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Diazoxide-induced insulin deficiency greatly reduced muscle protein synthesis in rats: involvement of eIF4E

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Sinaud, Sandrine, Michèle Balage, Gérard Bayle, Dominique Dardevet, Thomas C. Vary, Scot R. Kimball, Leonard S. Jefferson, and Jean Grizard. Diazoxide-induced insulin deficiency greatly reduced muscle protein synthesis in rats: involvement of eIF4E. *Am. J. Physiol.* 276 (*Endocrinol. Metab.* 39): E50–E61, 1999.—We have investigated the effect of a postprandial acute insulin deficiency induced by diazoxide injection on rat skeletal muscle protein synthesis. Diazoxide administration lowered plasma insulin >85% within 3 h after injection, whereas other hormones (insulin-like growth factor I, glucagon, corticosterone) involved in the regulation of muscle protein synthesis were not altered significantly compared with control animals. The fractional rate of muscle protein synthesis, measured *in vivo*, was reduced significantly ($P < 0.05$) in epitrochlearis (–46%), gastrocnemius (–41%), and soleus (–35%). The reduction in protein synthesis did not result from a reduced total RNA content but was associated with diminished translation efficiency. Analysis of ribosomal subunits revealed that the decreased translation efficiency resulted from an impairment in the initiation phase of protein synthesis. Diazoxide-induced insulin deficiency was associated with a dramatic decrease in eukaryotic initiation factor (eIF) 4G bound to eIF4E and a 2.5-fold increase in the amount of the eIF4E·4E-binding protein 1 (BP1) complex. In contrast, diazoxide injection did not change either the relative amount of eIF4E present in gastrocnemius or its phosphorylation state. These results indicate that an acute insulin deficiency significantly decreases postprandial muscle protein synthesis by modulating the interaction between 4E-BP1, eIF4G, and eIF4E to control translation initiation.

postprandial insulin deficiency; eukaryotic initiation factors 4E and 4G; 4E-binding protein 1; translation initiation

INSULIN PLAYS a major role in the regulation of muscle protein anabolism. *In vitro* studies have firmly established that insulin *per se* stimulates protein synthesis and inhibits protein degradation in skeletal muscle (9, 13, 19, 25). However, *in vivo* studies have been less conclusive, especially concerning the ability of insulin to stimulate protein synthesis. Most *in vivo* studies have failed to demonstrate a stimulatory effect of the hormone on muscle protein synthesis in either humans or animals (review in Ref. 38). In fact, only a few studies have shown an increase in skeletal muscle protein

synthesis after insulin administration in normal, healthy volunteers (5, 6) or young postabsorptive rats (1, 15). Consequently, the involvement of insulin in the regulation of muscle protein synthesis *in vivo* has been mainly investigated in insulin-deficient states.

Decreasing the plasma insulin concentration by either experimental diabetes or starvation usually causes a significant reduction in the rate of protein synthesis in rat skeletal muscles (1, 2, 11, 22, 27, 33), which results from an impairment in the initiation phase of protein synthesis (18, 37). Insulin replacement restores protein synthesis to control values. These observations have been interpreted to indicate a unique role for insulin in controlling protein synthesis. However, insulin deficiency is often associated with disturbances in plasma substrates (amino acids) and other hormones [insulin-like growth factor I (IGF-I), glucocorticoids, glucagon, etc.; review in Ref. 32], which could modify protein synthesis. Hypoinsulinemia causes a drastic decrease in plasma IGF-I concentrations and IGF-I mRNA content in most tissues of diabetic or fasted rats (7, 16, 42). Because rates of protein synthesis in gastrocnemius are linearly correlated with the muscle IGF-I content (26), decreases in protein synthesis observed in insulin-deficient states may be due to a decreased IGF-I content.

In the present study, we investigated the effect of an acute insulin deficiency in fed rats using a specific inhibitor of insulin secretion (the diazoxide). Skeletal muscle protein synthesis is greatly stimulated in response to food intake. However, the role of postprandial insulin secretion in this regulation is not clearly defined yet. Only few studies using anti-insulin antibodies (30, 36, 42) demonstrated that insulin was involved in the stimulation of muscle protein synthesis after oral feeding. Diazoxide injection, by depressing postprandial insulinemia, allows direct examination of effects of insulin on skeletal muscle protein synthesis *in vivo* and the mechanisms through which insulin modulates translation initiation. Other hormones involved in the regulation of protein synthesis (IGF-I, corticosterone, glucagon) were not altered significantly compared with control animals.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 70 g were purchased from IFFA CREDO (L'Arbresle, France) and were housed individually under controlled environmental conditions (temperature 22°C;

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12-h dark period starting at 0900). They were allowed food (composition given in g/kg dry matter in Ref. 3) and water ad libitum. Rats were acclimated to their surroundings for 5 days before the experimental protocol.

Experimental Procedure

The experimental design is shown in Fig. 1. Two types of experiments (I and II) were performed on the 6th day when the animals reached a mean body weight of 103.1 ± 1.4 g. In all experiments, 1 h after the beginning of the dark period, rats were fed for 1 h. They consumed 2.96 ± 0.17 and 3.04 ± 0.28 g (means $\pm$ SE) of dry matter in experiments of *type I* and *II*, respectively, on the day of the experiment. Food was then withdrawn, and the animals were randomly divided into the following two groups: the control group (C) and the diazoxide-injected group (DZ). The DZ group immediately received an intraperitoneal injection of diazoxide (20 mg in 3 ml of 0.05 N sodium hydroxide/100 g body wt; Sigma Chemical), whereas the C group received only the vehicle (Fig. 1).

Experiments of *type I* were designed to investigate the effect of diazoxide administration on 1) plasma hormones (insulin, IGF-I, glucagon, and corticosterone), plasma metabolites [glucose, β -hydroxybutyrate, nonesterified fatty acids (NEFA), and free amino acids], and in vitro protein metabolism at 1, 2, and 3 h after diazoxide injection or 2) ribosomal subunit distribution and initiation factor regulation 2 h after diazoxide injection. In these studies, animals were anesthetized with pentobarbital sodium (18 mg/100 g body wt) just before sample collection. For examination of in vitro protein metabolism, epitrochlearis muscles were dissected intact for incubations, immediately transferred to plastic tubes, preincubated for 30 min at 37°C in Krebs-Henseleit buffer supplemented with 5 mM glucose, 5 mM HEPES, 0.17 mM leucine,

0.20 mM valine, 0.10 mM isoleucine, and 0.1% BSA (99% fatty acid free), and saturated with a 95% O₂-5% CO₂ gas mixture as previously described (9, 10, 14). The muscles were then transferred to fresh medium of the same composition containing 0.5 mM [¹⁴C]phenylalanine (0.15 μ Ci/ml) and incubated again for 60 min at 37°C. To assess any direct effects of diazoxide on muscle protein metabolism, protein synthesis and breakdown were determined in vitro by incubating C muscles (epitrochlearis) in the absence or presence of an excess of diazoxide (0.4 mg/ml) according to the protocol as described in *Measurement of in vitro protein metabolism*. To measure plasma hormone and metabolite concentrations, blood samples were withdrawn by heart puncture in noninjected animals (*time 0*, basal values) and 1, 2, and 3 h after injection of vehicle or diazoxide and were rapidly centrifuged. The plasma was decanted and frozen until analyzed for hormones and metabolites. For ribosomal subunits and initiation factor analysis, gastrocnemius muscles were removed quickly, rinsed in ice-cold saline, weighed, and homogenized in the corresponding buffer. The homogenates were treated as described in *Ribosomal Subunit and Initiation Factor Analysis*.

Experiments of *type II* were performed to analyze the effect of diazoxide and exogenous insulin replacement on rates of skeletal muscle protein synthesis in vivo. The experimental design included the following four groups: 1) C; 2) DZ; 3) DZ group that received a low dose (see below) of insulin (Ins 1); and 4) DZ group that received a high dose of insulin (Ins 2). Muscle protein synthesis was measured in vivo by the incorporation of radioactive phenylalanine using the flooding dose method according to Garlick and Grant (15). This procedure reduces uncertainties about the labeling of the tracer amino acids in the precursor pool for protein synthesis.

Fig. 1. Experimental protocols. All animals received food 1 h after the beginning of the dark period and had free access to food for 1 h. At the end of feeding, animals were divided into the following 2 groups: the control (C) and the diazoxide-injected (DZ) group injected with vehicle or diazoxide according to the protocol described in MATERIALS AND METHODS. In type I experiments, an additional group that did not receive any injection was included to give basal values. Plasma metabolites and hormones were measured at *time 0* in nontreated rats (food is also removed at this time) and 1, 2, and 3 h after injections in vehicle or DZ rats. In vitro muscle protein turnover was only measured 1, 2, and 3 h after vehicle or diazoxide injections. Initiation factor analysis was only performed at 2 h after injections. In type II experiments, a second diazoxide injection was performed 1.5 h after the initial injection in DZ group, whereas C group received the vehicle. In addition, two additional groups were included [DZ group that received low dose of insulin (Ins 1) and DZ group that received high dose of insulin (Ins 2)], which were injected with insulin at the 1.5-h time point. In vivo protein synthesis was then measured using a flooding dose of phenylalanine, which was injected 20 min before killing. *t*₀, *t*₁, *t*_{1.5}, *t*₂, and *t*₃, Time at 0, 1, 1.5, 2, and 3 h, respectively.

A preliminary experiment showed that the flooding dose of phenylalanine significantly increased plasma insulin in the DZ group (from 6.2 ± 1.5 to 34.75 ± 10.3 μ U/ml before and 20 min after the bolus, respectively), whereas it did not in the C group. High doses of phenylalanine are known to transiently elevate plasma insulin, especially when given by rapid injection (29). Because diazoxide blocks insulin secretion without inhibiting its biosynthesis, insulin may have accumulated in β -cells and could have been liberated after the bolus injection of phenylalanine. Such a phenomenon could explain the rise in plasma insulin observed in DZ rats in contrast to the C group. To prevent the spike in plasma insulin due to the phenylalanine bolus, the DZ group received a second intraperitoneal injection of diazoxide (15 mg in 2 ml of 0.05 N sodium hydroxide/100 g body wt) 1.5 h after the first injection. Under these conditions, plasma insulin concentrations were not elevated by injection of the phenylalanine (6.5 ± 1.8 and 7.1 ± 2.5 μ U/ml before and after the bolus injection, respectively). At that time, Ins 1 and Ins 2 groups received a subcutaneous injection of insulin (Lente MC Insulin; Novo Pharmaceuticals) diluted in saline (197 ± 9 and 280 ± 6 mU in 0.5 ml/100 g body wt in Ins 1 and Ins 2 groups, respectively). These doses of insulin were chosen to partly or completely replace the plasma insulin concentrations (Table 1). The C group received the vehicle. The bolus injection of L-[U- 14 C]phenylalanine (30 μ Ci, 300 mM; 2 ml/100 g body wt) was given subcutaneously 2 h after the first diazoxide injection. Twenty minutes after the injection of radioactivity (i.e., 0.8 h after insulin or diazoxide injections and 2.3 h after the first diazoxide injection), the rats were anesthetized with pentobarbital sodium as described in preceding paragraphs. Soleus (in all groups) and gastrocnemius and epitrochlearis (only in C and DZ groups) were quickly excised, rinsed in cold saline, and blotted. They were then rapidly weighed and frozen in liquid nitrogen.

Analytical Methods

Free amino acid extraction. Plasma amino acids (*experiment I*) and tissue free amino acids (*experiment II*) were measured using the method described elsewhere (1). Briefly, plasma or frozen muscle samples were homogenized in 8 vol of ice-cold 10% trichloroacetic acid (TCA). The acid-soluble fraction containing free amino acids was separated from the protein precipitate by centrifugation (10,000 g for 20 min), and TCA was removed by chromatography on a column of cation exchange resin (AG50 X8, 100–200 mesh; Bio-Rad, Richmond, CA). For plasma amino acid determination, the NH_4OH effluent was dried and resuspended in 0.2 M lithium buffer (pH 2.2). Total plasma free amino acid concentrations were then determined by liquid chromatography (Resin BT 2410; Biotronik) with an automatic amino acid analyzer (LC

3000; Biotronik) using norleucine as an internal standard. For tissue free amino acids, the NH_4OH effluent was dried and resuspended in water (pH 7.0) for derivatization and HPLC analysis (see *Amino acid derivatization procedure and determination of phenylalanine specific radioactivity*).

Protein-bound amino acid extraction. Tissue protein precipitates were washed two more times in 10% TCA and finally one time in 0.2 M perchloric acid (PCA). Pellets were resuspended in 0.3 M NaOH, and an aliquot was used for protein measurement (see *Measurement of in vitro protein metabolism*). Pellets were precipitated again in 2 M PCA overnight at 4°C and centrifuged (10,000 g for 15 min). The corresponding supernatant was filtered, and RNA content was determined in this supernatant using the method described by Manchester and Harris (28). The protein pellets were then hydrolyzed in 6 N HCl for 24 h at 110°C, dried, and resuspended in water (pH 7.0) for amino acid derivatization.

Amino acid derivatization procedure and determination of phenylalanine specific radioactivity. Twenty microliters of tissue free amino acid extract and 500 μ l of protein-bound amino acid extract underwent the *o*-phthalaldehyde (OPA) derivatization at room temperature for 3 min using 40 μ l (for free amino acids) and 500 μ l (for protein-bound amino acids) of an OPA reagent. The OPA reagent was a mix of 50 mg OPA (Sigma Chemical) dissolved in 1 ml methanol with 9 ml borate buffer [800 mM, pH 10.4, Normapur RP (Prolabo)] and 100 μ l β -mercaptoethanol. Appropriate amino acid mixtures were used as standards over the range of 0.2–2.0 μ mol phenylalanine/ml. The derivatized amino acid extracts or standards were then injected in a Kontron HPLC system. Separation using a gradient of *solvent A* (phosphoric buffer containing 0.1 M $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ and 0.1 M H_3PO_4 adjusted to pH 7.0 using NaOH) and *solvent B* (*solvent A* supplemented with methanol, acetonitrile, and ultrapure water at 30, 35, 15, and 20%, respectively, vol/vol) was achieved at room temperature on a reverse-phase Interchim Spherisorb ODS2 column (5 μ m, 250×4.6 mm and 250×10 mm for free and for protein-bound amino acids, respectively). The corresponding flow rates were 1.00 and 4.25 ml/min, respectively. Chromatograms were processed by the integrator, and phenylalanine concentrations were calculated from peak areas of phenylalanine standards. The phenylalanine-associated radioactivity was determined by collecting the fractions corresponding to the phenylalanine peak. Radioactivity was measured by liquid scintillation spectrometry. The specific radioactivity (dpm/nmol) was calculated by dividing the phenylalanine-associated radioactivity (dpm/ml) by phenylalanine concentration (nmol/ml).

Measurement of in vitro protein metabolism. At the end of the incubation, muscles were blotted, weighed, and homogenized in 10% TCA. TCA insoluble material was washed three times with 10% TCA and solubilized for 1 h in 1 M NaOH at 37°C for determination of radioactivity incorporated into protein. Protein contents of tissues (*experiments I* and *II*) were determined in the NaOH solubilized material from TCA pellets (see *Protein-bound amino acid extraction*) by a colorimetric reaction using the bicinchoninic acid method (Pierce, Rockford, IL) with crystalline bovine serum albumin as a standard. Protein degradation was estimated as described elsewhere (9, 10, 44). Because tyrosine is neither synthesized nor degraded in muscle, release of the amino acid from muscle in the incubation medium reflects net protein breakdown. Tyrosine in the incubation medium was assessed fluorometrically (46).

Plasma substrates. Plasma glucose was determined enzymatically using glucose oxidase (Boehringer). Plasma NEFA and β -hydroxybutyrate were determined enzymatically using an autoanalyzer (4, 8).

Table 1. Plasma insulin and glucose concentrations in experiment II

Group	Plasma Insulin, μ U/ml	Plasma Glucose, mg/100 ml
C	66.2 ± 19.0	161 ± 0.1
DZ	$7.1 \pm 2.5^{*\dagger}$	$638 \pm 0.6^{*\ddagger}$
Ins 1	$22.4 \pm 3.8^*$	297 ± 0.6
Ins 2	41.6 ± 4.5	172 ± 0.1

Values are means $\pm$ SE. At time 0, animals were injected with either vehicle (C group) or diazoxide. Diazoxide-treated rats were divided into 3 groups 1.5 h later: the DZ group that received a second injection of diazoxide and Ins 1 and Ins 2 groups injected with low and high doses of exogenous insulin, respectively, as described in MATERIALS AND METHODS (*experiment II*). Plasma insulin and glucose were determined 50 min later at the time of death. There were 4 rats in C group, 7 rats in DZ group, and 5 rats in Ins 1 and Ins 2 groups. * $P < 0.05$ vs. C. $\dagger P < 0.05$ vs. Ins 2. $\ddagger P < 0.05$ vs. Ins 1.

Plasma hormones. Plasma insulin was determined by direct radioimmunoassay (RIA) with a commercial kit (ERIA Diagnostics Pasteur, Sanofi, France) using human insulin as standard. In the RIA, the insulin used as injection material (porcine-bovine mixture) to Ins 1 and Ins 2 groups cross-reacted with the antibody in a similar manner to human insulin standard, whereas rat insulin was more reactive. Plasma glucagon was determined by RIA with the Pharmacia kit (Serono). Total plasma IGF-I underwent a 0.5 M hydrochloric acid extraction process and a C₁₈ Sep Pak (octadecyl silica cartridge) purification before RIA, which was performed using a commercial kit (Sorin Biomedica). Plasma corticosterone was also determined by RIA (35).

Calculations

In vivo protein synthesis was expressed as fractional synthesis rates (FSR in %/day), which were calculated according to the method described (15): $FSR = 100 \times S_b / (S_a \times t)$, where S_b is the specific radioactivity of protein-bound phenylalanine, S_a the specific radioactivity of tissue free phenylalanine, and t the incorporation time (20 min), expressed in days. An estimate of translational efficiency was obtained by calculating the amount of protein synthesized per milligram RNA and per day ($\text{mg protein} \cdot \text{day}^{-1} \cdot \text{mg RNA}^{-1}$). Injection of phenylalanine resulted in elevated plasma free phenylalanine levels after 20 min ($772 \pm 30 \text{ nmol/ml}$ instead of $54 \pm 4 \text{ nmol/ml}$ after and before phenylalanine injection, respectively). In preliminary experiments using C and DZ rats, we verified that free phenylalanine specific radioactivity reached similar values in both plasma and muscle 10 min after injection of the same flooding dose and remained constant thereafter (data not shown). Due to this slow rate of equilibration, the calculated FSR likely underestimated the actual rates of protein synthesis in both groups. In the present experiments, the flooding dose was also effective in equalizing the free phenylalanine specific radioactivity in plasma and tissues; free phenylalanine specific radioactivity expressed in percent of the injected phenylalanine was 92.2 ± 5.0 and $84.1 \pm 4.4\%$ in plasma and muscle, respectively. No significant effect of diazoxide or insulin injections on the phenylalanine specific radioactivity was noted.

In vitro protein synthesis in epitrochlearis muscle was expressed as nanomoles of phenylalanine incorporated per milligram of protein per hour. Proteolysis was determined as previously described (9, 10, 44). It was then estimated as the sum of the net tyrosine release in the incubation medium and protein synthesis (phenylalanine incorporation in muscle proteins was expressed in terms of tyrosine equivalents; see Ref. 44). Proteolysis was expressed as nanomoles of tyrosine per milligram of protein per hour.

Ribosomal Subunit Analysis

For isolation of ribosomal subunits, fresh muscle (0.5 g of gastrocnemius) was homogenized in 6 vol of ice-cold homogenization buffer [in mM: 20 Tris, pH 7.4, 250 KCl, 5 MgCl₂, 1 dithiothreitol (DTT), and 2 vanadyl ribonucleoside] using a Polytron homogenizer. Simultaneously, 0.5 g of gastrocnemius of the second hindlimb of the rat was homogenized in the same buffer except vanadyl ribonucleoside was omitted for assay of total RNA. The homogenates were centrifuged at 10,000 *g* for 20 min at 4°C. Aliquots of the supernatants (0.8–1 ml), to which Triton X-100/deoxycholate had been added to final concentrations of 0.1%, were layered on 15–50% sucrose density gradients that were formed using homogenization buffer. Gradients were centrifuged for 18 h at 27,000 revolutions/min at 4°C in a Beckman SW28 rotor using a Beckman L8.70M ultracentrifuge. After centrifuga-

tion, the gradients were fractionated (~1 ml/fraction) with a peristaltic pump and a fraction collector (Gilson Medical Electronics, Middleton, WI). The absorbance at 260 nm was monitored with a Secoman spectrophotometer (Secoman, Domont, France). RNA contents of the homogenates and sucrose density gradient fractions were measured spectrophotometrically as the absorbance at 260 nm ($A_{260} \mu\text{g/ml} = 1$ at 260 nm), corrected for absorbance at 232 nm, after alkaline hydrolysis (12). The quantities of RNA in the 60S and 40S ribosomal subunit fractions were expressed relative to the total amount of RNA layered on the gradient ($\mu\text{g RNA/mg homogenate RNA}$).

Initiation Factor Analysis

For initiation factor analysis, 0.25 g of fresh muscle (gastrocnemius) were homogenized in 7 vol of *buffer A* [20 mM HEPES, pH 7.4, 100 mM KCl, 0.2 mM EDTA, 2 mM EGTA, 1 mM DTT, 50 mM NaF, 50 mM β -glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 0.5 mM sodium vanadate, and 1 μM microcystin LR] by use of a Polytron homogenizer. The homogenate was centrifuged at 10,000 *g* for 10 min at 4°C, and the supernatant was stored at –80°C until analysis.

Determination of the phosphorylation state of eukaryotic initiation factor 4E. The phosphorylated and unphosphorylated forms of eukaryotic initiation factor (eIF) 4E in gastrocnemius were separated by isoelectric focusing on a slab gel and quantitated by protein immunoblot analysis, as described previously (24, 41), using a monoclonal antibody against eIF4E. The blots were visualized using chemiluminescence as described by the manufacturer's instructions (Amersham). Films were scanned, and images were analyzed as described previously (41).

Quantification of 4E-BP1·eIF4E and eIF4G·eIF4E complexes. The association of eIF4E with 4E-BP1 and eIF4G was examined as described previously (24, 25). eIF4E and 4E-binding protein 1 (BP1)·eIF4E and eIF4G·eIF4E complexes were immunoprecipitated from aliquots of 10,000-*g* supernatants using an anti-eIF4E monoclonal antibody. The antibody-antigen complex was collected by incubation for 1 h with goat anti-mouse Biomag IgG beads (PerSeptive Diagnostics). Before use, the beads were washed in 1% nonfat dry milk in *buffer B* (50 mM Tris hydrochloride, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% β -mercaptoethanol, 0.5% Triton X-100, 50 mM NaF, 50 mM β -glycerophosphate, 0.1 mM PMSF, 1 mM benzamidine, and 0.5 mM sodium vanadate). The beads were captured by use of a magnetic stand and were washed two times with *buffer B* and one time with *buffer B* containing 500 mM, rather than 150 mM, NaCl. Protein bound to the beads was eluted by resuspending the beads in sodium dodecyl sulfate-sample buffer and boiling the sample for 5 min. The beads were collected by centrifugation, and the supernatants were subjected to electrophoresis either on a 7.5% polyacrylamide gel for quantification of eIF4G or on a 15% polyacrylamide gel for quantification of 4E-BP1 and eIF4E. Proteins were then electrophoretically transferred to a polyvinylidene difluoride membrane. The membranes were incubated with either a mouse anti-human eIF4E antibody, a rabbit anti-rat 4E-BP1 antibody, or a rabbit anti-eIF4G antibody for 1 h at room temperature. The blots were then developed using an enhanced chemiluminescence Western blotting kit (Amersham). Films were scanned and quantitated as described previously (41).

Statistics

Data are expressed as means $\pm$ SE. The significance of differences between groups in each experiment was analyzed

by ANOVA to test for overall differences among groups followed by a Student's *t*-test to test differences among the means only when the ANOVA indicated a significant difference among group means. Differences among means were considered significant at $P < 0.05$.

RESULTS

Plasma Hormones and Metabolites After Injection of Diazoxide

The time course (*experiment I*) for the effect of diazoxide on plasma insulin concentrations is shown in Fig. 2. Before diazoxide or vehicle injections, the postprandial mean plasma insulin concentration was 49.0 ± 10.3 $\mu\text{U/ml}$ in all animals (see *time 0* on Fig. 2). Diazoxide administration induced a significant decrease in postprandial plasma insulin concentration at each time after injection (3- to 4-fold decrease vs. C group, $P < 0.05$, Fig. 2). In the C group, the plasma insulin concentration remained unchanged over the 3 h. Pilot experiments showed that the decrease in plasma insulin in the DZ group was reversible. Insulin concentrations returned to values observed in control fed rats 5 h after the diazoxide injection (30.5 ± 3.0 and 36.7 ± 10.3 $\mu\text{U/ml}$ in DZ and C groups, respectively). In *experiment II*, plasma insulin was also very low in DZ rats compared with C (Table 1). Insulin injection of the DZ rats resulted in a dose-dependent increase in plasma insulin in the Ins 1 and Ins 2 groups (Table 1). As expected, the higher dose of insulin (Ins 2 group) restored plasma insulin concentration closer to that of the C group, whereas the low dose did not.

Other plasma hormones (IGF-I, glucagon, and corticosterone) were measured just at the end of feeding in nontreated animals (basal values) and 1, 2, and 3 h

after vehicle or diazoxide injections in *experiment I*. Unlike insulin, plasma hormone levels were not significantly different between C and DZ groups at any of the times examined after injections (Table 2). However, plasma IGF-I and corticosterone were both significantly increased 1 and 3 h after injections of either vehicle or diazoxide compared with basal values. Plasma glucagon did not change except 2 h after vehicle injection in the C group. Plasma glucose was constant in the C group in *experiment I* for the 0- to 3-h period (1.68 ± 0.08 g/l). Diazoxide injection induced hyperglycemia at 1, 2, and 3 h ($P < 0.05$ vs. C group) with a maximum value at 2 h (6.79 ± 0.40 g/l, $P < 0.05$ vs. values at 1 and 3 h, Fig. 2). Similar glucose concentrations were also observed in the DZ groups during *experiment II* at tissue harvest, suggesting that neither the phenylalanine injection nor the second diazoxide injection had an effect on plasma glucose. Injection of insulin in DZ animals partly corrected the diazoxide-induced hyperglycemic state (Table 1). Plasma glucose remained elevated with the lower insulin dose, whereas it reached a value observed in the C group with the higher dose.

Plasma β -hydroxybutyrate remained constant in the C group during the 0- to 3-h period. It was greatly increased in the DZ group at all times ($P < 0.05$, Fig. 3A). Plasma NEFA showed an increase in the C group during the 0- to 2-h period and then decreased at 3 h. The same temporal pattern was observed in the DZ group, but the peak occurred earlier (at 1 h), and the values at 2 and 3 h remained elevated. As a result, only values at 1 and 3 h were significantly higher in the DZ group than in the C group ($P < 0.05$, Fig. 3B).

Plasma amino acids were also assessed at 1, 2, and 3 h after diazoxide injection (Table 3). Most essential amino acids were similar in the DZ and C groups at 1 h. The decrease ($P < 0.05$) in plasma arginine in the DZ group was an exception. Two hours after diazoxide injection, there was a significant increase in plasma leucine along with a decrease in plasma methionine and tyrosine in the DZ compared with the C group. The change in plasma leucine was maintained at 3 h with additional increases in other branched-chain amino acids (valine and isoleucine) and a decrease in plasma arginine in the DZ compared with C group (Table 3). The effect of diazoxide injection to modulate amino acid concentrations was more pronounced on nonessential than on essential plasma amino acids, whatever the time after diazoxide injection (Table 3).

Muscle Protein Synthesis

The effect of diazoxide injection on muscle protein synthesis was measured *in vivo* 2 h after injection of diazoxide. Diazoxide-induced hypoinsulinemia reduced protein synthesis in each muscle examined (-35.2% , $P < 0.005$ vs. C in soleus; -41.2% , $P < 0.005$ vs. C in gastrocnemius; -46.5% , $P < 0.0001$ vs. C in epitrochlearis; Fig. 4A). Diazoxide per se had no direct effect on muscle protein metabolism because incubation of C muscles in medium with a high diazoxide concentration did not induce any change in either muscle protein

Fig. 2. Time course of plasma insulin and glucose in diazoxide-injected (○) and control (●) rats in *experiment I*. Rats were injected intraperitoneally with diazoxide (DZ group) or vehicle (C group) at *time 0*, as described in MATERIALS AND METHODS, whereas some animals did not receive any injection to give basal values (*time 0*). Plasma insulin was measured by radioimmunoassay at 0, 1, 2, and 3 h after injection. Plasma glucose was determined by an enzymatic method using the same samples and is shown in *inset*. Values are means $\pm$ SE of 5 animals in each group at each time. * $P < 0.05$ between C and DZ groups at the same time.

Table 2. Plasma IGF-I, glucagon, and corticosterone concentrations in DZ group and C group in experiment I

	Basal Values (time 0)	C Group			DZ Group		
		1 h	2 h	3 h	1 h	2 h	3 h
IGF-I, nmol/l	5.98 ± 0.95	11.3 ± 1.4*	9.7 ± 1.8	9.9 ± 1.1*	13.2 ± 1.9*	7.7 ± 1.0	11.6 ± 0.4*
Glucagon, pg/ml	369 ± 29	430 ± 63	527 ± 46*	349 ± 19	308 ± 17	488 ± 50	431 ± 73
Corticosterone, ng/ml	41 ± 15	105 ± 10*	84 ± 14	116 ± 27*	152 ± 6*	87 ± 11	125 ± 29*

Values represent means ± SE of 5 rats at each time in each group. IGF-I, insulin-like growth factor I. At the end of food intake, animals were divided into 3 groups: DZ group, C group, and a group that did not receive any injection (this group yielded basal values). In treated groups, plasma hormones were measured 1, 2, and 3 h after injections. *Significantly different from basal values at $P < 0.05$ (ANOVA); no statistically significant difference between DZ and C groups was observed at the same time.

synthesis (0.40 ± 0.06 vs. 0.37 ± 0.04 nmol phenylalanine · mg protein⁻¹ · h⁻¹ in the absence or presence of diazoxide, respectively) or breakdown (1.30 ± 0.06 vs. 1.33 ± 0.13 nmol tyrosine · mg protein⁻¹ · h⁻¹, in the absence or presence of diazoxide, respectively) measured in vitro. These data indicate that impaired protein synthesis observed in skeletal muscles from DZ-treated rats in vivo resulted from DZ-induced postprandial insulin deficiency. To further examine the specific role of insulin on protein synthesis in DZ-induced hypoinsulinemia, insulin was injected 1.5 h after diazoxide injection (Ins 1 and Ins 2 groups). Injection of insulin restored plasma insulin concentrations (Table 1). Because all three muscles studied in the DZ group exhibited the same reduction in protein synthesis after diazoxide injection, fractional protein synthesis rates were only determined in soleus muscle from rats subjected to insulin replacement. As shown in Fig. 4A, *inset*, insulin injections to DZ-treated rats normalized FSR to values observed in C rats (i.e., values from Ins 1 and Ins 2 groups did not differ significantly from the C group). Although FSR was not statistically significantly different between Ins 1 and Ins 2 groups, the increase of protein synthesis after insulin injection was more pronounced with the larger insulin dose.

Rates of protein synthesis are dependent on both the muscle ribosome content and the translational efficiency of ribosomes. Therefore, the content of RNA in the muscles was determined. No significant differences in the RNA content were observed between any of the muscles (Fig. 4B) or treatments studied (Fig. 4B, *inset*),

indicating that a decrease in the abundance of ribosomes for protein synthesis was not responsible for the changes in muscle protein synthesis. In contrast to RNA content, translational efficiency was significantly reduced after treatment with DZ (Fig. 4C). The magnitude of the decrease in translational efficiency paralleled the reduction in protein synthesis. Furthermore, administration of insulin to DZ-treated rats resulted in a stimulation of translation efficiency. Consequently, the changes observed in in vivo FSR were entirely explained by changes in the ribosomal efficiency (Fig. 4C, *inset*).

To examine potential mechanisms through which DZ-induced hypoinsulinemia reduced the translational efficiency, the content of free 40S and 60S ribosomal subunits was measured in gastrocnemius from C and DZ groups 2 h after injections. As shown in Fig. 5, diazoxide injection increased the RNA contents of the 60S and 40S subunit fractions, displaying an accumulation of 40S and 60S ribosomal subunits in DZ animals compared with C animals.

To further investigate the mechanism through which acute postprandial insulin deficiency modulated translation initiation, the amount and extent of phosphorylation of eIF4E was examined in gastrocnemius from C and DZ rats 2 h after the injection of diazoxide. As shown in Table 4, neither the content nor phosphorylation state of eIF4E was significantly altered by diazoxide treatment of the animals. Another mechanism through which protein synthesis might be regulated is the association of eIF4E with eIF4G or 4E-BP1. Western blot analysis of eIF4E immunoprecipitates with

Fig. 3. Time course of plasma β -hydroxybutyrate (β -OH; A) and nonesterified fatty acids (NEFA; B) in diazoxide-injected (○) and control (●) rats in experiment I. Rats were injected with diazoxide (DZ group) or vehicle (C group) at time 0, as described in MATERIALS AND METHODS, whereas some animals did not receive any injection to give basal values (time 0). Values are means ± SE of 5 animals in each group at each time. * $P < 0.05$ between C and DZ groups at the same time.

Table 3. Individual plasma amino acid concentrations ($\mu\text{mol/l}$) in control and diazoxide groups 1, 2, and 3 h after injections in experiment I

	C Group			DZ Group		
	1 h	2 h	3 h	1 h	2 h	3 h
Threonine	307.8 $\pm$ 35.6	349.6 $\pm$ 34.5	261.0 $\pm$ 25.7	251.4 $\pm$ 47.3	267.7 $\pm$ 14.3	271.4 $\pm$ 27.2
Valine	132.7 $\pm$ 17.5	128.1 $\pm$ 14.0	133.6 $\pm$ 19.1	101.1 $\pm$ 11.7	144.4 $\pm$ 13.7	203.6 $\pm$ 12.7*
Isoleucine	78.6 $\pm$ 6.9	74.7 $\pm$ 3.4	66.3 $\pm$ 5.4	69.1 $\pm$ 3.8	91.8 $\pm$ 9.2	122.2 $\pm$ 8.5*
Leucine	109.7 $\pm$ 24.5	81.9 $\pm$ 8.9	110.1 $\pm$ 13.0	90.0 $\pm$ 11.3	139.9 $\pm$ 18.0*	183.5 $\pm$ 13.8*
Phenylalanine	60.3 $\pm$ 6.9	53.6 $\pm$ 3.0	72.1 $\pm$ 2.7	54.7 $\pm$ 2.8	61.8 $\pm$ 3.3	60.6 $\pm$ 3.1
Lysine	634.3 $\pm$ 34.4	530.1 $\pm$ 87.9	628.7 $\pm$ 38.1	500.6 $\pm$ 28.0	567.7 $\pm$ 17.8	558.3 $\pm$ 25.8
Methionine	55.3 $\pm$ 8.1	59.7 $\pm$ 10.3	45.4 $\pm$ 7.0	39.2 $\pm$ 8.1	27.1 $\pm$ 4.3*	26.6 $\pm$ 3.6
Arginine	123.8 $\pm$ 12.2	90.7 $\pm$ 20.1	124.4 $\pm$ 6.5	85.2 $\pm$ 11.3*	73.0 $\pm$ 10.3	81.4 $\pm$ 6.3*
Histidine	72.4 $\pm$ 5.9	76.6 $\pm$ 15.1	106.1 $\pm$ 15.6	59.0 $\pm$ 6.0	86.8 $\pm$ 9.2	101.1 $\pm$ 5.2
Tyrosine	50.1 $\pm$ 7.5	47.1 $\pm$ 2.0	52.4 $\pm$ 4.2	39.0 $\pm$ 4.3	32.9 $\pm$ 2.8*	45.3 $\pm$ 4.6
Serine	345.5 $\pm$ 20.2	366.2 $\pm$ 29.4	385.3 $\pm$ 38.2	325.8 $\pm$ 19.7	396.5 $\pm$ 25.0	345.0 $\pm$ 23.2
Alanine	766.5 $\pm$ 55.0	844.2 $\pm$ 52.5	703.1 $\pm$ 57.2	534.1 $\pm$ 59.9*	442.0 $\pm$ 77.8*	374.6 $\pm$ 54.2*
Aspartic acid	19.9 $\pm$ 2.2	20.9 $\pm$ 0.7	18.8 $\pm$ 1.7	14.5 $\pm$ 2.5*	10.5 $\pm$ 1.6*	12.4 $\pm$ 1.5*
Asparagine	92.6 $\pm$ 11.4	97.8 $\pm$ 3.0	97.7 $\pm$ 7.9	67.7 $\pm$ 7.1*	79.0 $\pm$ 3.9	86.7 $\pm$ 8.4
Glutamic acid	211.7 $\pm$ 19.1	203.8 $\pm$ 21.2	212.4 $\pm$ 29.3	123.0 $\pm$ 6.6*	107.8 $\pm$ 18.2*	102.2 $\pm$ 4.3*
Glutamine	472.5 $\pm$ 22.5	485.3 $\pm$ 19.5	577.7 $\pm$ 55.7	438.1 $\pm$ 26.2	498.5 $\pm$ 29.7	513.6 $\pm$ 24.6
Proline	ND	232.3 $\pm$ 9.3	ND	ND	180.8 $\pm$ 5.7	ND
Glycine	454.2 $\pm$ 22.0	551.3 $\pm$ 28.7	494.4 $\pm$ 52.8	496.1 $\pm$ 24.1	572.8 $\pm$ 43.7	702.2 $\pm$ 81.6*
Citrulline	116.1 $\pm$ 10.8	116.7 $\pm$ 7.1	135.0 $\pm$ 12.2	123.8 $\pm$ 5.7	136.9 $\pm$ 4.9	151.8 $\pm$ 7.0
Ornithine	52.5 $\pm$ 6.9	53.1 $\pm$ 7.7	67.2 $\pm$ 3.1	38.3 $\pm$ 4.2	52.5 $\pm$ 9.0	53.9 $\pm$ 2.4

Data are means $\pm$ SE in $\mu\text{mol/l}$. In treated groups, plasma hormones were measured 1, 2, and 3 h after injections. * $P < 0.05$ (ANOVA) between C and DZ groups at the same time. ND, not determined.

either eIF4G or 4E-BP1 antibodies revealed that eIF4G associated with eIF4E was not detectable in gastrocnemius from DZ rats compared with C (Table 4). In contrast, diazoxide injection caused a 2.5-fold increase in the amount of 4E-BP1, which coprecipitated with eIF4E (Table 4). Taken together, these findings demonstrate that an acute postprandial insulin deficiency dramatically depressed the association of eIF4G, with eIF4E favoring formation of the 4E-BP1·eIF4E complex.

Using incubated muscles from DZ and C rats, we assessed the effect of in vivo diazoxide-induced insulin deficiency on protein breakdown measured in vitro. As expected, protein degradation was increased ($P < 0.05$) in muscles from DZ-treated rats compared with C animals (Fig. 6A). Protein synthesis determined in vitro was significantly decreased in incubated epitrochlearis from rats injected with diazoxide compared with C although the difference between the two groups was less pronounced than in vivo (Fig. 6B).

DISCUSSION

Insulin deficiency in vivo induced by diabetes or starvation (1, 2, 11, 22, 27, 33) is commonly associated with a reduction in the rate of skeletal muscle protein synthesis. Insulin replacement therapy or refeeding generally restores skeletal muscle protein synthesis to C values. These observations suggest that insulin modulates protein synthesis. However, it is not clear from such studies whether the effect of insulin deficiency on protein synthesis is directly related to changes in insulin per se or to metabolic disturbances as a consequence of the hypoinsulinemia. Insulin deficiency is associated with changes in circulating hormones (e.g., IGF-I, glucocorticoids, glucagon) and plasma amino acids (see Ref. 32 for a review), which could also

regulate protein synthesis. In the present study, using diazoxide as a specific inhibitor of insulin secretion, we show that suppression of plasma insulin by diazoxide did not significantly change any of the other hormones investigated (glucagon, IGF-I, corticosterone) compared with vehicle-treated animals. However, diazoxide treatment induced a dramatic reduction in skeletal muscle protein synthesis in growing rats. Exogenous insulin replacement abolished this defect. These results demonstrate that insulin is intimately involved in the regulation of skeletal muscle protein synthesis after feeding.

IGF-I is a potent stimulator of muscle protein synthesis (9). Tissue IGF-I mRNA and plasma IGF-I are usually gradually decreased after hypoinsulinemia (7, 15, 32, 42). In the present experiment, plasma IGF-I exhibited the same time course in DZ and C groups. Presumably due to feeding, postprandial values were higher than basal values ($P < 0.05$ only at 1 and 3 h). In spite of low values in the DZ group at 2 h, postprandial plasma IGF-I was not significantly different between the two groups at any time considered. It is unlikely that such a change in plasma IGF-I could affect protein synthesis. Indeed, in vitro protein synthesis did not show any change in either group whatever the time after injections. Thus the lack of difference in plasma IGF-I concentrations between C and DZ rats precludes the involvement of this growth factor in the decrease in protein synthesis. It is well known that plasma IGF-I is associated with binding proteins, which could provide important modulation of the effects of IGF-I without changing total plasma values. Unfortunately, we have no information on these binding proteins in the present experiment.

Insulin deprivation also generally results in elevated plasma glucocorticoids, which are known to decrease

Fig. 4. Rates of protein synthesis (A), RNA content (B), and protein synthesis efficiency (C) in skeletal muscles from diazoxide-injected (open bars) and control (filled bars) rats. Animals received 2 injections of either diazoxide (DZ group) or vehicle (C group) according to the experimental design presented in Fig. 1 for *type II* experiments. In vivo protein synthesis was determined in soleus, gastrocnemius (Gastroc), and epitrochlearis (Epitro) muscles using the phenylalanine flooding dose method. Protein synthesis is expressed as fractional synthetic rate (K_s in %/day), and protein synthesis efficiency is expressed as mg protein synthesized $\cdot$ mg RNA $^{-1} \cdot$ day $^{-1}$. Protein content in soleus, gastrocnemius, and epitrochlearis were, respectively, 184.2 ± 4.8 vs. 171.8 ± 4.4 , 188.6 ± 7.8 vs. 175.1 ± 5.9 , and 186.4 ± 3.2 vs. 172.5 ± 5.4 mg/g muscle in C and DZ groups, respectively (means $\pm$ SE for 4 and 7 rats in each group). Insets represent the effect of exogenous insulin replacement on rates of protein synthesis, RNA content, and protein synthesis efficiency in soleus of some diazoxide-injected rats. According to the experimental design presented on Fig. 1 for *type II* experiments and described in MATERIALS AND METHODS, some diazoxide-injected rats received an injection of exogenous insulin instead of the second diazoxide injection at low (Ins 1 group) or high dose (Ins 2 group). Values are presented as a percentage of values in control group. Protein content in soleus from Ins 1 and Ins 2 groups were, respectively, 187.9 ± 3.8 and 180.3 ± 5.2 mg/g muscle (means $\pm$ SE of 5 rats in each group). Values are means $\pm$ SE of 4 (C group), 7 (DZ group), and 5 (Ins 1 and Ins 2 groups) animals. * $P < 0.05$ compared with C group; † $P < 0.05$ compared with Ins 2 group.

protein synthesis (30, 45). Plasma corticosterone was not significantly different between DZ and C animals, suggesting that glucocorticoids most likely are not the cause of alterations in the protein synthesis observed in DZ rats. Both vehicle and diazoxide injections induced

an increase in plasma corticosterone (not statistically significant 2 h after injection). However, corticosterone levels were still in the range of physiological values compared with stress values (20, 39). Moreover, plasma corticosterone required to inhibit muscle protein synthesis is generally much more elevated than in the present experiment (40, 45). There was no change in plasma

Fig. 5. Effect of diazoxide injection on levels of free ribosomal subunits in gastrocnemius. Muscle samples were taken from control or diazoxide-treated rats 2 h after injection. 60S and 40S ribosomal subunits were isolated on sucrose density gradients as described in MATERIALS AND METHODS. Values are means $\pm$ SE of 6 animals in each group. * $P < 0.05$ between C and DZ groups.

Table 4. Effect of diazoxide injection on phosphorylation of eIF4E, content of eIF4E, and eIF4E associated with either 4E-BP1 or eIF4G in experiment I

	C Group	DZ Group
eIF4E phosphorylation, % of total	55.40 $\pm$ 2.49	47.97 $\pm$ 5.33
eIF4E content, arbitrary units	2,129 $\pm$ 95	1,685 $\pm$ 181
eIF4E associated with 4E-BP1, arbitrary units	32.83 $\pm$ 1.47	80.03 $\pm$ 6.94*
eIF4E associated with eIF4G, arbitrary units	221.14 $\pm$ 73.4	ND

Values are means $\pm$ SE of 4–6 animals in each group. Initiation factors in rat gastrocnemius were analyzed 2 h after injection of either diazoxide (DZ group) or vehicle (C group). Phosphorylation of eukaryotic initiation factor (eIF)4E was expressed as a percentage of total eIF4E in skeletal muscle. * $P < 0.001$, DZ vs. C group. ND, not detectable.

Fig. 6. Effect of diazoxide injection on in vitro protein degradation (A) and synthesis (B). Animals were injected with either diazoxide (DZ group, open bars) or vehicle (C group, filled bars) and at 1, 2, and 3 h later; epitrochlearis muscles were isolated and incubated as described in MATERIALS AND METHODS. Proteolysis is expressed as nmol of tyrosine released $\cdot$ mg protein $^{-1} \cdot$ h $^{-1}$, and protein synthesis is expressed as nmol phenylalanine incorporated $\cdot$ mg protein $^{-1} \cdot$ h $^{-1}$. Values represent means $\pm$ SE of 5 animals in each group at each time. * $P < 0.05$ between C and DZ groups at each time.

glucagon between C and DZ rats, which excluded any contribution of this hormone in the impairment of muscle protein synthesis.

Besides hormones, plasma metabolites, especially amino acids, can regulate muscle protein synthesis (review in Ref. 43). Insulin deprivation induced by starvation or insulin infusion without concomitant administration of exogenous amino acids results in a decrease in plasma amino acids (38, 43). Under such conditions, a decrease in the availability of amino acids is associated with a reduction in protein synthesis. Conversely, reduced protein synthesis caused by insulin deficiency in experimental diabetes is associated with a marked increase in amino acids (especially branched-chain amino acids; see Ref. 47). In the present experiment, moderate changes in essential plasma

amino acids were observed 2 h after diazoxide injection (time at which protein synthesis was determined). The most significant increase in plasma branched-chain amino acids occurred at 3 h after diazoxide injection. However, if such an increase affected protein synthesis, it would tend to underestimate the differences observed between C and DZ groups because amino acids would tend to increase rather than decrease protein synthesis (15, 43). Thus it is also unlikely that changes in the concentration of plasma amino acids were responsible for the alterations in protein metabolism after diazoxide treatment.

As expected, diazoxide-induced hypoinsulinemia resulted in a large increase in plasma glucose. However, glucose per se does not have any effect on muscle protein synthesis either in vivo (15) or in the perfused rat hemi-corpus (19). DZ rats also showed an increase in plasma NEFA and β -hydroxybutyrate. Although infusion of these metabolites has been shown to modify whole body leucine fluxes (oxidation is decreased with NEFA; oxidation and protein synthesis are increased with β -hydroxybutyrate; see Ref. 32), their direct effect on muscle protein synthesis has not been demonstrated clearly. Moreover, the increase in β -hydroxybutyrate that occurs in insulin-deficient rats would be expected to stimulate rather than inhibit muscle protein synthesis. Note that the vehicle per se was able to increase NEFA at 2 h in the C group. This increase might represent a catecholamine effect in response to NaOH injection in the peritoneum. However, no chemical peritonitis was visible at the time the animals were killed.

Even though vehicle or diazoxide injections induced some hormonal and metabolic perturbations, it seems likely that diazoxide-induced hypoinsulinemia is a suitable model to study the relationship between plasma insulin and skeletal muscle protein synthesis in vivo. Indeed, these alterations are in the physiological range and are not statistically significantly different between DZ and C rats. Moreover, the decrease in blood pressure resulting from diazoxide injection was not statistically significant compared with vehicle injection (45 ± 5 vs. 58 ± 2 mmHg in DZ and C group under anesthesia, means $\pm$ SE of 4 and 3 animals in each group obtained in a side experiment).

Because insulin deficiency can be induced in the postprandial state with diazoxide, the signal that elicits the stimulation of protein synthesis in response to feeding in skeletal muscle can be defined more clearly. Before the studies presented herein, the rise in plasma insulin caused by refeeding has been prevented either by using diabetic animals or by intravenous injection of anti-insulin antibodies. Such experiments have yielded conflicting results. In one experiment, anti-insulin antibodies completely suppressed the response of protein synthesis to feeding (36). In contrast, in two other studies (30, 42), the response was attenuated but was not prevented. Such studies imply that plasma insulin is an important component in the protein synthetic response to feeding. In contrast, a recent study using diabetic mice (42) showed that protein synthesis is

stimulated by feeding in the absence of a rise in plasma insulin. This apparent discrepancy is probably a result of different plasma insulin concentrations in various studies. Thus anti-insulin antibodies cause an almost complete deprivation of insulin, whereas the plasma insulin in the diabetic animals used by Svanberg et al. (42) was only reduced to 50% of the value observed in control, fed animals. Overall, the results suggest that insulin is required, but is not sufficient alone, to stimulate protein synthesis.

In the present study, the potential mechanisms responsible for the inhibition of protein synthesis after injection of diazoxide were examined. As would be expected, total RNA content was not altered by the 2-h insulin deficiency caused by diazoxide. A decline in RNA content and therefore in ribosomes occurs only after longer durations of insulin deficiency. Consequently, the reduction in protein synthesis observed in DZ rats could be explained by an impairment of translational efficiency without any alteration in ribosome number. This observation is consistent with other reports using insulin-deficient states. For example, short-term fasting causes a reduction in translation initiation, whereas long-term fasts (beyond 24 h) induce an additional decrease in ribosome numbers (1, 27, 37). Similarly, experimental diabetes (2- to 7-day alloxan-treated rats) causes both an impairment in translational efficiency and a decrease in ribosomal capacity; insulin replacement for 50 min restores translation efficiency but not RNA content (11).

The decreased translational efficiency may result from an inhibition of peptide-chain initiation or elongation/termination. Analysis of the distribution of ribosomal subunits between free particles and polysomes revealed an accumulation of free subunits in gastrocnemius from DZ rats compared with C rats. Hence, diazoxide-induced hypoinsulinemia impaired the rate of protein synthesis in skeletal muscles through inhibition of initiation relative to elongation. Indeed, initiation of protein synthesis is a complex sequence of reactions that leads to the association of the 40S and 60S ribosomal subunits (i.e., formation of the 80S initiation complex). If initiation becomes rate limiting for protein synthesis, free ribosomal subunits accumulate, and polysomes are depleted. Conversely, when initiation is accelerated, subunits are depleted and polysomes accumulate (review in Ref. 23). The 40S and 60S subunit accumulation in diazoxide-induced insulin deficiency agrees with other insulin-deficient states such as diabetes or starvation, where the inhibition of protein synthesis occurs by decreasing the rate of translation initiation (18, 21, 27).

One of the most regulated steps in translation initiation is the binding of mRNA to the 40S subunit (34). This step involves several initiation factors constituting eIF4F, which catalyze the association. The component of eIF4F that binds to the m⁷GTP cap at the 5' end of the mRNA is eIF4E. The subsequent binding of the eIF4E-mRNA complex to another initiation factor, eIF4G, is a critical step in the formation of the 48S preinitiation complex. Regulation of the binding of

eIF4E to eIF4G involves another protein, 4E-BP1. In vitro, eIF4E can bind to either 4E-BP1 or eIF4G but not at the same time (17). This finding in combination with the observation that the amino acid sequence of the eIF4E binding domain of the two proteins is homologous suggests that eIF4G and 4E-BP1 compete for binding to eIF4E. The results of the present study demonstrate a reciprocal relationship between eIF4E binding to 4E-BP1 and eIF4G. Moreover, the inhibition of protein synthesis observed in DZ rats results in an increase in 4E-BP1 bound to eIF4E concomitant with a dramatic decrease in binding of eIF4E to eIF4G. Similarly, long-term insulin deficiency, such as experimental diabetes (3-day alloxan treatment) or starvation, also results in an increase in the association of 4E-BP1 with eIF4E, which is completely reversed by either insulin administration to diabetic rats or refeeding starved mice (24, 41). Taken together, these results suggest that insulin is involved directly in the association of eIF4E with either 4E-BP1 or eIF4G to regulate the initiation of protein synthesis.

Another mechanism through which the binding of mRNA to the 40S ribosomal subunit is regulated involves phosphorylation of eIF4E. It has been shown that phosphorylation of eIF4E in vitro increases its affinity for the m⁷GTP cap of mRNA (31), suggesting that phosphorylation of eIF4E might enhance translation initiation. As observed in previous studies using experimental diabetic rats and insulin-treated rats (24), diazoxide-induced insulin deficiency did not alter the phosphorylation state of eIF4E compared with C rats, suggesting that, in vivo, insulin does not regulate translation initiation by modulating the phosphorylation of eIF4E.

In conclusion, the present study has shown that skeletal muscle protein synthesis is greatly depressed in fed rats subjected to postprandial insulin deprivation caused by injection of a specific inhibitor of insulin secretion (i.e., diazoxide). The reduced skeletal muscle protein synthesis observed during postprandial diazoxide-induced insulin deficiency results from an inhibition of translation initiation. It is associated with increased formation of the 4E-BP1·eIF4E complex and decreased formation of the eIF4G·eIF4E complex without alteration of the amount or phosphorylation state of eIF4E. The results allow us to conclude that short-term insulin deficiency (2 h) inhibits postprandial muscle protein synthesis by sequestering eIF4E into an inactive 4E-BP1·eIF4E complex. Such an animal model of hypoinsulinemia might be used to define the specific role of insulin in the regulation of skeletal muscle protein synthesis and to investigate insulin signaling.

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