Muscle Protein Turnover in the Perfused Hindquarters of Lean and Genetically Obese-Diabetic (*db/db*) Mice

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SUMMARY

The stability of the perfused mouse hindquarter was assessed for a period of 5 h, and the preparation shown to remain stable for metabolic studies over this time period. Muscle protein synthesis and degradation rates in lean and diabetic-obese (*db/db*) mice were measured using the in situ perfused hindquarter preparation. The rates of protein synthesis were 48% lower in the muscles of intact *db/db* mice than in the lean controls when expressed per gram TCA precipitable protein and 46% lower when expressed per gram dry weight. Adrenalectomy, which has been shown to restore the lean body mass of the *db/db* mice to normal, had the effect of returning protein synthesis rate in muscle of *db/db* mice to lean control values. Insulin at a dose of 1 mU/ml stimulated protein synthesis in lean mice only, showing that the process of protein synthesis in the *db/db* mice is also insensitive to insulin. Measurements of the rates of degradation of muscle protein showed no differences between lean and *db/db* mice. These findings suggest that the decreased lean body mass of *db/db* mice is the result of a defect in protein synthesis rather than due to altered degradation. DIABETES 1984; 33:1160–64.

Obesity in the commonly used animal models, the Zucker fatty (*fa/fa*) rat, the obese (*ob/ob*) mouse, and the diabetic-obese (*db/db*) mouse is inherited as an autosomal Mendelian recessive trait.[1] The obesity is characterized by excess lipid deposition, hyperinsulinemia, insulin resistance, and a decrease in the lean body mass.[1,2]

Manipulations that have been used to reduce body weight have been unsuccessful in changing the body composition of these animals.[1,3–6] This has led to the suggestion that the mechanism responsible for the imbalance in lipid and protein deposition may be an important lesion in these mutants. In the *ob/ob* mouse, Trostler et al.,[7] using 3-methylhistidine content as a measure of lean body mass, showed that the 3-methylhistidine decreased in these mice as a function of age. Using this method, these authors showed that there was no difference in the fractional synthesis rate of myofibrillar proteins between lean and obese mice, but there was an increase in the fractional breakdown rate of lean tissue in the obese mice. These authors concluded that the decreased lean body mass of the obese mice was a result of increased protein breakdown. They further related this increase in protein degradation rate to an increase in the activity of alkaline, myofibril-bound protease in the muscles of *ob/ob* mice.[8] However, it has to be noted that, at this stage, muscle mass was already decreased, and so the fractional synthesis rate or the fractional breakdown rate does not explain the differential development of lean body mass.

In the Zucker fatty rats, Dunn and Hartsook demonstrated that after an injection of a mixture of [U-^{14}C]-amino acids, there was more incorporation of the labeled carbons into body lipids than into the lean tissues of the obese rats.[9] Thus as yet, the reason(s) for the decreased lean body mass in genetically obese rodents is unclear, and the issue of protein turnover rate in the obesity syndrome remains unresolved. In the present study, we have measured both protein synthesis and degradation rates in the muscles of lean and diabetic-obese (*db/db*) mice using the perfused hindquarter preparation.

MATERIALS AND METHODS

Animals. Male C57BL/KsJ and C57/Ks-*db/db* mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) at 4–5 wk of age. They were fed ad libitum on laboratory chow and kept in a 12-h light/12-h dark cycle in a constant temperature (24°C) controlled room.

Adrenalectomy was performed through bilateral flank incisions under ether anesthesia.[10] Adrenalectomized mice

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were given 0.9% saline to drink, had free access to chow, and were studied 2 wk after surgery. Successful adrenalectomies were checked by following weight gains and by visual inspection for adrenal tissue at time of killing.

The mouse hindquarter perfusion procedure was as described by Chan and Dehaye,[11] which is a modification of the perfusion procedure described by Ruderman et al. for rats.[12] The standard perfusion medium was made up of Krebs-Henseleit bicarbonate buffer, pH 7.4, containing 3% bovine serum albumin (Pentax, fraction V), 20% thoroughly washed (with saline) aged human erythrocytes, and 7 mM glucose.

For the measurement of protein synthesis, the following mixture of amino acids, as described by Rannels and Jefferson,[13] was added to the perfusion medium: aspartate 118 μM, threonine 283 μM, serine 254 μM, asparagine 80 μM, glutamine 596 μM, proline 161 μM, glutamate 303 μM, glycine 370 μM, alanine 465 μM, valine 181 μM, methionine 54 μM, isoleucine 90 μM, leucine 159 μM, tyrosine 88 μM, lysine 540 μM, histidine 71 μM, arginine 228 μM, tryptophan 100 μM, and phenylalanine 320 μM. The standard perfusion medium with no added amino acids was used to measure protein degradation.

Analytic techniques. For the measurement of glucose uptake, blood samples were obtained every 20 min from both the arterial and venous cannulae after a 30-min equilibration period. The samples were centrifuged and glucose levels of the supernatant were determined by the glucose-oxidase method using a Beckman Glucose Analyzer (Fullerton, California). Oxygen tension in the arterial and venous samples was measured using a Corning Blood Gas Analyzer (Medfield, Massachusetts). Phenylalanine was assayed fluorometrically using a kit obtained from Sigma (Sigma Technical Bulletin 60-F, St. Louis, Missouri). Tissue dry weights were determined by drying a fixed amount of tissue in an oven at 80°C to a constant weight. For the measurement of trichloroacetic acid (TCA)-precipitable protein, about 100 mg tissue was homogenized in 1 ml of 10% TCA. The homogenate was spun in a bench centrifuge, the supernatant decanted, and the pellet dried in an oven to a constant weight.

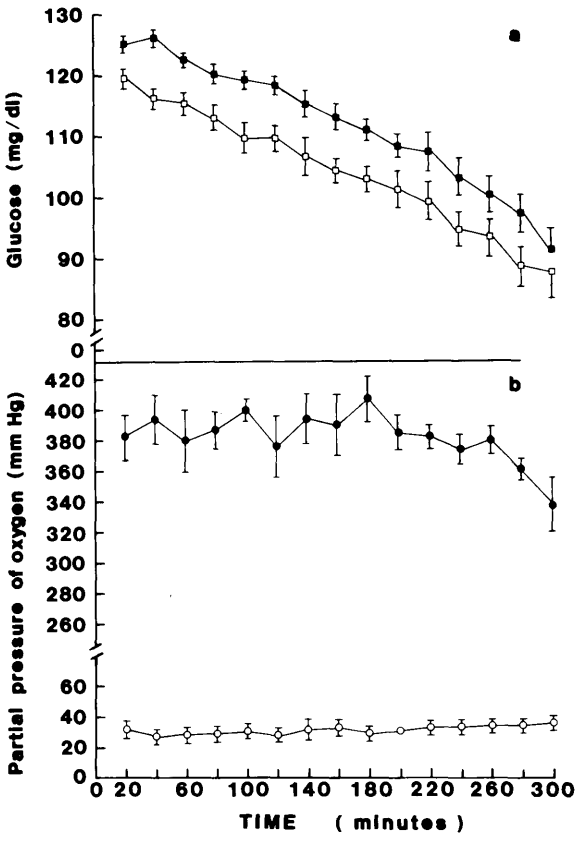

FIGURE 1. Characteristics of the perfused mouse hindquarter preparation. Lean mice weighing 19–23 g were used in these experiments. Values are mean ± SEM for 8 perfusions. Closed symbols (●,■) are arterial and open symbols (○,□) are venous levels. The preparation was allowed to equilibrate for 30 min after which time a recirculating perfusion was established. Arterial and venous samples were taken from this point.

Protein synthesis. Protein synthesis was measured essentially by the method described by Jefferson et al.[14] After a 30-min equilibration period during which the perfusion medium was not recirculated, a recirculating perfusion was set up and 5 μCi [U-^{14}C]-phenylalanine was injected into the reservoir. The perfusion medium contained a mixture of all amino acids,[13] and phenylalanine at five times the normal concentration (320 μM). The hindquarters were perfused for 180 min, at which time the tissue was removed and freeze-clamped as reported earlier.[11] Muscle tissue was then pulverized under liquid nitrogen, and a known amount precipitated in 10% TCA. The TCA-precipitated protein was solubilized in formic acid and counted in a liquid scintillation spectrometer to determine the radioactivity incorporated. Rates of protein synthesis were calculated on the basis of the specific activity of ^{14}C-phenylalanine in the perfusion medium.[14] Phenylalanine incorporated (nmol/g protein/3 h) = ([dpm incorporated/g protein]/3 h)/^{14}C-phenylalanine sp. act.

Protein degradation. Protein degradation was determined by measuring the net release of phenylalanine from the muscle in the presence of 100 μM cycloheximide, an inhibitor of protein synthesis, which prevented the reincorporation of phenylalanine into protein.[13-15] Again, a 3-h recirculating perfusion was used after a 30-min equilibration period. No amino acids were added to the perfusion medium. It is assumed that under these conditions, the net phenylalanine released equals protein degradation.

Source of chemicals. Bovine serum albumin (Pentax fraction V) was obtained from Miles Laboratories (Elkhart, Indiana). Glucose was obtained from Fisher Scientific (Tustin, California). All amino acids and the phenylalanine assay kit were from Sigma. L-[U-^{14}C]-phenylalanine (sp. act. 415 mCi/mmol) was purchased from ICN Pharmaceuticals, Inc. (Irvine, California).

Statistical analysis of data. All data are expressed as mean ± SEM. The numbers in parentheses indicate the number of animals perfused. The level of significance was determined using Student's *t*-test for unpaired data.

RESULTS

The stability of the perfused hindquarter preparation during a 5-h perfusion. Previous studies using the perfused hindquarter of the mouse have been over a time period of about 2 h.[11,16] The present experiments required longer periods. Glucose uptake and oxygen consumption by the per-

TABLE 1
Protein synthesis rates in the perfused hindquarters of intact and adrenalectomized lean and *db*/*db* mice

	Protein synthesis rate			
	nmol PA incorporated/3 h/g protein*		nmol PA incorporated/3 h/g dry weight	
	− Insulin	+ Insulin	− Insulin	+ Insulin
Lean				
Intact	277.5 ± 40.1 (5)	416.3 ± 37.9 (7)§	270.4 ± 20.5 (5)	384.1 ± 45.4 (8)§
Adx	412.6 ± 36.6 (5)‖	426.3 ± 30.1 (6)	343.8 ± 24.6 (5)‖	363.1 ± 23.0 (6)
Diabetic-obese				
Intact	143.1 ± 20.7 (5)†	167.8 ± 13.2 (8)†	147.2 ± 24.0 (5)†	171.0 ± 11.7 (8)†
Adx	380.1 ± 39.7 (5)‡	324.5 ± 40.6 (6)‡	347.8 ± 36.4 (6)‡	299.6 ± 34.2 (6)‡

PA = Phenylalanine. Protein synthesis was measured as described in the text. The values represent mean ± SEM for the number of observations shown in parentheses. Adx = adrenalectomy.
*TCA-precipitable protein.
†$P < 0.005$ compared with intact lean mice in the same group.
‡$P < 0.005$ compared with intact *db*/*db* mice in the same group.
§$P < 0.005$ compared with basal in the same group.
‖$P < 0.05$ compared with intact mice in the same group.

fused mouse hindquarter during 5 h of perfusion are shown in Figure 1. In a recirculating system, the glucose concentration in the perfusion medium steadily falls with time. Figure 1a shows that the extraction of glucose is constant over the 5-h period (8–9 mg/dl) at every point measured. Figure 1b shows the partial pressure of oxygen in the arterial and venous samples. A constant extraction of oxygen by the tissue (A-V difference of about 360 mm Hg) during the 5 h shows that the preparation remains stable for this duration. A slight fall in the arterial pO_2 in the last hour is probably due to the deterioration of some red blood cells. A relatively constant rate of lactate release (1.3 nmol/min) was maintained throughout the 5-h period. These parameters clearly indicate that, under the conditions used, the perfused hindquarter maintained metabolic integrity over the 5-h period. This is the first demonstration that the preparation can be used for studies up to 5 h.

Although the data presented in Figure 1 are for lean mice, similar data have been obtained using age-matched obese (*db*/*db*) mice, showing that the preparation is likewise stable for this genotype.

Basal and insulin-stimulated protein synthesis in intact lean and *db*/*db* mice. Table 1 shows that, under basal conditions, protein synthesis, expressed per gram of TCA-precipitable protein or tissue dry weight, was 48% lower in skeletal muscle of *db*/*db* mice compared with the lean control mice as measured by the incorporation of ^{14}C-phenylalanine. No significant differences were obtained between the TCA-precipitable protein from lean or obese tissues, and the values compared well with the dry weights determined for the same tissues. Therefore, it is quite evident that the rates of protein synthesis expressed per dry weight or per TCA-precipitable protein reflect true differences between the phenotypes, and are not due to any differences in the fat content of the muscles. Additions of 1 mU/ml insulin to the perfusion medium significantly increased protein synthesis in intact lean mice from 277.5 ± 40.1 nmol phenylalanine incorporated/g protein/3 h to 416.3 ± 37.9 nmol. Tissue from *db*/*db* mice did not respond to insulin. The lack of insulin stimulation in *db*/*db* mice reflects the insulin resistance that develops in the genetically obese rodents, which are hyperinsulinemic.[1] Using a similar preparation, we have previously reported decreased glucose uptake in both the basal and insulin-stimulated states by muscle of *db*/*db* mice[11] and *ob*/*ob* mice.[16] The present observation shows that, like glucose uptake, protein synthesis in muscle also exhibits insulin resistance.

Effect of adrenalectomy on muscle protein synthesis. Adrenalectomy has been reported to normalize most of the defects observed in the genetically obese rodents,[6,10,16–22] including an increase in the muscle mass.[20–22] Using the perfused preparation, we have recently shown that in the *ob*/*ob* mice, adrenalectomy restores glucose uptake and insulin sensitivity in muscles from these animals.[16] As shown in Table 1, adrenalectomy increases protein synthesis rates in both lean and *db*/*db* mice, namely by 50% in the lean and by 166% in the *db*/*db* mice. The rate of muscle protein synthesis in adrenalectomized *db*/*db* mice was similar to the rate seen in the adrenalectomized lean mice. The presence of insulin in the medium had no effect on the rate of protein synthesis in either adrenalectomized lean or *db*/*db* mice.

Protein degradation in muscles of lean and *db*/*db* mice. Protein degradation by the perfused hindquarter was assessed by measuring the release of phenylalanine from muscle in the presence of 100 μM cycloheximide, an inhibitor of protein synthesis. Such an approach has previously been used in the rat by many researchers.[13–15] The data in Table 2 indicate that there is no difference in the rates of protein degradation in muscles of lean and *db*/*db* mice.

DISCUSSION

The technique of labeling proteins with radioactive amino acids has been used extensively to study both protein synthesis and degradation of muscle proteins in the rats.[13,14] However, studies on protein turnover in the muscles of mice are scarce. The small size of the animal may be the reason for this. Monier et al.[23] have used isolated soleus muscle from lean and goldthioglucose (GTG) obese mice to measure protein turnover. Using this in vitro preparation, they concluded that the protein synthesis machinery was not impaired in GTG-induced obesity. The hyperinsulinemia that

TABLE 2
Protein degradation rates in the perfused hindquarters of lean and *db/db* mice

	Lean	*db/db*
Body wt (g)	21.5 ± 1.2 (6)	33.0 ± 1.8 (5)*
Wt of tissue perfused (g)	1.72 ± 0.12 (6)	1.55 ± 0.12 (5)
Protein degradation rate (nmol phenylalanine released/g tissue/3 h)	1120 ± 115 (6)	1330 ± 200 (5)

Values are mean ± SEM for the number of perfusions shown in parentheses. Phenylalanine concentration in the medium increased linearly with time. In blank runs, phenylalanine released from red blood cells alone in the presence of 100 μM cycloheximide did not increase with time, but remained constant (~1000 nmol). Net phenylalanine released from muscle proteolysis was taken as the difference between the phenylalanine released during the perfusion and that due to red blood cells alone.
*$P < 0.001$ compared with the lean group.

is a characteristic of the GTG and other obese models[1] is generally associated with the development of insulin resistance. However, in the study of Monier et al.,[23] the authors showed that the process of protein synthesis was not associated with insulin resistance. Trostler et al.,[7] using 3-methylhistidine content as a measure of lean body mass, showed a decrease in 3-methylhistidine in the *ob/ob* mice as a function of age. Although the urinary excretion of 3-methylhistidine was similar in lean and obese mice, the fractional breakdown rate (expressed as a percentage of total 3-methylhistidine content) was increased in the *ob/ob* mice. In the same study, the fractional synthesis rate of myofibrillar protein was calculated and was shown to be similar in lean and obese mice. This led these authors to conclude that the decreased lean body mass of obese mutants was a result of increased protein breakdown.

The contribution of different tissues to 3-methylhistidine is unclear. It has been shown, for example, that in the rat the skin contributes 20% or more to the total 3-methylhistidine that is excreted.[24] Wassner et al.,[25] using the perfused hemicorpus and perfused intestine preparations, showed that in the rat the turnover of the gastrointestinal tract actomyosin is much greater than that of skeletal muscle and is responsible for over 40% of the 3-methylhistidine that is excreted in the urine. In a recent report, Millward and Bates[26] elaborate further on this and show that a substantial amount of urinary 3-methylhistidine is derived from non-muscle sources. Therefore, the existing evidence clearly suggests that 3-methylhistidine excretion, particularly in the intact animal, does not represent protein turnover of skeletal muscle or other lean tissues.

The perfused hindquarter preparation provides a more physiologic model and is more representative of the collective tissue rather than individual muscles. The use of this preparation has enabled a direct measurement of protein synthesis by the mixed muscles of lean and *db/db* mice in both the basal and insulin-stimulated states. The values obtained compare favorably with the rates of protein synthesis reported by others in the perfused hindquarter of rats.[13-15] *Db/db* mice show a lower rate of protein synthesis than do lean controls. Data calculated on the basis of tissue dry weight or TCA-precipitable protein dry weight clearly indicate that the reduced protein synthesis rate in the *db/db* mice is not attributable to a lower percent of lean mass in these animals. The presence of 1 mU/ml insulin caused a 50% stimulation of protein synthesis in lean mice and only 17% in the *db/db* mice. The insulin stimulation observed in lean mice compares well with the data of others.[13-15] The difference in protein synthesis between lean and *db/db* mice does not appear to be due to differences in the availability of amino acids for incorporation. In a separate set of experiments (data not shown) the initial rates of amino acid transport measured by the uptake of α-aminoisobutyric acid did not appear to be different in muscles from lean and *db/db* mice. Although insulin has been clearly shown to stimulate transport of amino acids into cells,[27,28] its effect on protein synthesis does not appear to be secondary to the transport process. This has been demonstrated in hepatoma cells in culture[29] and in the isolated soleus muscle preparation.[23]

The normal accretion of protein in the body is a balance between the rates of protein synthesis and degradation. After the inhibition of protein synthesis with cycloheximide, the accumulation of phenylalanine in the perfusion medium has been assumed to represent muscle protein degradation. Using this approach, we are unable to show a difference between the rates of protein degradation in lean and *db/db* muscle. Protein degradation rates obtained here are several fold higher than the synthesis rates in both lean and *db/db* mice. This observation is consistent with all other reports.[13-15,30,31] The values shown in Table 2, however, should only be taken as a relative measure, as the technique has some limitations. The measurements have been made in the absence of amino acids and complete inhibition of protein synthesis. The presence of circulating amino acids is known to play an important role in the control of protein turnover.[32]

Many recent reports have shown the absolute requirement of adrenal glucocorticoids for the development of the obesity in both the genetically obese[6,10,16-22] and in ventromedial hypothalamus (VMH)-lesioned[33,34] rodents. The *db/db* mouse exhibits adrenal hypertrophy and increased circulating levels of corticosterone.[35] Adrenalectomy caused an increase in the protein synthesis rate in lean mice, and to an even greater extent in the *db/db* mice. This increased rate of protein synthesis in muscle of adrenalectomized *db/db* mice may be responsible for the increase in lean tissue seen in obese mice after adrenalectomy.[20-22] In contrast to the glucose transport process, in which adrenalectomy improved the insulin action in the obese (*ob/ob*) mice but not in the lean mice,[16] protein synthesis in adrenalectomized mice failed to respond to insulin regardless of the phenotype. To our knowledge, there have been no studies on the effect of insulin on protein synthesis in adrenalectomized rodents, and the observation here is probably the first of its kind in muscle. The mechanism by which steroid deficiency influences insulin stimulation of protein synthesis in skeletal muscle remains to be investigated.

In conclusion, the data reported here strongly suggest that the decreased protein accretion in the genetically obese-diabetic (*db/db*) mouse is a result of a lower rate of protein synthesis and not due to a difference in the rate of degradation. We have shown that the process of protein synthesis also exhibits insulin resistance, and that the adrenal glu-

cocorticoids play an important role in the metabolism of muscle protein. Further studies need to be carried out to evaluate protein turnover more precisely, and to understand the hormone interaction with regard to protein turnover.

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