Orally Administered Leucine Enhances Protein Synthesis in Skeletal Muscle of Diabetic Rats in the Absence of Increases in 4E-BP1 or S6K1 Phosphorylation

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In this study, food-deprived (18 h) control rats and rats with alloxan-induced diabetes were orally administered saline or the amino acid leucine to assess whether it regulates protein synthesis independently of a change in serum insulin concentrations. Immediately after leucine administration, diabetic rats were infused with insulin (0.0, 4.0, or 20 pmol · min^{-1} · kg^{-1}) for 1 h to examine the role of the hormone in the protein synthetic response to leucine. In control rats, leucine stimulated protein synthesis by 58% and increased phosphorylation of the translational repressor, eukaryotic initiation factor (eIF) 4E-binding protein (BP)-1, 4E-BP1, fivefold. Consequently, association of the mRNA cap-binding protein eukaryotic initiation factor (eIF)4E with 4E-BP1 was reduced to 50% of control values, and eIF4G•eIF4E complex assembly was increased 80%. Furthermore, leucine increased the phosphorylation of the 70-kDa ribosomal protein S6 (rp S6) and the ribosomal protein S6 kinase (S6K1). Diabetes attenuated protein synthesis compared with control rats. Nonetheless, in diabetic rats, leucine increased protein synthesis by 53% without concomitant changes in the phosphorylation of 4E-BP1 or S6K1. Skeletal muscle protein synthesis was stimulated in diabetic rats infused with insulin, but rates of synthesis remained less than values in nondiabetic controls that were administered leucine. Phosphorylation of 4E-BP1 and S6K1 was increased in diabetic rats infused with insulin in a dose-dependent manner, and the response was enhanced by leucine. The results suggest that leucine enhances protein synthesis in skeletal muscle through both insulin-dependent and -independent mechanisms. The insulin-dependent mechanism is associated with increased phosphorylation of 4E-BP1 and S6K1. In contrast, the insulin-independent effect on protein synthesis is mediated by an unknown mechanism. *Diabetes* 51: 928–936, 2002

After consumption of a protein-containing meal, the fractional rate of protein synthesis of total mixed proteins in skeletal muscle of growing animals is upregulated. Two vital components of this response are elevations in circulating concentrations of the hormone insulin and an increase in amino acid supply. The relative importance of each of these components in regulating protein synthesis continues to be a topic of investigation and controversy. Several reports indicate that physiological increases in circulating insulin concentrations are not sufficient to stimulate protein synthesis in food-deprived rats (1–4). When postabsorptive rats are administered an oral bolus of carbohydrate alone, no change in protein synthesis is observed. In contrast, when food-deprived rats are administered an isocaloric macronutrient-mixed meal, rates of protein synthesis are stimulated (1). Plasma insulin concentrations in rats administered either meal are similar; hence, the enhanced rate of recovery in rats administered a mixed meal cannot be attributed to a differential insulin response between the groups. Likewise, when fasted rats are refed a diet devoid of protein, no change in rates of muscle protein synthesis is observed. However, refeeding a diet containing 20% protein stimulates rates of protein synthesis in skeletal muscle compared with fasted controls (4). Thus, dietary protein appears to play a pivotal role in regulating protein synthesis after food intake.

Additional studies suggest that the anabolic effect of dietary protein may be attributable to specific amino acids. Garlick and Grant (5) reported that infusion of glucose, in addition to the branched-chain amino acids, leucine, isoleucine, and valine, stimulates protein synthesis in skeletal muscle of rats that were food deprived overnight. Most, and perhaps all, of the effect of the branched-chain amino acids to enhance rates of protein synthesis in skeletal muscle may be attributed to leucine alone. Leucine independently enhances protein synthesis in isolated muscle preparations (6–8) and in perfused rat hindlimb preparations (9). More recently, it was demonstrated that oral administration of leucine stimulates protein synthesis in skeletal muscle of food-deprived rats (10,11). However, a time course analysis revealed that oral administration of leucine also results in a transient elevation in circulating

concentrations of insulin (12). Furthermore, when insulin is stabilized at fasting levels by somatostatin infusion, rates of protein synthesis in skeletal muscle are refractory to oral administration of leucine (12). Therefore, although physiological increases in serum insulin do not independently stimulate protein synthesis in skeletal muscle of food-deprived rats, a transient increase in the circulating concentration of the hormone may be permissive for the leucine-induced stimulation of protein synthesis.

The mechanism through which muscle cells recognize and respond to oral administration of leucine remains to be determined. Nonetheless, it has been established that the effect of leucine to regulate protein synthesis involves the initiation of mRNA translation (6,9). Oral administration of leucine to food-deprived rats enhances protein synthesis in skeletal muscle in association with hyperphosphorylation of the translational repressor, eukaryotic initiation factor (eIF) 4E-binding protein (BP)-1, and enhances the availability of the mRNA cap-BP eIF4E for binding eIF4G and assembly of the initiation complex known as eIF4F (13–15). The eIF4F complex collectively serves to recognize, unfold, and guide the mRNA to the 40 S ribosomal subunit. Additionally, leucine administration increases the phosphorylation state of the 70-kDa ribosomal protein S6 kinase (S6K1) (11). Phosphorylation of S6K1 is associated with its activation and results in the hyperphosphorylation of ribosomal protein S6 (rp S6) (16,17). Activation of S6K1 facilitates the translation of a class of mRNAs containing terminal oligopyrimidine tracts at the 5′ end of the message (TOP mRNAs) (18). TOP mRNAs encode elements of the translational apparatus, including ribosomal proteins and elongation factors. Thus, by promoting hyperphosphorylation of S6K1, leucine may enhance the synthesis of proteins involved in translation and ribosome biogenesis.

Transient elevations in circulating insulin concentrations may also facilitate increases in the phosphorylation states of both 4E-BP1 and S6K1 after administration of leucine. Infusion of somatostatin partially attenuates but does not altogether prevent the leucine-induced hyperphosphorylation of these proteins in skeletal muscle of food-deprived rats (12). No study has examined whether leucine can modulate the phosphorylation of 4E-BP1 and S6K1 independently of changes in circulating insulin concentrations. Thus, the contribution of basal levels of insulin to alterations in translation initiation factor function remains to be determined.

In the present study, alloxan-treated diabetic rats were orally administered leucine to assess whether dietary amino acids regulate protein synthesis and translation initiation factor function in skeletal muscle independently of insulin. To determine the contribution of insulin to the protein synthetic response to leucine, diabetic rats were infused with the hormone at two different rates. The first infusion rate ($4.0 \, \text{pmol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) was chosen to restore circulating insulin concentrations to values observed in nondiabetic control rats. The other rate of infusion ($20 \, \text{pmol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) was designed to increase serum insulin concentrations to values observed in control rats after oral administration of leucine. The results suggest that oral administration of leucine enhances protein synthesis in skeletal muscle through both insulin-dependent and -inde-

pendent mechanisms. The insulin-dependent mechanism occurs in association with hyperphosphorylation of 4E-BP1 and enhanced assembly of the eIF4G•eIF4E complex as well as increased phosphorylation of S6K1. In contrast, leucine also enhances protein synthesis in diabetic rats independent of alterations in serum insulin and without concomitant changes in the phosphorylation states of 4E-BP1 or S6K1.

RESEARCH DESIGN AND METHODS

Animals. The animal facilities and protocol were reviewed and approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine. Male Sprague-Dawley rats were housed at a constant temperature, exposed to a 12-h light-dark cycle, and maintained on standard rodent chow (Harlan-Teklad Rodent Chow; Harlan, Madison, WI) and water ad libitum for at least 1 week before the initiation of experimental procedures. All experiments were performed at the beginning of the light cycle. Unless otherwise noted, rats weighed ~200 g on the day of the experiment.

Experimental design. Diabetes was induced in postabsorptive rats by intravenous injection of alloxan (48 mg/kg body wt in 0.155 mol/l saline) (Sigma, St. Louis, MO) as previously described (19,20). A blood sample was taken daily from the tail vein to confirm the presence of hyperglycemia (>17 mmol/l glucose). Experiments were performed 3 days after alloxan administration. The average weight of diabetic rats (173 ± 5 g) on the day of the experiment was less than controls (198 ± 8 g, $P < 0.05$).

Both control and diabetic rats were food deprived for 18 h before the day of the experiment and then administered saline (0.155 mol/l) or 1.35 g/kg body wt leucine (prepared as 54.0 g/l L-amino acid in distilled water) by oral gavage. The volume of the leucine suspension administered was 2.5 ml/100 g body wt. The amount of leucine administered was equivalent to that consumed in a 24-h period when rats of this age and strain are provided free access to food (10). Immediately after gavage, rats were immobilized in a perforated cloth, and a 27-gauge needle, attached to a length of polypropylene tubing, was inserted into the lateral tail vein as previously described (21). Human insulin (Eli Lilly, Indianapolis, IN) was administered intravenously as a primed constant infusion ($0 \, \text{pmol/kg} + 0 \, \text{pmol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, $160 \, \text{pmol/kg} + 4.0 \, \text{pmol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, or $800 \, \text{pmol/kg} + 20 \, \text{pmol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) to rapidly raise serum insulin concentrations to the desired level (C.H.L., unpublished observations). Control rats were similarly infused with vehicle (0.155 mol/l NaCl, 0.2% BSA). The infusion rates of 4.0 and $20 \, \text{pmol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ were chosen to restore circulating insulin concentrations in diabetic rats to those observed in nondiabetic control rats that were either food deprived or orally administered leucine, respectively.

Administration of metabolic tracer and sample collection. A flooding dose (1.0 ml per 100 g body wt) of L-[2,3,4,5,6-³H]phenylalanine (150 mmol/l containing 100 µCi/ml) was injected via the tail vein 10 min before sample collection for the measurement of synthesis of total mixed proteins in skeletal muscle (22). Rats were killed by decapitation at 60 min after oral gavage. Blood was collected and centrifuged at 1,800*g* for 10 min at 4°C to obtain serum. The left gastrocnemius and plantaris were excised as a unit, quickly frozen in liquid N_2, and used for the determination of muscle protein and RNA contents as described below. The contralateral hindlimb muscles were excised as a unit, weighed, and homogenized in seven volumes of buffer consisting of (in mmol/l) 20 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (pH 7.4), 100 KCl, 0.2 EDTA, 2 ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 1 dithiothreitol, 50 sodium fluoride, 50 β-glycerophosphate, 0.1 phenylmethylsulfonyl fluoride, 1 benzamidine, and 0.5 sodium vanadate. An aliquot (0.5 ml) was used for the measurement of skeletal muscle protein synthesis as described below. The remainder of the homogenate was immediately centrifuged at 10,000*g* for 10 min at 4°C. The supernatant was used for analysis of translation initiation factor function and phosphorylation status as described below.

Serum measurements. Serum glucose was analyzed by a glucose oxidase-peroxidase automated method (YSI Model 2300 analyzer; Yellow Springs Instruments, Yellow Springs, OH). Serum insulin concentrations were analyzed using a commercial radioimmunoassay kit for rat insulin (Linco Research, St. Charles, MO). Serum amino acid concentrations were determined by derivatizing with phenylisothiocyanate, followed by high-performance liquid chromatography analysis as previously described (22a).

Measurement of protein and RNA contents in skeletal muscle. Protein and RNA contents in frozen muscle samples were analyzed according to the method of Schmidt and Thannhauser (23), which was later modified by Fleck and Munro (24) and Gautsch et al. (25). Frozen muscle samples (100 mg) were

placed in 17×100-mm polypropylene tubes containing 4 ml distilled water and homogenized using a Polytron (Kinematica, Luzern, Switzerland) for 10 s at speed 5. The polytron was rinsed with 4 ml distilled water followed by 4 ml of 0.6 mol/l $HClO_4$, and then the rinses were added to the original homogenate. The centrifuge tubes were placed on ice for 10 min to allow proteins and RNA to precipitate and were then centrifuged at $6,000g$ for 15 min. The supernatant was discarded, and the pellet was washed twice with 4 ml of 0.2 mol/l $HClO_4$. Next, the pellet was solubilized in 6 ml of 0.3 mol/l KOH for 1 h at 37°C. The samples were then divided into three 2-ml portions. One portion was used to ascertain protein content in duplicate using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA), with crystalline BSA as a standard. The remaining portions were used to determine tissue RNA content. $HClO_4$ (1.2 ml of 1.2 mol/l) was added to the duplicate samples, and the tubes were placed on ice for 10 min and centrifuged at $6,000g$ to precipitate DNA. The supernatant containing RNA was transferred into a new centrifuge tube. The pellet was washed with 3 ml of 0.2 mol/l $HClO_4$, and the supernatant was combined with the first. The RNA concentration of the supernatant was calculated as follows: μg RNA/ml = $[32.6 (A_{260}) - 6.11(A_{232})] \times 6.2$ (sample volume in milliliters) $\times 3$ (dilution factor).

Measurement of protein synthesis in skeletal muscle. Fractional rates of skeletal muscle protein synthesis were estimated from the rate of incorporation of radioactive phenylalanine into muscle protein using the specific radioactivity of serum phenylalanine as representative of the precursor pool (26). Previous studies have demonstrated that aminoacyl-tRNA and blood- and tissue-free amino acid pools are rapidly equilibrated after administration of a flooding dose of phenylalanine in vivo (27). The elapsed time from injection of the metabolic tracer until homogenization of muscle was recorded as the actual time for incorporation of radiolabeled amino acid into protein.

The rate of protein synthesis, expressed as nmol/l of phenylalanine incorporated into protein per hour per milligram of muscle protein (nmol phe/[mg protein · h]), was calculated by dividing the disintegrations per hour incorporated into protein by the serum phenylalanine specific radioactivity (26). Translational efficiency expressed as nmol/l of phenylalanine incorporated into protein per milligram of RNA (nmol phe/[mg RNA · h]) was determined by dividing the rates of protein synthesis by the amount of RNA per milligram muscle protein.

Phosphorylation of protein kinase B on Ser 473. Phosphorylation of protein kinase B (PKB) on Ser 473 was examined in $10,000g$ supernatants by protein immunoblot analysis as previously described (28). Duplicate sets of samples were resolved on polyacrylamide gels. The proteins in the gels were transferred to two separate polyvinylidene difluoride membranes. One membrane was incubated with an anti-PKB antibody (New England Biolabs, Beverly, MA). The second membrane was incubated with a rabbit polyclonal antibody, which specifically recognizes phosphorylation of PKB on Ser 473 (New England Biolabs). The amount of phosphorylation on Ser 473 was normalized for the total amount of PKB in the muscle homogenate.

4E-BP1 phosphorylation state. An aliquot of the $10,000g$ supernatant from skeletal muscle was boiled for 10 min, cooled to room temperature, and then centrifuged at $10,000g$ for 30 min at 4°C. The supernatant was then used for protein immunoblot analysis using a rabbit anti–rat 4E-BP1 antibody as previously described (29).

Analysis of 4E-BP1•eIF4E and eIF4G•eIF4E complexes. eIF4E was immunoprecipitated from $10,000g$ supernatants of muscle homogenates using a monoclonal antibody to eIF4E (29). Next, samples were subjected to immunoblot analysis using polyclonal antibodies to either 4E-BP1 or eIF4G to determine the association of 4E-BP1 and eIF4G with eIF4E, respectively (29). Results were normalized to the amount of eIF4E in the immunoprecipitates.

Phosphorylation of S6K1. The phosphorylation state of S6K1 was analyzed in $10,000g$ supernatants by protein immunoblot analysis using a rabbit polycolonal S6K1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (1).

Phosphorylation of S6K1 on Thr 389 was determined in $10,000g$ supernatants by protein immunoblot analysis as previously described (29). Membranes were incubated with a rabbit polyclonal antibody, which specifically recognizes phosphorylation of S6K1 on Thr 389 (New England Biolabs).

Phosphorylation of rp S6. Phosphorylation of rp S6 was examined in $10,000g$ supernatants by protein immunoblot analysis as previously described (28). Membranes were incubated with an anti-phosphopeptide antibody specific for phosphorylated rp S6 (a kind gift from Dr. Morris J. Birnbaum, Department of Medicine, University of Pennsylvania, Philadelphia).

Statistical analysis. All data were analyzed by the STATISTICA statistical software package for the Macintosh, volume II (StatSoft, Tulsa, OK). All data were analyzed using a one-way ANOVA to assess main effects, with the treatment group as the independent variable. When a significant overall effect was detected, differences among individual means were assessed with Dun-
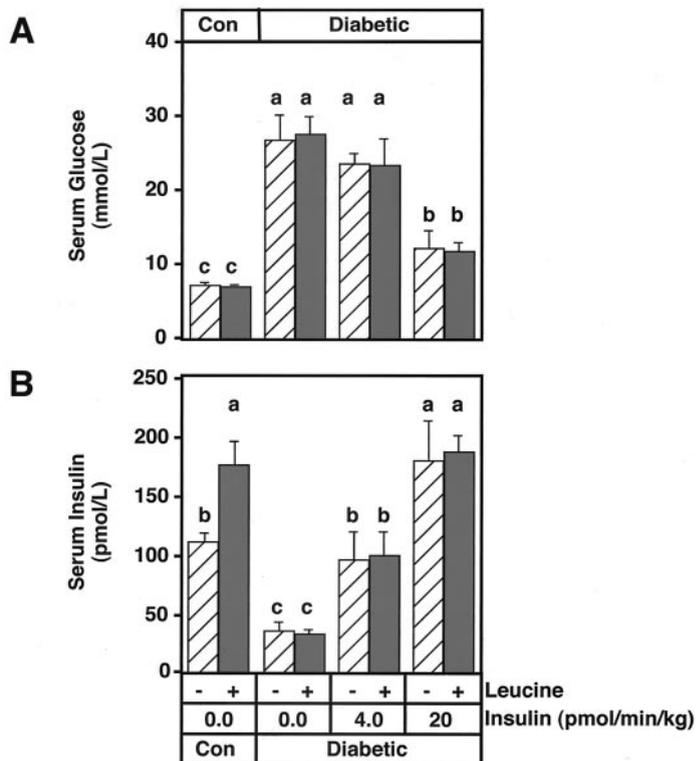

FIG. 1. Serum glucose *(A)* and insulin *(B)* concentrations in food-deprived control and diabetic rats that were administered leucine and intravenously infused with insulin. Control (Con) and diabetic rats were food deprived (hatched bars) or orally administered leucine (solid bars). Immediately after leucine administration, insulin was infused into diabetic rats via a tail vein catheter at rates of 0.0, 4.0, or 20 pmol · min^{-1} · kg^{-1} for 1 h. Control rats were similarly infused with vehicle (0.155 mol/l NaCl, 0.2% BSA). Values are means ± SE, $n = 4-8$. Means not sharing a superscript are different, $P < 0.05$.

can's Multiple Range posthoc test. The level of significance was set at $P < 0.05$ for all statistical tests.

RESULTS

In the present study, diabetes was confirmed by the presence of hyperglycemia (Fig. 1*A*) and insulinopenia (Fig. 1*B*). Serum glucose concentrations were increased more than threefold in diabetic compared with control rats. Infusion of insulin at 4.0 pmol · min^{-1} · kg^{-1} did not significantly reduce concentrations of glucose in the diabetic rats. In contrast, infusion of insulin at 20 pmol · min^{-1} · kg^{-1} for 1 h tended to normalize serum glucose; the values were statistically intermediate between nondiabetic controls and diabetic rats that were not infused with insulin. Oral administration of leucine did not alter serum glucose concentrations in either control or diabetic rats. In diabetic rats, serum insulin concentrations were 25% of food-deprived control values. The infusion rates of 4.0 and 20 pmol · min^{-1} · kg^{-1} restored circulating insulin concentrations in diabetic rats to levels observed in nondiabetic control rats that were either food deprived or orally administered leucine, respectively. Oral administration of leucine increased circulating concentrations of insulin in food-deprived control rats, but it did not alter insulin in diabetic animals.

In food-deprived diabetic rats, rates of protein synthesis in skeletal muscle were reduced to 35% of the values observed in food-deprived nondiabetic controls (Fig. 2*A*).
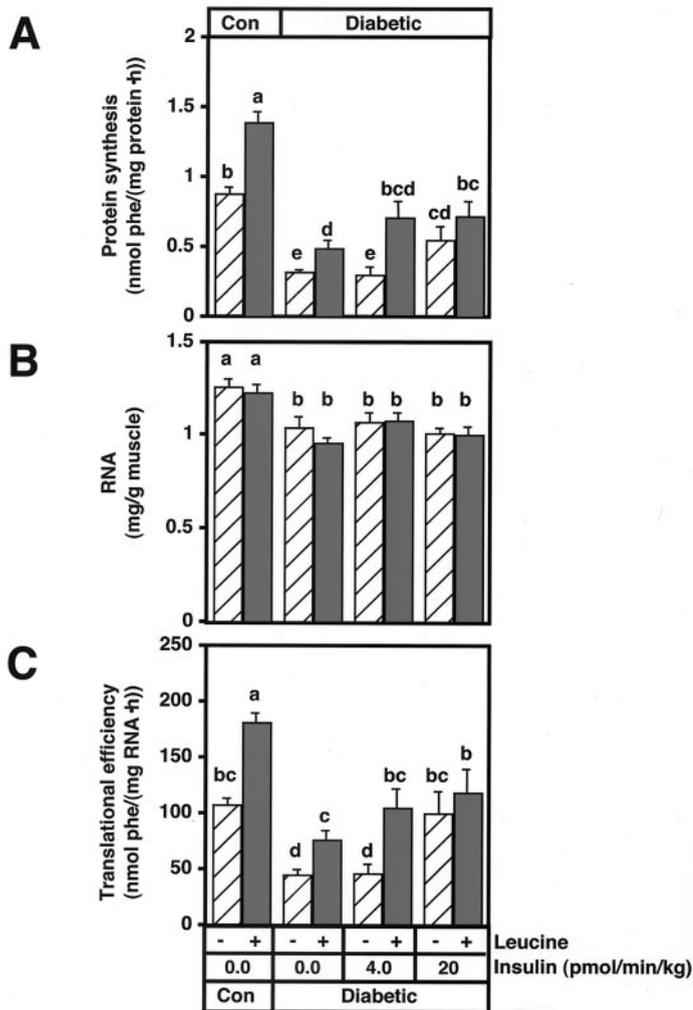
FIG. 2. Protein synthesis (*A*), RNA concentration (*B*), and translational efficiency (*C*) in skeletal muscle of food-deprived control and diabetic rats that were administered leucine and intravenously infused with insulin. Control (Con) and diabetic rats were food-deprived (hatched bars) or orally administered leucine (solid bars). Immediately after leucine administration, insulin was infused into diabetic rats via a tail vein catheter at rates of 0.0, 4.0, or 20 pmol · min^{-1} · kg^{-1} for 1 h. Control rats were similarly infused with vehicle (0.155 mol/l NaCl, 0.2% BSA). Values are means ± SE, $n = 4$–8. Means not sharing a superscript are different, $P < 0.05$.

Infusion of insulin at 4.0 pmol · min^{-1} · kg^{-1} did not enhance muscle protein synthesis in food-deprived diabetic rats. In contrast, replacing insulin at 20 pmol · min^{-1} · kg^{-1} independently elevated rates of protein synthesis compared with food-deprived diabetic rats, but protein synthesis remained less than values observed in food-deprived nondiabetic controls.

In control rats, oral administration of leucine resulted in a 58% increase in the rate of protein synthesis in skeletal muscle. Leucine administration also resulted in a 53% increase in protein synthesis in skeletal muscle of diabetic rats. The protein synthetic response to leucine was enhanced in diabetic rats infused with insulin. However, recovery of muscle protein synthesis remained incomplete. When diabetic rats were orally administered leucine and then infused with insulin at either 4.0 or 20 pmol · min^{-1} · kg^{-1}, rates of muscle protein synthesis were increased 46% ($P = 0.06$) and 48% ($P < 0.05$), respectively,

above leucine-treated diabetic rats that were not infused with insulin. These values were equivalent to food-deprived control values but substantially less than values observed in control rats administered leucine.

Both translational capacity (tissue ribosomal content) and translational efficiency (protein synthesized per ribosome) have been shown to contribute to the attenuation of muscle protein synthesis in diabetic rats (19). Therefore, these parameters were examined to assess whether alterations in tissue ribosomal content could explain the incomplete recovery of muscle protein synthesis in diabetic rats infused with insulin. Total muscle RNA content was used as an index of changes in translational capacity because >80% of total RNA is ribosomal. RNA content per gram of skeletal muscle was reduced to ~85% of control values and was unchanged by either leucine or insulin treatment (Fig. 2*B*), suggesting that only a small portion of the decline in muscle protein synthesis rates in diabetic rats can be attributed to alterations in translational capacity. As an index of translational efficiency, rates of protein synthesis were expressed relative to skeletal muscle RNA concentrations (Fig. 2*C*). Changes in translational efficiency in diabetic rats resembled changes in protein synthesis, i.e., insulin infusion enhanced translational efficiency in a dose-dependent manner. However, rates of translational efficiency in diabetic rats administered leucine and infused with insulin remained attenuated compared with control. The results suggest that the inability of insulin to restore rates of protein synthesis in diabetic rats administered leucine to control values cannot be entirely attributed to a decline in translational capacity. Rather, the results indicate that impaired translational efficiency is a key contributor. The impaired translational efficiency observed in the presence of both leucine and insulin administration indicates a role for other regulatory factors that are altered as a result of 3 days of diabetes and that are not corrected by 1 h of hormone replacement. Thus, the experimental model used in these experiments should not be viewed as one in which insulin is simply removed and then replaced.

To assess whether reduced translational efficiency in skeletal muscle of diabetic rats resulted from resistance to insulin action on early insulin signaling events, we examined phosphorylation of PKB on Ser 473, a residue whose phosphorylation is associated with increased activation of the protein (30). In both control and diabetic rats, oral administration of leucine did not enhance the phosphorylation of PKB on Ser 473 (Fig. 3). Rather, changes in PKB phosphorylation reflected alterations in circulating insulin concentrations in diabetic rats. Thus, in diabetic rats, PKB phosphorylation was reduced compared with control. Restoration of circulating insulin concentrations to fasting levels enhanced the phosphorylation of PKB, resulting in values that were equivalent to control rats. The phosphorylation of PKB was further enhanced in diabetic rats infused with insulin at a rate that restored circulating concentrations of the hormone to values observed in control rats administered leucine. The reason for the dramatic increase in PKB phosphorylation in the latter group of diabetic rats is unknown. However, the results would suggest that insulin signaling through PKB is not impaired with diabetes. In fact, phosphorylation of PKB in
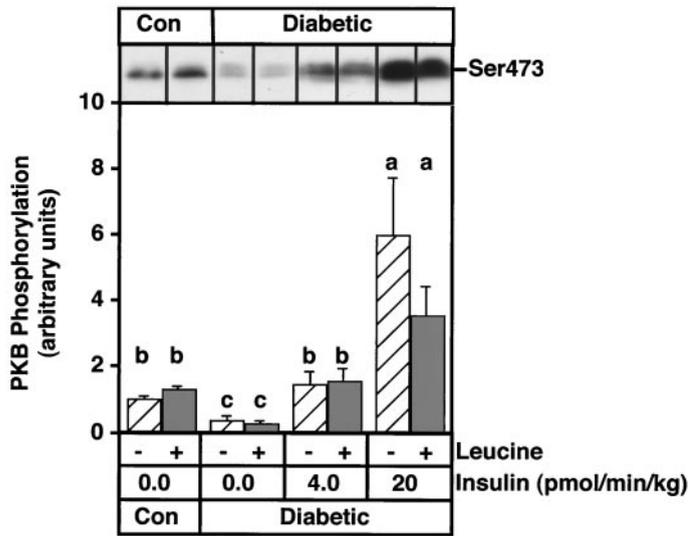
FIG. 3. Phosphorylation of PKB on Ser 473 in skeletal muscle of food-deprived control and diabetic rats that were administered leucine and infused intravenously with insulin. Control (Con) and diabetic rats were food deprived (hatched bars) or orally administered leucine (solid bars). Immediately after leucine administration, insulin was infused into diabetic rats via a tail vein catheter at rates of 0.0, 4.0, or 20 pmol · min^{-1} · kg^{-1} for 1 h. Control rats were similarly infused with vehicle (0.155 mol/l NaCl, 0.2% BSA). Inset shows a representative immunoblot with phosphorylated Ser 473 noted to the right. The amount of phosphorylation on Ser 473 was normalized for the total amount of PKB in the muscle homogenate. Values are means ± SE, n = 4–8. Means not sharing a superscript are different, $P < 0.05$.

the diabetic rats appeared more sensitive to changes in insulin availability than in control rats because the elevation in serum insulin associated with leucine administration did not enhance PKB phosphorylation in skeletal muscle of food-deprived controls.

To further define the mechanism(s) by which leucine stimulates protein synthesis in diabetic rats, alterations in translation initiation factor phosphorylation and/or association in skeletal muscle were assessed. In control rats, oral administration of leucine increased the phosphorylation of 4E-BP1 (Fig. 4A), and the amount of eIF4E bound in an inactive complex with the BP was reduced (Fig. 4B). Likewise, leucine administration enhanced the assembly of the eIF4G•eIF4E complex (Fig. 4C). In diabetic rats, phosphorylation of 4E-BP1 was reduced compared with food-deprived controls, resulting in an increase in the association of 4E-BP1 with eIF4E and a decrease in the amount of the eIF4G•eIF4E complex. Leucine administration did not alter the phosphorylation of 4E-BP1 or the association of eIF4E with 4E-BP1 or eIF4G in diabetic rats that were not infused with insulin. However, it is noteworthy that the association of eIF4G with eIF4E tended to be greater in diabetic rats administered leucine compared with food-deprived diabetic controls ($P = 0.08$). As a note of caution, it should be mentioned that methodological limitations may have contributed to the failure to achieve statistically significant differences in translation initiation factor phosphorylation and/or association under conditions in which a modest but significant change in translational efficiency occurred (Fig. 2A). Insulin, infused into diabetic rats at a rate that restored circulating concentrations of the hormone to levels observed in food-deprived control rats, had no effect on the phosphorylation of
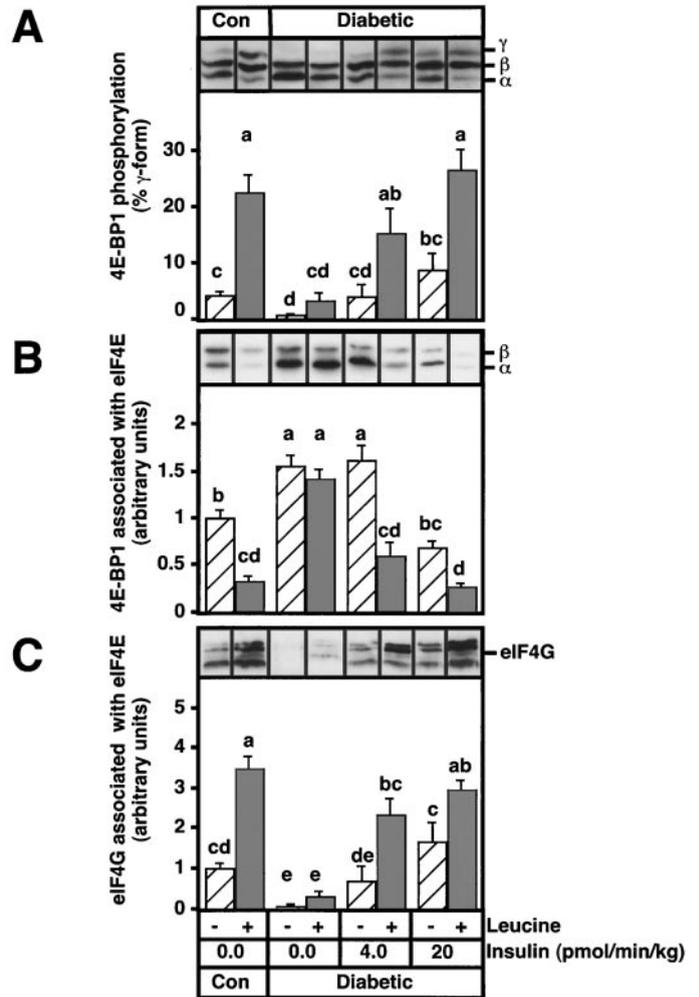

FIG. 4. Phosphorylation state of eIF 4E-BP1, and the amount of 4E-BP1 and eIF4G associated with eIF4E in skeletal muscle of food-deprived control and diabetic rats that were administered leucine and infused intravenously with insulin. Control (Con) and diabetic rats were food deprived (hatched bars) or orally administered leucine (solid bars). Immediately after leucine administration, insulin was infused into diabetic rats via a tail vein catheter at rates of 0.0, 4.0, or 20 pmol · min^{-1} · kg^{-1} for 1 h. Control rats were similarly infused with vehicle (0.155 mol/l NaCl, 0.2% BSA). A: Amount of 4E-BP1 in the γ-phosphorylated form as a percentage of the total 4E-BP1. Inset shows a representative immunoblot with positions of α-, β-, and γ-forms of 4E-BP1 noted to the right. The most highly phosphorylated form, i.e., the γ-form, exhibits the slowest electrophoretic mobility and does not bind eIF4E. Therefore, phosphorylation of 4E-BP1 was expressed as the percentage of the protein in the γ-form. B: Amount of 4E-BP1 associated with eIF4E. Inset shows a representative immunoblot with positions of α- and β-forms of 4E-BP1 noted to the right. C: Amount of eIF4G associated with eIF4E. Inset shows a representative immunoblot with eIF4G noted to the right. Values are means ± SE, n = 4–8. Means not sharing a superscript are different, $P < 0.05$.

4E-BP1 or the association of eIF4E with 4E-BP1 and eIF4G. Conversely, when infused at 20 pmol · min^{-1} · kg^{-1}, insulin tended to increase 4E-BP1 phosphorylation in skeletal muscle ($P = 0.09$). As a result, the amount of the inactive 4E-BP1•eIF4E complex was decreased and the availability of eIF4E for binding eIF4G was increased compared with diabetic rats that did not receive insulin and equivalent to values observed in food-deprived controls. Furthermore, complete recovery of 4E-BP1 phosphorylation was observed in diabetic rats administered leucine and infused with insulin at the higher rate. Moreover, the amount of eIF4E associated with 4E-BP1 and
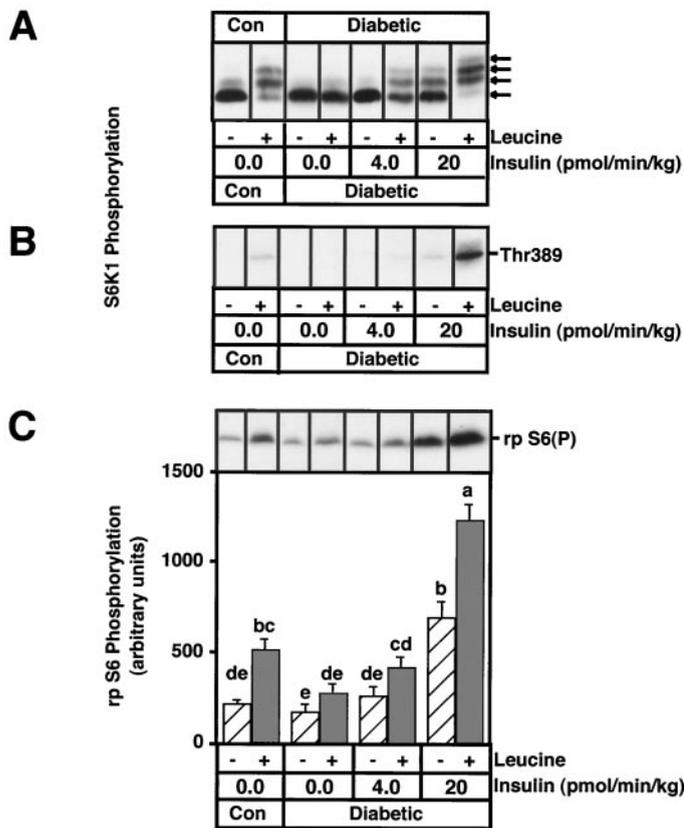
FIG. 5. Phosphorylation states of the 70-kDa S6K1 and rp S6 in skeletal muscle of food-deprived control and diabetic rats that were administered leucine and infused intravenously with insulin. Control (Con) and diabetic rats were food deprived (hatched bars) or orally administered leucine (solid bars). Immediately after leucine administration, insulin was infused into diabetic rats via a tail vein catheter at rates of 0.0, 4.0, or 20 pmol · min⁻¹ · kg⁻¹ for 1 h. Control rats were similarly infused with vehicle (0.155 mol/l NaCl, 0.2% BSA). A: **Phosphorylation of S6K1. Arrows indicate multiple electrophoretic forms of S6K1 with the most highly phosphorylated forms exhibiting the slowest electrophoretic mobility. Data shown are representative of four to eight rats per condition.** B: **Phosphorylation of S6K1 on Thr 389, a residue whose phosphorylation is associated with increased activation of the protein. Data shown are representative of four to eight rats per condition.** C: **Phosphorylation of rp S6. Inset shows a representative immunoblot with phosphorylated rp S6 noted to the right. Values are means ± SE, $n = 4$–8. Means not sharing a superscript are different, $P < 0.05$.**

eIF4G was equivalent to values obtained in muscle extracts from control rats administered leucine.

Oral administration of leucine also promoted increased phosphorylation of S6K1 (Fig. 5A). The leucine-induced hyperphosphorylation of S6K1 was associated with increased phosphorylation of the kinase on Thr 389, a residue whose phosphorylation is associated with increased activation of the protein (Fig. 5B) (31). As a result, oral administration of leucine enhanced the phosphorylation of rp S6 in food-deprived controls (Fig. 5C). In diabetic rats the phosphorylation states of S6K1 and rp S6 were similar to food-deprived controls, and leucine did not affect the phosphorylation of either protein. Infusion of insulin into diabetic rats enhanced the phosphorylation of both S6K1 and rp S6 in a dose-dependent manner, and in diabetic rats administered leucine and infused with insulin at 20 pmol · min⁻¹ · kg⁻¹, phosphorylation of these proteins exceeded values observed in control rats. These results suggest that the inhibition of protein synthesis in diabetic rats administered leucine and infused with insulin cannot be explained by impaired signaling of insulin to 4E-BP1

and S6K1 and may suggest an impaired ability to recognize and respond to changes in leucine supply in diabetic rats. Overall, the data therefore demonstrate that the mTOR pathway responds appropriately to leucine and insulin administration in the diabetic rat. Yet, translational efficiency is not fully restored, implicating the involvement of additional unidentified mechanisms.

DISCUSSION

Adequate insulin availability is necessary for the maintenance of protein synthesis in skeletal muscle. In experimental models of diabetes, rates of protein synthesis are reduced to 25–75% of control values, with the greatest inhibition in skeletal muscle containing a high proportion of fast-twitch fibers (19,32). The reduced rate of muscle protein synthesis that accompanies diabetes is due in part to downregulation of the initiation step of mRNA translation (19). Several translation initiation factors appear to be involved in mediating the downregulation. Insulinopenic diabetes is reported to decrease the phosphorylation state of 4E-BP1 and increase the amount of eIF4E associated in an inactive complex with the BP in skeletal muscle, thereby reducing the availability of eIF4E for assembly the eIF4F mRNA cap-binding complex (20). In the present study, phosphorylation of 4E-BP1 was reduced in skeletal muscle of diabetic rats compared with food-deprived controls, resulting in an increase in the association of 4E-BP1 with eIF4E and a decrease in eIF4G•eIF4E complex formation. The reduction in eIF4F complex assembly may explain, in part, the reduced translation efficiency in skeletal muscle of diabetic rats.

Alterations in the phosphorylation state of rp S6 also affects the rate of translation of those messages containing TOP sequences in their 5′-untranslated region, which includes messages encoding ribosomal proteins. In the present study, changes in the phosphorylation state of either S6K1 or rp S6 were not observed in diabetic compared with food-deprived nondiabetic rats, suggesting that the S6K1 signaling pathway is not attenuated in skeletal muscle during diabetes. However, a small but significant decrease in muscle ribosomal content was observed in skeletal muscle of diabetic rats compared with control rats. These results may suggest that tissue ribosome content is principally regulated at the level of degradation in the diabetic rats. Alternatively, S6K1 signaling may be downregulated in the fed state in diabetic rats compared with control.

Few studies have examined whether amino acids regulate muscle protein synthesis in diabetic rats (32,33). Nakano and Hara (32) refed streptozotocin diabetic rats a diet containing 25% casein and found that the incorporation rate of labeled phenylalanine into isolated gastrocnemius preparations was increased compared with food-deprived diabetic controls. Furthermore, the addition of leucine to the incubation medium stimulates the incorporation of labeled precursors into muscle proteins in hemidiaphragms isolated from diabetic rats compared with hemidiaphragms incubated in the absence of the amino acid (33). In the current study, oral administration of leucine to diabetic rats enhanced muscle protein synthesis compared with diabetic controls. Therefore, some portion

of the protein synthetic response to dietary leucine may occur in the absence of insulin availability.

The protein synthetic response to leucine in diabetic rats occurred without alterations in the phosphorylation state of 4E-BP1 or significant alterations in eIF4G•eIF4E complex assembly. Additionally, leucine did not promote the hyperphosphorylation of S6K1 in diabetic rats. Accordingly, rp S6 phosphorylation was not increased in diabetic rats receiving leucine compared with diabetic rats that were food deprived. The data underscore the fact that an adequate basal insulin concentration is required to facilitate the stimulatory effect of dietary leucine on 4E-BP1 and S6K1 phosphorylation. Nonetheless, leucine stimulated protein synthesis in diabetic rats. The results imply unique regulation of translation control of muscle protein synthesis by leucine through an uncharacterized insulin-independent mechanism.

A number of reports have suggested that diabetes results in insulin resistance with regard to protein synthesis (16,29,30). Pain and Garlick (34) reported that subcutaneous administration of pharmacological concentrations of insulin to freely fed streptozotocin-induced diabetic rats only increases the fractional rate of protein synthesis in gastrocnemius muscle to values observed in food-deprived nondiabetic controls. These findings are corroborated by the results presented herein. Insulin replacement only partially restored rates of muscle protein synthesis in diabetic rats. However, the available evidence implies that insulin resistance with regard to protein synthesis in skeletal muscle of diabetic rats does not result from defects in the proximal signaling events in the phosphoinositol 3-kinase (PI 3-K) signaling cascade. Phosphorylation of insulin receptor substrate (IRS) proteins, IRS-1 and -2, in response to insulin is not impaired in skeletal muscle in streptozotocin-treated diabetic rats (35–37). Furthermore, the amount of PI 3-K and PKB is not altered in skeletal muscle of type 2 diabetic subjects (38). Though some studies suggest that the insulin-induced activation is impaired in diabetic rats (36) and humans (39), this may only be true when insulin is used at pharmacological doses. Krook et al. (39) reported that when muscle from type 2 diabetic subjects is incubated in vitro with physiological concentrations of insulin, activation of PKB is normal (39). Likewise, in the present study, restoration of circulating insulin concentrations in diabetic rats to physiological levels enhanced the phosphorylation of PKB. Hence, molecular defects resulting in insulin resistance with respect to protein synthesis may involve more distal steps in the PI 3-K signaling pathway.

Accumulating evidence points to the mammalian target of rapamycin kinase, mTOR, which lies downstream of PKB in the PI 3-K signaling pathway, as a convergence point for both amino acid– and insulin-mediated effects on translation initiation. mTOR serves as a bifurcation point in the control of translation initiation, regulating the phosphorylation of both 4E-BP1 and S6K1. Experiments in HEK-293 cells demonstrate insulin treatment to induce phosphorylation of mTOR at Ser 2448, a site that is considered crucial in the activation of the kinase (40). On the other hand, amino acid starvation reduces the phosphorylation of mTOR at Ser 2448 and makes the phosphorylation of this site refractory to insulin. Recently, the

contribution of mTOR to the leucine-induced stimulation of protein synthesis and translation initiation was investigated (41). Food-deprived rats were injected intravenously with the immunosuppressant drug rapamycin, a specific inhibitor of mTOR, 2 h before oral leucine administration. It was reported that rapamycin completely prevents the leucine-induced hyperphosphorylation of both 4E-BP1 and S6K1. Furthermore, it was recently demonstrated that although leucine enhances the phosphorylation of 4E-BP1 and S6K1 in the presence of fasting levels of insulin, a maximal response requires an indirect effect of leucine on pancreatic insulin release (12). The results presented here underscore these earlier findings. In diabetic rats administered leucine and infused with insulin to restore circulating concentrations of the hormone to levels observed in fasting controls, a partial restitution of mTOR-mediated signaling events in translation initiation was observed in skeletal muscle. In comparison, complete recovery of the phosphorylation of 4E-BP1 and S6K1 was observed in diabetic rats administered leucine and then infused with insulin to mimic concentrations of the hormone observed in control rats given leucine. Thus, the possibility exists that mTOR may integrate both leucine- and insulin-mediated signals and thereby contribute to the regulation of protein synthesis in skeletal muscle.

Prior investigations do not suggest that a defect in mTOR signaling accounts for insulin resistance with respect to translational efficiency in skeletal muscle of diabetic rats. Grzelkowska et al. (42) reported that rapamycin, an inhibitor of mTOR, reduces insulin-stimulated rates of protein synthesis in isolated epitrochlearis muscle of control rats to 64% of values obtained in muscles incubated in the absence of the inhibitor. In contrast, rapamycin further attenuates rates of insulin-stimulated protein synthesis in muscle isolated from streptozotocin-induced diabetic rats to 32% of values reported in diabetic controls. Thus, a rapamycin-sensitive pathway makes a greater contribution to the stimulatory effects of insulin on protein synthesis in skeletal muscle of diabetic compared with control rats. Furthermore, Kimball et al. (20) reported that intraperitoneal injections of insulin enhance the phosphorylation state of 4E-BP1, reducing the amount of the inactive 4E-BP1•eIF4E complex in skeletal muscle of alloxan-treated diabetic rats to values observed in nondiabetic controls. However, the doses of insulin used to stimulate protein synthesis in the aforementioned studies were at pharmacological levels. One must consider that supraphysiological levels of the hormone could potentially compensate for defects in insulin signaling to the translational apparatus through mTOR. The results presented herein suggest that physiological concentrations of insulin promote recovery of mTOR-mediated events in translation initiation. Infusion of insulin enhanced 4E-BP1 phosphorylation. Moreover, insulin promoted hyperphosphorylation of S6K1. Taken together, these studies imply that the impaired action of insulin on muscle protein synthesis in diabetic rats does not result from a defect in mTOR signaling. Collectively, the results indicate that the inhibition of protein synthesis observed in diabetic rats cannot be explained by impaired signaling to the translational apparatus through the PI 3-K/mTOR signaling cascade and

suggest that signaling through an alternative pathway by leucine and/or insulin must be dampened.

In summary, oral administration of leucine stimulated protein synthesis in skeletal muscle of control rats, in association with enhanced assembly of the mRNA cap-binding complex, and increased phosphorylation of rp S6. Leucine administration also elevated circulating concentrations of insulin. This increase in insulin enhanced phosphorylation of 4E-BP1 and S6K1 and contributed to the leucine-dependent regulation of protein synthesis. However, a portion of the protein synthetic response to leucine occurred through an insulin-independent pathway because rates of protein synthesis in diabetic rats administered leucine were greater than in diabetic controls. The stimulatory effect of leucine on muscle protein synthesis in diabetic rats occurred in the absence of changes in the phosphorylation states of 4E-BP1 and S6K1 and implies a unique mechanism through which leucine regulates protein synthesis independently of insulin.

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