams*	*grams*
7 day	Control	78.1±3.1	79.4±4.3	97.3±3.1	106.7±5.9	1.10±0.04	6.39±0.19	0.37±0.02
	Insulinopenic	44.5±1.5*	66.1±3.0‡	43.0±1.6*	74.2±3.2§	0.48±0.02*	3.91±0.06*	NF*
3 day	Control	76.7±3.1	91.4±3.0	81.0±1.6	133.9±7.4	0.993±0.04	5.52±0.16	0.49±0.02
	Insulinopenic	62.9±4.0‖	85.3±3.0	62.2±3.0§	112.6±1.5‖	0.763±0.03§	5.07±0.23	0.23±0.03*

Values are mean±SEM of measurements made at 3 ($n = 8$) or 7 d ($n = 9$) after STZ injection; *NF*, none found. ‡$P < 0.05$ vs. control, ‖$P < 0.01$; §$P < 0.001$; *$P < 0.0001$.

control rats (Table I). Finally, the epididymal fat pads in insulinopenic rats were undetectable in rats after 7 d compared with those of pair-fed, control rats (Table I).

To determine which proteolytic pathway is activated in muscle, we measured protein degradation in the mixed-fiber epitrochlearis muscle of insulinopenic and pair-fed, control rats (31). First, we assessed the role of lysosomal and calcium-activated proteases by incubating one muscle under basal conditions and the contralateral muscle under conditions that inhibit lysosomal function and calcium-activated proteases (16). The basal rate of protein degradation was higher in muscles of insulinopenic rats and this difference was not eliminated by blocking lysosomal and calcium-dependent proteases (Fig. 2). Blocking these proteases reduced protein degradation 21% in muscles of control rats and 16% in muscles of insulinopenic rats (P = NS). The differences in tyrosine release between control and insulinopenic rat muscles cannot be attributed to accumulation of intracellular tyrosine because we measured the free tyrosine concentration in muscles incubated with inhibitors of lysosomal function, calcium-activated proteases, and ATP production. There was no significant difference in the values found in muscles of control and insulinopenic rats in muscles incubated without inhibitors (118±15 and 121±16 nmol tyrosine/gram of wet weight of muscle, respectively); in muscles incubated with inhibitors, these values were 109±8 and 102±13 nmol tyrosine/gram of wet weight, respectively. Thus, the rate of tyrosine release reflects protein degradation. Similar findings were obtained in muscles from rats with CRF and their pair-fed control rats (14) and in septic rats (15).

To investigate whether the increase in proteolysis was related to activation of an ATP-dependent pathway, one muscle from an insulinopenic or pair-fed, control rat was incubated with inhibitors of lysosomal and calcium-dependent proteases and the contralateral muscle of each rat was incubated under identical conditions except that inhibitors of ATP synthesis were added. As in the previous experiment, blocking lysosomal and calcium-dependent proteases did not prevent the increase in protein degradation in muscles of insulinopenic rats. This difference was blocked by the inhibitors of ATP synthesis (Fig. 3).

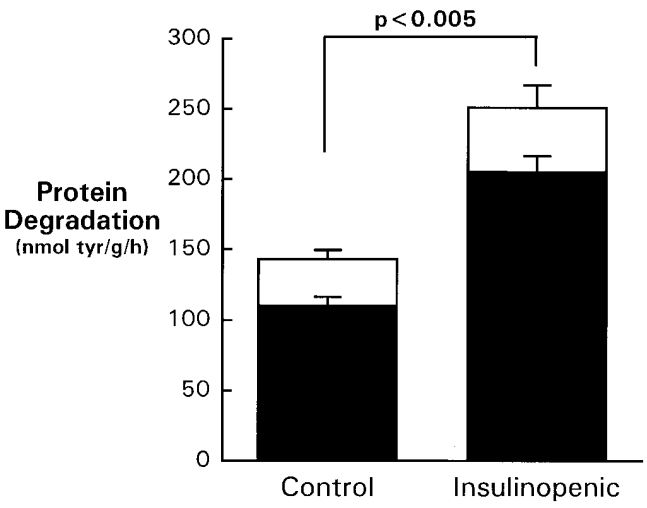

Figure 2. Insulinopenia does not increase muscle proteolysis through lysosomal and calcium-activated protease pathways. At 7 d after STZ injection, total protein degradation (*open bars*) was measured in one epitrochlearis muscle of six pairs of insulinopenic and pair-fed control rats while the contralateral muscle was incubated with inhibitors of lysosomal and calcium-dependent proteases (*solid bars*). Total proteolysis was increased by insulinopenia ($P < 0.005$) and this response persisted after inhibiting lysosomal and calcium-dependent proteolysis ($P < 0.005$).

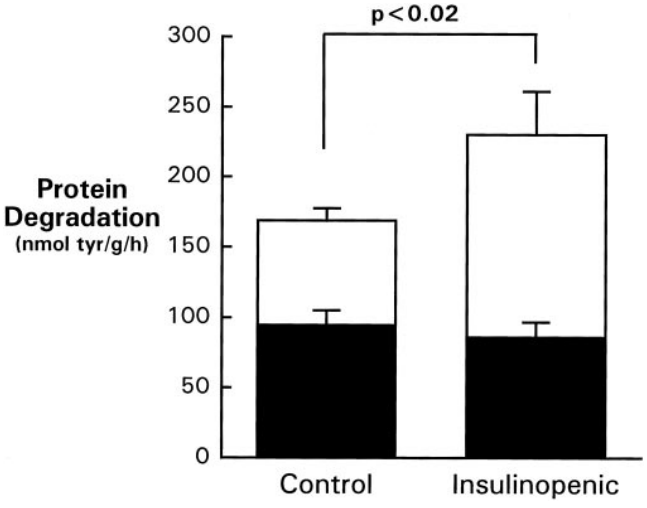

Figure 3. Insulinopenia stimulates an ATP-dependent proteolytic pathway in muscle. At 7 d after STZ injection, protein degradation was measured in one epitrochlearis muscle of six pairs of insulinopenic and pair-fed control rats incubated with inhibitors of lysosomal function and calcium-dependent proteases (*open bars*) while the contralateral muscles were incubated with the same inhibitors plus 2-deoxyglucose and dinitrophenol to prevent ATP synthesis (*solid bars*). When ATP production was halted, the increase in muscle protein degradation ($P < 0.02$) was eliminated.

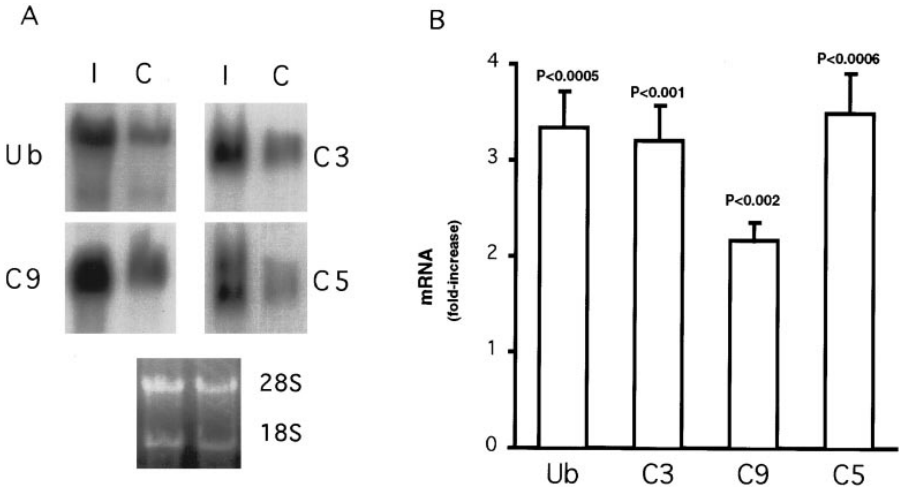

Figure 4. mRNAs encoding proteins of the ubiquitin–proteasome pathway are increased in muscles of insulinopenic rats after 7 d. At 7 d after STZ injection, RNA from muscles of pair-fed control (*C*) and insulinopenic (*I*) rats was hybridized with cDNA probes for human ubiquitin and C3, C5, and C9 subunits of the rat proteasome. The corresponding ethidium-stained 18S and 28S ribosomal RNA bands are also shown as are densitometric measurements (mean±SE, $n = 6$) of the mRNA values normalized to the corresponding 18S values. Results are expressed as the fold increase in the value found in the muscles of pair-fed, control rats.

To provide evidence for involvement of the ubiquitin–proteasome pathway in the proteolytic response to insulinopenia, we compared the levels of mRNAs encoding components of this pathway in muscles of experimental and control rats. 7 d after STZ injection, there was an increase in mRNAs for ubiquitin, C3, C5, and C9 proteasome subunits in muscles of insulinopenic rats (Fig. 4). Ubiquitin mRNA was increased 3.3±0.4 fold ($P < 0.0005$) while mRNA levels of the proteasome C3, C5, and C9 subunits were increased 3.2±0.4 ($P < 0.001$), 3.5±0.4 ($P < 0.0006$), and 2.2±0.2 ($P < 0.002$) fold, respectively.

To determine if shorter periods of insulinopenia also stimulate the ubiquitin–proteasome pathway in muscle, we studied rats 3 d after STZ injection. Both muscles and fat pads were smaller at 3 d after STZ; the protein content of the white-fiber EDL muscle was lower in insulinopenic rats but the protein content in the red-fiber soleus muscle was not different from that of control rats (Table I). To document involvement of the proteasome, one muscle was incubated under conditions that block lysosomal and calcium-activated protein degradation while the contralateral muscle was incubated under identical conditions except that MG132, an inhibitor of proteasome activity, was added (14, 26, 28). Inhibition of lysosomal and calcium-dependent proteases did not eliminate the increase in muscle protein degradation at 3 d after STZ (Fig. 5); the magnitude of the increase in muscle proteolysis was comparable with that at 7 d after STZ (Figs. 2 and 3). In the presence of MG132, the increase in muscle proteolysis was eliminated. The

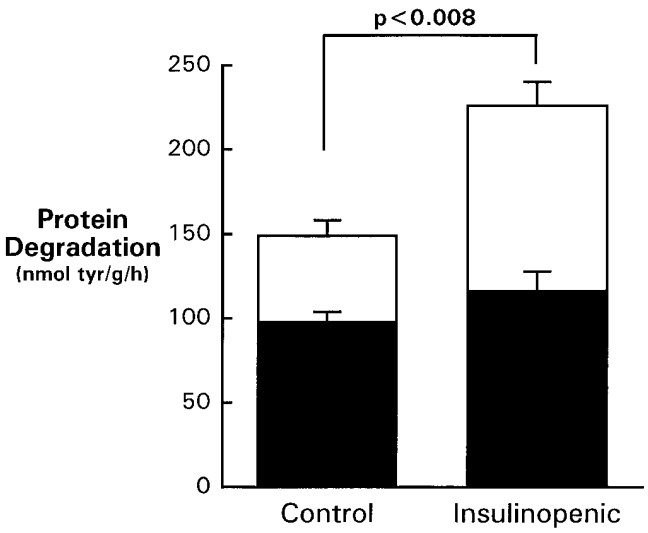

Figure 5. Insulinopenia increases proteasome-dependent protein degradation in muscle. At 3 d after STZ injection, protein degradation was measured in one epitrochlearis muscle of eight pairs of insulinopenic and pair-fed control rats incubated with inhibitors of lysosomal function and calcium-dependent proteases (*open bars*); the contralateral muscle was incubated with the same inhibitors plus the proteasome inhibitor MG132 (*solid bars*). This inhibitor blocked the increase ($P < 0.008$) in protein degradation induced by insulinopenia.

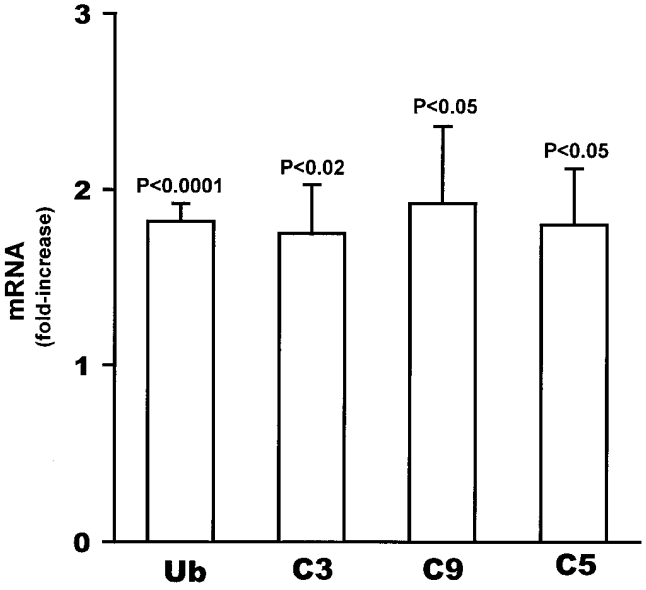

Figure 6. 3 d of insulinopenia increases mRNAs encoding proteins of the ubiquitin–proteasome pathway in muscle. At 3 d after STZ injection, RNA from muscles of pair-fed control and insulinopenic rats was hybridized with cDNA probes for human ubiquitin and C3, C5, and C9 subunits of the rat proteasome. Shown are results of densitometric measurements (mean±SEM, $n = 5$ pairs) of the mRNA (normalized to the corresponding 18S RNA) which are expressed as the fold increase in the value found in muscles of control rats.

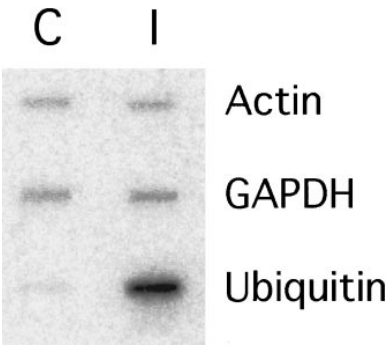

Figure 7. Insulinopenia increases ubiquitin gene transcription in muscle. Nuclei were isolated from muscles of insulinopenic (*I*) and pair-fed, control (*C*) rats to perform a nuclear run-on assay. Radiolabeled transcripts were hybridized with γ-actin, GAPDH, or ubiquitin cDNA inserts (2 μg each) and the results from one pair of insulinopenic and control rats are shown; identical results were obtained from three pairs of rats.

stimulation of muscle protein degradation was also eliminated by giving insulin to STZ-injected rats, suggesting that insulin may directly or indirectly suppress activity of this pathway (Price, S.R., manuscript in preparation). Concurrent with the increase in protein degradation at 3 d after STZ injection, the levels of mRNAs encoding ubiquitin and proteasome subunits were increased (Fig. 6).

To determine the mechanism for the increase in levels of mRNAs encoding components of the ubiquitin–proteasome pathway, we performed a nuclear run-on assay using nuclei isolated from muscles from rats 3 d after STZ injection and from control rats. We found that insulinopenia stimulates transcription of the ubiquitin gene (Fig. 7). In contrast, the transcription of two genes encoding proteins that do not participate in protein degradation (GAPDH and γ-actin) was unchanged (Fig. 7).

Discussion

We found that insulinopenia induced by STZ causes substantial loss of body weight and the mass of muscle, liver, and adipose tissue. Others have provided evidence that the loss of muscle mass with insulinopenia is related to accelerated protein degradation. For example, urinary 3-methylhistidine excretion is increased in diabetic patients (4) and rats (3) and the rate of protein degradation is increased in the perfused hindquarter muscles of insulinopenic rats (5). The latter experiments were performed in rats the first day after injection with 65 mg/kg STZ, but accelerated muscle proteolysis was not present at 3, 7, or 28 d after the same dose of STZ, nor was the proteolytic pathway stimulated by insulinopenia identified (32). When rats were given a higher dose of STZ (125 mg/kg), muscle protein degradation increased but it was concluded that this response was due in large part to decreased food intake because control rats given the same amount of food also had increased muscle protein degradation (32). In the present studies, we controlled for this factor by using a pair-feeding protocol. Moreover, the food intake was sufficient to support growth in pair-fed, control rats (Fig. 1).

What proteolytic pathway is activated by insulinopenia? It is well-established that insulin can suppress lysosomal proteolysis (12), so it was surprising to find that inhibition of lysosomal function had no significant impact on muscle proteolysis. Kettelhut et al. (33) reported that calcium- and ATP-dependent muscle proteolysis were increased during the acute phase of insulinopenia (1–3 d after injection of STZ) but by day 5, muscle protein breakdown returned to normal. We did not find that calcium-dependent proteolysis played an important role. However, ATP-dependent muscle proteolysis was activated early and persisted for at least 7 d. Recently, there has been an explosion of interest in the role of the ATP-dependent, ubiquitin–proteasome proteolytic system in degrading muscle proteins. The ATP-dependent, ubiquitin–proteasome pathway in muscle is activated in several states associated with muscle wasting including acidosis (13), uremia (14), sepsis (15), fasting (16), cancer (17, 18, 34), muscle denervation (19), and burn injury (35). The present results indicate that insulinopenia also stimulates this pathway in muscle. As in the other catabolic conditions, the increase in protein degradation was accompanied by increased levels of mRNAs encoding components of this pathway in muscle. We determined that this response is due in part to stimulation of gene expression because results from a nuclear run-on experiment showed increased transcription of the ubiquitin gene in muscle. The only other condition in which increased transcription of components of this pathway has been documented is chronic uremia (CRF) (14). In CRF, we found that the response is related to the presence of acidosis and/or glucocorticoids because rats with CRF are acidotic and have increased production of glucocorticoids (10) and correcting the acidemia of CRF blocks the increase in mRNAs encoding components of the ubiquitin–proteasome pathway (14). The link to glucocorticoids arises from our results showing that acidosis in normal rats increases glucocorticoid excretion, muscle proteolysis, and the levels of mRNAs of components of this pathway (11, 13), while adrenalectomy blocks the rise in these mRNAs (29). In the latter experiment, we found that a combination of acidosis plus a physiologic dose of glucocorticoids was required for the increase in mRNAs. In the present studies, we found high levels of the mRNAs encoding ubiquitin and the C3, C5, and C9 subunits of the proteasome at 7 d after STZ injection even though the serum bicarbonate was normal. Consequently, it is difficult to ascribe these responses to acidosis especially since our earlier report showed that in metabolic acidosis the ubiquitin mRNA level returns to control values within 24 h of correcting metabolic acidosis (13).

Do the changes in protein degradation, levels of mRNAs for ubiquitin and proteasome subunits, and gene transcription in response to insulinopenia occur in all fiber types of skeletal muscle? The answer to this question cannot be determined easily because the amount of muscle and the methods required to evaluate these responses preclude us from making these measurements in the same muscle. We predict that these responses occur in most muscles because: (*a*) epitrochlearis muscles used to measure proteolysis and gastrocnemius muscle used to measure mRNAs are mixed-fiber muscles (36, 37) and the proteolytic responses to acidosis, CRF, and starvation are similar in both muscles (13, 14, 16); (*b*) we found that mRNAs for ubiquitin and subunits of the proteasome are increased in white-fiber EDL and mixed-fiber gastrocnemius muscles in response to acidosis and glucocorticoids (29); and (*c*) the increased proteolysis and mRNA levels of ubiquitin in response to starvation and denervation occur in both the white-fiber EDL and red-fiber soleus muscles (16, 38).

In conclusion, the present findings indicate that the loss of lean body mass in insulinopenia results in part from muscle wasting due to activation of the ubiquitin–proteasome pro-

teolytic pathway. This activation includes increased gene transcription, resulting in an increase in mRNAs encoding components of this pathway. Characterization of the factors that regulate the expression of these genes will be an important step in understanding the mechanism(s) causing protein catabolism in disease states characterized by muscle wasting.

Acknowledgments

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