Involvement of the rapamycin-sensitive pathway in the insulin regulation of muscle protein synthesis in streptozotocin-diabetic rats

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Abstract

Insulin resistance in 3-day streptozotocin (STZ)-treated rats was manifested by the lack of antiproteolytic action of insulin as well as by a reduction of its stimulatory effect on protein synthesis (−60% compared with the control group) in epitrochlearis muscle incubated *in vitro*. In the present study, we have investigated the diabetes-associated alterations in the insulin signalling cascade, especially the phosphatidylinositol-3 kinase (PI-3 kinase)/p70 S6 kinase (p70^{S6K}) pathway, in rat skeletal muscle. LY 294002, a specific inhibitor of PI-3 kinase, markedly decreased the basal rate of protein synthesis and completely prevented insulin-mediated stimulation of this process both in control and diabetic rats. Thus, PI-3 kinase is required for insulin-stimulated muscle protein synthesis in diabetic rats as in the controls. Rapamycin, an inhibitor of mammalian

target of rapamycin (mTOR), had no effect on the basal rate of protein synthesis in either of the experimental groups. In control rats, the stimulatory action of insulin on muscle protein synthesis was diminished by 36% in the presence of rapamycin, whereas in diabetic muscles this reduction amounted to 68%. The rapamycin-sensitive pathway makes a relatively greater contribution to the stimulatory effect of insulin on muscle protein synthesis in diabetic rats compared with the controls, due presumably to the preferential decrease in the rapamycin-insensitive component of protein synthesis. Neither basal nor insulin-stimulated p70^{S6K} activity, a signalling element lying downstream of mTOR, were modified by STZ-diabetes.

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Introduction

A characteristic feature of uncontrolled diabetes mellitus is the decrease in body weight and marked muscle atrophy. This net loss of body protein results from both an increase in proteolysis and a decrease in protein synthesis. Enhanced protein breakdown has been described in skeletal muscle of rats with experimentally-induced diabetes (Pepato *et al.* 1996, Bailey *et al.* 1997) as well as in type 1 diabetic patients (reviewed in Tessari *et al.* 1992). The inhibition of elevated proteolysis in diabetic humans requires higher plasma insulin levels than those normally occurring *in vivo* (Tessari *et al.* 1992), which reflects an insensitivity to the antiproteolytic effect of insulin. In numerous studies performed *in vivo* (Pain & Garlick 1974, Odedra *et al.* 1982, Pain *et al.* 1983, Karinch *et al.* 1993), and on perfused muscle preparations (Flaim *et al.* 1980, Williams *et al.* 1980), insulin treatment only partially restored an impaired protein synthesis in diabetic rats, further suggesting alterations of the action of this hormone on muscle protein synthesis. Taken together these data clearly indicate that insulin resistance with respect to

protein metabolism may contribute, along with insulin deficiency *per se*, to the loss of muscle protein in diabetic subjects.

There has been much recent progress in the elucidation of the signalling cascade acting downstream of the insulin receptor. A critical event is the activation of the insulin receptor tyrosine kinase and resultant phosphorylation of insulin receptor substrate-1 (IRS-1) (White & Kahn 1994). This leads to the recruitment of several further signalling components such as phosphatidylinositol-3 kinase (PI-3 kinase), ras, and the tyrosine phosphatase SHPTP2 which, in turn, trigger multiple effector pathways. Although the mechanism of insulin action on skeletal muscle metabolism is not exactly defined, several lines of evidence point to the role of PI-3 kinase and the pathway including p70 S6 kinase (p70^{S6K}). It has been demonstrated that PI-3 kinase is required for insulin-regulated glucose uptake (Le Marchand-Brustel *et al.* 1995, Jullien *et al.* 1996), glycogen synthesis (Shepherd *et al.* 1997), as well as protein synthesis and proteolysis (Dardevet *et al.* 1996). p70^{S6K} is usually regarded as one of the intermediates situated downstream of PI-3 kinase

(Chung *et al.* 1994) on the pathway implicated in the stimulation of muscle protein synthesis (Dardevet *et al.* 1996).

In parallel to the investigation of the insulin signalling cascade in skeletal muscle, analysis of the possible defects leading to insulin resistance has recently been developed. Decreased autophosphorylation of the receptor kinase, previously proposed as the most straightforward explanation for the insulin resistance (Kadowaki *et al.* 1984, Block *et al.* 1991), seems to have limited biological significance in view of the increased insulin binding to various tissues of insulinopaenic diabetic rats (reviewed in Sechi *et al.* 1992). Moreover, the early postreceptor events e.g. IRS-1 protein content and its phosphorylation in response to insulin were not impaired during diabetes (Giorgino *et al.* 1992, Saad *et al.* 1992). Molecular defects leading to insulin resistance may therefore involve more distal steps in the insulin signalling cascade.

In the present study we have examined the possible contribution of the PI-3 kinase/p70^{S6K} pathway to the insulin resistance of skeletal muscle protein turnover in streptozotocin-diabetic rats. We have investigated the effect of LY 294002 and rapamycin, specific inhibitors of PI-3 kinase and p70^{S6K} respectively, on insulin action in epitrochlearis muscle *in vitro*. The activity of p70^{S6K} was also analysed.

Materials and Methods

Chemicals

L-[U-¹⁴C]phenylalanine (450 mCi/mmol) and [γ -³²P]ATP (3000 Ci/mmol) were obtained from Amersham (Aylesbury, Bucks, UK). Porcine insulin was supplied by Novo (Bagsvaerd, Denmark).

Long-acting Lente MC insulin (a mixture of porcine and bovine insulin) and Ultratard HMge human insulin were obtained from Novo Nordisk Pharmaceutique SA (Boulogne, France). Rabbit polyclonal anti-p70^{S6K} antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Streptozotocin and other chemicals were supplied by Sigma Chemical Co. (St Louis, MO, USA). Rapamycin (ICN, Costa Mesa, CA, USA) and LY 294002 (Tebu, Le Peray en Yvelines, France) were dissolved in dimethyl sulphoxide (DMSO) and stored at -20 °C.

Plasma insulin was determined by direct radioimmunoassay with a commercial kit (ERIA Diagnostic Pasteur, Sanofi, France), according to the manufacturer's protocol. Protein content was assayed by the bicinchoninic acid (BCA, Pierce Chemical Co., Rockford, IL, USA) or Bradford (Bio-Rad, Richmond, CA, USA) reaction.

Animal procedure

These experiments were carried out in accordance with current legislation on animal experiments in France.

Young (4-week-old) male Sprague-Dawley rats were purchased from IFFA-Credo (L'Arbresle, France) and housed under controlled environmental conditions (temperature 22 °C, 12-h darkness period starting at 1800 h). Animals were given free access to water and standard laboratory chow diet 3–4 days before the beginning of the experiment. Diabetes was induced with streptozotocin (STZ) dissolved in 0.01 M citrate buffer, pH 4.5, administered intravenously in a single dose of 110 mg/kg, at 0900–1000 h, to overnight-fasted rats. Control animals received an equal volume of vehicle. All animals were returned to *ad libitum* feeding conditions 6 h after injection. Glycosuria was verified by Clinistix (Bayer Diagnostics, Puteau, France) the following morning and was 3+ (i.e. high on arbitrary scale) in STZ-treated rats. Preliminary observations have shown that the maximum effect of diabetes on protein turnover in skeletal muscle (e.g. insulin resistance of protein synthesis and degradation *in vitro*) occurred three days after STZ injection (data not shown). This time was therefore chosen for further experimental procedures. Rats were killed under anaesthesia with sodium pentobarbital (6.0 mg/100 g body weight) after an overnight fast. Epitrochlearis muscles were dissected intact for incubation (see below). Blood was drawn from the aorta to determine the plasma glucose and insulin levels.

Assessment of protein metabolism

Muscle incubation was performed in Krebs–Hensleit buffer (KHB) (120 mM NaCl, 4.8 mM KCl, 25 mM NaHCO₃, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, and 1.2 mM MgSO₄, pH 7.4) supplemented with 5 mM HEPES, 5 mM glucose, 0.1% BSA (99% fatty acid free), 0.17 mM leucine, 0.20 mM valine, 0.10 mM isoleucine, and saturated with 95%O₂/5%CO₂ gas mixture. Epitrochlearis muscles were preincubated for 30 min in KHB supplemented with 100 μ M LY 294002 or 200 nM rapamycin, and then transferred for a further 3 h into fresh medium of the same composition, without or with 30 nM insulin (corresponding to approx 4 mU/ml). This dose of insulin has already been shown to exert a maximal stimulatory effect on protein synthesis in rat epitrochlearis muscle incubated *in vitro* (Dardevet *et al.* 1994). The appropriate controls with the diluent of the inhibitors (DMSO, final concentration 0.01%) were also performed. Incubation medium was changed at 1-h intervals. In order to measure the rate of protein synthesis, 0.5 mM L-[U-¹⁴C]phenylalanine (0.15 μ Ci/ml) was added to the medium during the last hour of incubation. Since anti-proteolytic action of insulin on epitrochlearis muscle *in vitro* requires prolonged treatment with the hormone (Stirewalt & Low 1983), in the third hour of incubation it was possible to measure both protein synthesis and protein breakdown in the same muscle preparations. Previous observations showed that rat epitrochlearis muscle maintained linear rates of protein synthesis for 3 h of incubation

(see above and D Dardevet, unpublished observations). At the end of the incubation muscles were blotted and homogenized in 10% trichloroacetic acid (TCA). TCA-precipitated material was washed three times with 10% TCA and solubilized in 1 M NaOH at 37 °C. These extracts were used for the estimation of protein content as well as the radioactivity incorporated into muscle proteins. The rate of protein synthesis was expressed as nanomoles phenylalanine (Phe)/mg protein.h. Protein degradation was determined as described in detail by Tischler *et al.* (1982). Briefly, since tyrosine is neither synthesized nor degraded in skeletal muscle (discussed in Fulks *et al.* 1975), the appearance of this amino acid in the medium provides a measure of net protein breakdown. Re-utilization of tyrosine was estimated from the rate of protein synthesis by using the ratio of tyrosine/phenylalanine content in total muscle protein (0.77). The rate of muscle protein degradation was therefore calculated as follows: total proteolysis (nanomoles Tyr/mg protein.h)=(incorporation of [14 C] Phe into protein) (0.77)+(release of tyrosine into the medium). It should be noted that incubated muscles *in vitro* are in a negative nitrogen balance, with protein breakdown rates exceeding protein synthesis rates, even in the presence of insulin. Under these conditions the pool of intracellular amino acids resulting from protein degradation and supplying the precursors for new proteins is rather unlikely to limit the rate of protein synthesis.

Measurement of p70^{S6K} activity

Epitrochlearis muscles were preincubated for 30 min in KHB and transferred into fresh medium without or with insulin (30 nM) for a further 20 min. Afterwards, muscles were homogenized in ice-cold extraction buffer (50 mM Tris-acetate, 50 mM NaF, 2.5 mM EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 5 mM β -glycerophosphate, 1 mM Na₃VO₄, 2 mM dithiothreitol (DTT), 1 mM benzamidine, 4 μ g leupeptin, and 0.1% Triton X-100, pH 7.2) and centrifuged at 10 000 *g* for 15 min at 4 °C. The activity of p70^{S6K} was measured by an immune complex kinase assay. Briefly, aliquots of supernatant containing 100 μ g total protein were incubated for 4 h at 4 °C with 3 μ l p70^{S6K} antibody preadsorbed to protein A-agarose beads. The immune complexes were collected by centrifugation, washed three times with kinase assay buffer (25 mM MOPS, 15 mM MgCl₂, 1 mM DTT, 0.1% BSA, pH 7.2) and then resuspended in the same buffer supplemented with 2 μ M inhibitory peptide of cAMP-dependent protein kinase, 0.1 mM S6 peptide (RRRLSSLRA) and 100 μ M [γ - 32 P]ATP (specific activity 3000 d.p.m./pmol). The reaction was conducted for 40 min at 30 °C and was terminated by adding 50 mM unlabelled ATP. The reaction contents were spotted onto phosphocellulose filter paper (Whatman P-81) and washed three times with 75 mM phosphoric acid prior to counting in a β -scintillation counter. Pilot

experiments showed that under these conditions, the incorporation of 32 P into S6 peptide is linear and proportional to the amount of proteins used (100–200 μ g).

Western blotting

Equal amounts of muscle protein (25 μ g) were subjected to SDS-PAGE under reducing conditions. Electrotransfer of proteins to nylon membranes (Millipore Corp., Bedford, MA, USA) was carried out for 1 h at 100 V, followed by blocking in TBS buffer (20 mM Tris, 500 mM NaCl, pH 7.5) supplemented with 5% nonfat powdered milk. Membranes were then probed with primary antibody (1 μ g/ml) for 16 h at 4 °C, washed three times in TBS containing 0.05% Tween-20 and were incubated with goat anti-rabbit antibody conjugated with horseradish peroxidase. The blots were developed using the enhanced chemiluminescence (ECL) detection system (Amersham) according to the manufacturer's protocol.

Statistical analysis

The data were expressed as means $\pm$ s.e.m. The results were statistically evaluated using ANOVA. Values of $P < 0.05$ were considered significant.

Results

Animal characteristics

STZ-treated rats lost on average 5 g/day in body weight over the study period, whereas rats in the control group gained approximately 6 g/day. At the end of the experiment, the plasma insulin level in STZ-treated rats was markedly reduced when compared with the control group (10.3 ± 0.6 vs 32.1 ± 3.0 μ U/ml, $P < 0.05$). This drop in the insulin level in diabetic rats was accompanied by a 4-fold increase in fasting plasma glucose (32.1 ± 1.4 vs 7.7 ± 0.2 mmol/l for diabetic and control rats respectively, $P < 0.05$). Diabetic rats exhibited a marked muscle atrophy reflected by a reduction in the weight of epitrochlearis (21.5 ± 0.6 vs 16.2 ± 0.5 mg for control and diabetic rats respectively, $P < 0.05$) and gastrocnemius (734.2 ± 16.2 vs 528.4 ± 26.2 mg for control and diabetic rats respectively, $P < 0.05$) muscle.

Effect of LY 294002 on insulin-regulated muscle protein turnover

Treatment of the epitrochlearis muscles taken from control rats with insulin caused a significant increase in the rate of protein synthesis (0.274 ± 0.020 vs 0.506 ± 0.021 nmoles Phe/mg protein.h for muscles incubated without and with insulin respectively, $P < 0.05$) (Fig. 1). LY 294002 markedly decreased the rate of protein synthesis detected under

Figure 1 Effect of LY 294002 on basal (solid bars) and insulin-stimulated (hatched bars) muscle protein synthesis in control and diabetic rats. Incubation was performed as described in Materials and Methods. When present, LY 294002 (LY) was added 30 min before insulin. Values represent the mean $\pm$ S.E.M. of nine to twelve individual epitrochlearis muscles. * $P < 0.05$, insulin-stimulated vs basal for the same group; ** $P < 0.05$, compared with corresponding value without inhibitor for the same group; † $P < 0.05$, difference between control and diabetic rats for the same muscle treatment.

basal conditions (0.178 ± 0.005 nmoles Phe/mg protein.h) and totally prevented the hormonal stimulation of protein synthesis (0.180 ± 0.010 nmoles Phe/mg protein.h).

The basal rate of muscle protein synthesis was not altered in diabetic rats (0.274 ± 0.020 nmoles Phe/mg protein.h). Incubation with insulin also led to a significant increase in the rate of protein synthesis (0.373 ± 0.20 nmoles Phe/mg protein.h, $P < 0.05$). However, the magnitude of this stimulation was significantly lower than in the control group (+36% above basal value vs +84% respectively, $P < 0.05$). As in the case of control muscles LY 294002 inhibited basal protein synthesis. It should be noticed, however, that the fall in basal protein synthesis was more pronounced in the diabetic than in the control group (decrease by 54% vs 35% in the controls, $P < 0.05$). The presence of LY 294002 in the medium containing insulin also totally blocked the stimulatory effect of the hormone on protein synthesis. It thus appeared that both in control and diabetic animals, PI-3 kinase plays a major role in the regulation of basal and insulin-stimulated protein synthesis in rat epitrochlearis muscle.

As shown in Fig. 2, insulin significantly attenuated proteolysis in control muscles (1.57 ± 0.07 vs 1.10 ± 0.10 nmoles Tyr/mg protein.h in the absence and in the presence of insulin respectively, $P < 0.05$). Supplementation of the medium with LY 294002 had no effect on the basal rate of protein breakdown (1.75 ± 0.16 nmoles Tyr/mg protein.h). When used in combination with insulin, this inhibitor completely reversed its antiproteolytic effect (1.65 ± 0.15 nmoles Tyr/mg protein.h). In basal conditions, proteolysis in muscles isolated from diabetic rats was markedly elevated compared with the control group (+30%, $P < 0.05$). Moreover, the rate of

Figure 2 Effect of LY 294002 on basal (solid bars) and insulin-stimulated (hatched bars) muscle proteolysis in control and diabetic rats. Incubation was performed as described in Materials and Methods. When present, LY 294002 (LY) was added 30 min before insulin. Values represent the mean $\pm$ S.E.M. of seven to twelve individual epitrochlearis muscles. * $P < 0.05$, insulin-treated vs basal for the same group; ** $P < 0.05$, compared with corresponding value without inhibitor for the same group; † $P < 0.05$, difference between control and diabetic rats for the same muscle treatment.

proteolysis was not affected by the availability of insulin (2.01 ± 0.11 vs 1.83 ± 0.09 nmoles Tyr/mg protein.h in the absence and presence of insulin respectively), indicating total resistance to the antiproteolytic action of the hormone. LY 294002 was without significant effect on the rate of proteolysis in both the absence (1.95 ± 0.23 nmoles Tyr/mg protein.h) and the presence (1.78 ± 0.14 nmoles Tyr/mg protein.h) of insulin.

In order to verify whether the observed changes in insulin action *in vitro* result from diabetes-associated insulin deficiency and not from the effect of STZ *per se*, an additional experiment was performed in which diabetic rats underwent insulin replacement by exogenous insulin (Table 1). Because impairment of insulin-stimulated protein synthesis as well as resistance to the antiproteolytic action of the hormone *in vitro* could be prevented by insulin therapy, the changes may be attributable to insulin deficiency rather than toxicity of STZ.

Effect of rapamycin on insulin-regulated muscle protein turnover

Addition of rapamycin to the incubation medium did not significantly modify the basal rate of protein synthesis both in control and diabetic muscles (Fig. 3). The stimulatory effect of insulin on protein synthesis was still detected in the presence of rapamycin. In the case of control muscles, protein synthesis activation amounted to 64% of the value detected in the absence of the inhibitor (as reflected by the increment above basal: 0.170 and 0.109 nmoles Phe/mg protein.h measured without and with rapamycin respectively). In other words, 36% of overall insulin-stimulated protein synthesis may be

Table 1 Effect of diabetes and insulin treatment on protein metabolism in rat epitrochlearis muscle. Values represent the mean $\pm$ S.E.M. of five individual epitrochlearis muscles

Group	Protein synthesis (nmoles Phe/mg protein.h)		Protein breakdown (nmoles Tyr/mg protein.h)	
	Basal	Insulin	Basal	Insulin
Control	0.237 $\pm$ 0.007	0.437 $\pm$ 0.045	2.00 $\pm$ 0.14	1.45 $\pm$ 0.09**
Diabetic	0.232 $\pm$ 0.019	0.363 $\pm$ 0.018*	2.22 $\pm$ 0.16	1.92 $\pm$ 0.08*
Diabetic + insulin	0.225 $\pm$ 0.011	0.405 $\pm$ 0.013**	1.84 $\pm$ 0.05**	1.46 $\pm$ 0.08*

Rats were rendered diabetic with STZ, as described in Materials and Methods. One unit Ultratard HMge insulin was then injected subcutaneously each morning and evening, except for the last injection (2 h before killing) when Lente MC insulin was used. This protocol of insulin treatment completely normalized the plasma glucose levels of the animals (not shown). Protein synthesis and breakdown was measured in incubated epitrochlearis muscles.

* $P < 0.05$ vs control; ** $P < 0.05$ vs untreated diabetic.

attributed to the rapamycin-dependent pathway. In comparison with the control group, insulin-stimulated protein synthesis in muscles from diabetic rats was more profoundly attenuated by the presence of rapamycin, as it represents only 32% of the value recorded in the absence of inhibitor (demonstrated by increment above basal of 0.060 and 0.019 nmoles Phe/mg protein.h without and with addition of rapamycin respectively). The main portion (68%) of insulin action could be attributed to the rapamycin-dependent pathway in this case. These results indicate that the rapamycin-sensitive pathway plays a more important role in the insulin-stimulated muscle protein synthesis of diabetic rats compared with the control rats. The presence of rapamycin in the incubation medium failed to modify the rate of protein breakdown under any experimental conditions (data not shown), suggesting that

the rapamycinsensitive pathway is not involved in the regulation of proteolysis in epitrochlearis muscle.

Basal and insulin-stimulated p70^{S6K} activity

When extracted from untreated muscles and subjected to SDS-PAGE, the p70^{S6K} appeared as a single band of apparent molecular mass 70 kDa (Fig. 4, top). Activation of p70^{S6K} caused by multiple phosphorylation results in several phosphorylated forms that exhibit decreased

Figure 3 Effect of rapamycin on basal (solid bars) and insulin-stimulated (hatched bars) muscle protein synthesis in control and diabetic rats. Data for the absence of inhibitor represent new experiments paired with the rapamycin treatment. Values represent the mean $\pm$ S.E.M. of eight to twelve individual epitrochlearis muscles. * $P < 0.05$, insulin-stimulated vs basal for the same group; ** $P < 0.05$, compared with corresponding value without inhibitor for the same group; † $P < 0.05$, difference between control and diabetic rats for the same muscle treatment.

Figure 4 Insulin stimulation of p70^{S6K} in epitrochlearis muscles of control and diabetic rats. (Top) Western blot analysis was performed as described in Materials and Methods. Presented data are representative of three separate experiments. (Bottom) The results of the immune kinase assay are expressed as the mean $\pm$ S.E.M. of ten to twelve individual muscles. * $P < 0.05$, insulin-stimulated vs basal for the same group.

electrophoretic mobility (Chung *et al.* 1994). Treatment of muscles with insulin led to the gel mobility retardation of p70^{S6K} indicating its phosphorylation and activation.

There were no differences between control and diabetic rats regarding the quantity of p70^{S6K} protein, as well as the basal p70^{S6K} activity (2.59 ± 0.20 and 2.35 ± 0.19 pmoles ATP/100 μ g protein·40 min respectively) in the epitrochlearis muscles. Consistent with the results from the bandshift experiments, the immune complex kinase assay showed that the p70^{S6K} activation by insulin was similar in both experimental groups (3.89 ± 0.37 and 3.61 ± 0.29 pmoles ATP/100 μ g protein·40 min for control and diabetic rats respectively).

Discussion

The diabetes-associated muscle atrophy is a consequence of both decreased protein synthesis and increased protein degradation. Several lines of evidence showed that muscle protein synthesis in diabetic rats is markedly reduced, especially in fast-twitch muscle, and that this effect is attributed to an impairment of peptide chain initiation (Flaim *et al.* 1980, Pain *et al.* 1983). Elevated proteolysis has already been claimed in skeletal muscle of diabetic rats (Pepato *et al.* 1996, Bailey *et al.* 1997). It appeared that enhanced protein breakdown in skeletal muscle observed during acute phase of diabetes results from activation of the ATP-dependent proteolytic system (Pepato *et al.* 1996, Bailey *et al.* 1997).

In the present study, the basal rate of muscle protein synthesis estimated *in vitro* was not altered in diabetic rats. However, the extent of protein synthesis activation by insulin was markedly reduced in diabetic muscles (-60% , as reflected by the increment above basal value), when compared with the control group. In other words, 3-day STZ-diabetic rats have developed insulin resistance which cannot be overcome by adding surplus insulin *in vitro*. This impaired responsiveness of muscle protein synthesis to insulin *in vitro* is consistent with the diabetes-associated decrease in protein synthesis in skeletal muscle, observed in numerous *in vivo* studies.

As expected, supplementation of insulin *in vitro* significantly diminished the rate of proteolysis in control epitrochlearis muscle. In diabetic rats, basal proteolysis was not only elevated compared with the controls, but it was also unaffected by availability of insulin *in vitro*, indicating an insensitivity to the antiproteolytic action of this hormone. Thus, it is clear that the resistance to the anabolic effect of insulin, along with insulin deficiency, facilitates muscle wasting during diabetes.

Until now, the mechanism underlying insulin resistance has not been adequately explored. A reduction in the receptor kinase activity seems to be insufficient to explain this phenomenon and might be challenged by a number of studies showing that: (i) in STZ-diabetic rats a decrease in

insulin secretion is accompanied by an increased insulin binding in adipose tissue, liver, muscles, and kidney (see Sechi *et al.* 1992, for review); (ii) the level of the IRS-1 protein is higher in liver of diabetic rats and only slightly diminished in muscles; the latter does not explain insulin resistance, because (iii) after acute insulin stimulation *in vivo*, IRS-1 phosphorylation is several-fold higher and markedly prolonged, compared with the controls (Giorgino *et al.* 1992, Saad *et al.* 1992). Recent work has suggested the role of tumour necrosis factor- α (TNF- α) interaction with the insulin transduction cascade in inducing insulin resistance (Feinstein *et al.* 1993, Hotamisligil *et al.* 1994). In STZ-diabetes accompanied by a high rate of lipolysis, elevated production of TNF- α by adipocytes scattered through the muscle tissue could lead to increased insulin receptor dephosphorylation as well as decreased IRS-1 activity via serine phosphorylation. These effects could account for impaired insulin sensitivity modifying different signalling pathways. A 3-day-period of diabetes in the present experiment allows sufficient exposure to TNF- α to result in alterations in insulin action. In contrast, short-term (few hours) treatment of rat soleus muscles with TNF- α *in vitro* had no inhibitory effect on either early steps of insulin signal transduction or glucose transport (Nolte *et al.* 1998).

We have demonstrated that pretreatment of muscles with LY 294002, the specific inhibitor of PI-3 kinase, completely prevented both the stimulatory effect of insulin on protein synthesis and the antiproteolytic action of this hormone in control muscles, confirming the well-described role of PI-3 kinase in several metabolic effects exerted by insulin in skeletal muscles (Le Marchand-Brustel *et al.* 1995, Dardevet *et al.* 1996, Jullien *et al.* 1996, Shepherd *et al.* 1997). Since PI-3 kinase represents one of the early events in insulin action, it might be considered as a potential element involved in insulin resistance. Indeed, several lines of evidence indicate that attenuated responsiveness to insulin observed in non-insulin-dependent diabetes mellitus (NIDDM) (Bjornholm *et al.* 1997), gold thioglucose-induced obesity (Heydrick *et al.* 1993), as well as after glucocorticoid administration (Saad *et al.* 1993), may be, at least partially, explained by the impairment of the PI-3 kinase/IRS-1 association/activation.

It should be noticed, however, that NIDDM, obesity and treatment with glucocorticoids are associated with hyperinsulinaemia and, thus, the alterations in the insulin transduction system which occur in these insulin-resistant states cannot be directly compared with the conditions of insulin deficiency. Instead, the opposite regulation of IRS-1-associated PI-3 kinase activity by insulin in hyperinsulinaemic and hypoinsulinaemic states has already been documented by Folli *et al.* (1993). Whereas, in skeletal muscle and liver tissue of hyperinsulinaemic *ob/ob* mice insulin-mediated PI-3 kinase activity was profoundly impaired, in tissues of STZ-diabetic rats insulin-mediated PI-3 kinase/IRS association was markedly higher, relative

to control animals. Although the PI-3 kinase activity was not measured in our study and thus its potential alteration cannot be excluded, taking into account the results obtained with LY 294002 it becomes clear, that also in diabetic rats this kinase mediates overall stimulation of muscle protein synthesis exerted by insulin. Thus, the insulin resistance observed in STZ-treated rats is probably due to impairment of the element(s) lying downstream and dependent on PI-3 kinase.

In fact, PI-3 kinase is usually considered as one of the initial steps in the mammalian target of rapamycin (mTOR) pathway (Chung *et al.* 1994). Moreover, it has already been reported that the effects of insulin on increasing the synthesis of glycogen and protein in rat skeletal muscle, two of the most important actions of the hormone, involve a rapamycin-dependent input (Azpiazu *et al.* 1996). In the present experiments, rapamycin did not modify the basal rate of muscle protein synthesis either in control or in diabetic rats. Furthermore, this inhibitor only attenuated but did not abolish the stimulatory action of insulin in control muscles, confirming the well-established concept that the inhibition of protein synthesis exerted by rapamycin is incomplete. Indeed, in the cell culture system rapamycin has been shown to have only a partial inhibitory influence on the serum-stimulated protein synthesis, despite the fact that this drug blocks, at least in part, three of the main regulatory mechanisms controlling translation in animal cells. One of them may be the phosphorylation of ribosomal protein S6, since it appears to lie in the ribosome's mRNA binding site (Ferrari & Thomas 1994). Likewise, phosphorylation of the translational repressor PHAS-I (also known as 4E-BP1) is rapamycin-dependent (Beretta *et al.* 1996). Finally, the dephosphorylation and consequent activation of elongation factor eEF-2 in response to insulin involves a rapamycin-sensitive mechanism (Redpath *et al.* 1996). The limited effect of rapamycin may reflect the fact that both S6 protein (or p70 S6 kinase) and PHAS-I regulate primarily cap-dependent translation rather than the overall rate of translation (Jefferies *et al.* 1994, Terada *et al.* 1994, Mendez *et al.* 1996, Pedersen *et al.* 1997). Furthermore, another protein of the 40S ribosomal subunit, S17, is subjected to rapamycin-sensitive phosphorylation, and probably plays a role in the regulation of translation of specific mRNAs (Patel *et al.* 1996).

Interestingly, the signalling model in which PI-3 kinase acts as an upstream regulator of mTOR has been challenged by a recent finding showing that the function of mTOR is directly inhibited by wortmannin and LY 294002 (Brunn *et al.* 1996). Indeed, such an observation provides a plausible explanation for the parallel effects of rapamycin, wortmannin and LY 294002 on certain cellular processes. On the other hand, it might question the interpretation of earlier data obtained with these PI-3 kinase inhibitors. In the present study, however, LY 294002 and rapamycin exhibited quantitatively distinct

effects on insulin-stimulated muscle protein synthesis (complete vs partial inhibition). It suggests that even if total blockage of insulin action by LY 294002 occurs partially via direct mTOR inhibition, other signalling components are involved. These components are sensitive to LY 294002, but mTOR-independent. Regarding skeletal muscle, Azpiazu *et al.* (1996) have recently shown that in rat diaphragm incubated *in vitro*, basal activity of p70^{S6K} and the phosphorylation state of PHAS-I were not affected by rapamycin. In the presence of the drug, insulin-stimulated PHAS-I phosphorylation was markedly diminished, but not abolished. The above observations again suggest the presence of further, insulin-activated but rapamycin-independent pathway(s) mediating a high portion of the stimulation of protein synthesis. Interestingly, these potential intermediate signalling events, bypassing rapamycin-sensitive input, are likely to be altered during diabetes because, in the present study, the insulin-stimulated muscle protein synthesis was more profoundly impaired by rapamycin in diabetic rats than in the controls. It means that the rapamycin-dependent mechanism, which in control conditions makes a quantitatively minor contribution in insulin-regulated protein synthesis, becomes more important when other signalling pathways have been suppressed. In fact, a similar phenomenon has already been described by Chang *et al.* (1995). In their study, rapamycin was apparently unable to inhibit the insulin stimulation of glycogen synthase in normal mice. However, in mutant insulin-receptor-transgenic mice, when mitogen activating protein (MAP) kinase and RSK2 activation were defective, the insulin-stimulated increment in glycogen synthase fractional velocity was partially reduced by this drug.

Although in diabetic rats the rapamycin-sensitive pathway is essential for insulin-mediated muscle protein synthesis, diabetes-associated alterations of this pathway have recently been reported. It is well known that insulin-promoted phosphorylation of PHAS-I decreases its affinity for eukaryotic initiation factor 4E (eIF-4E) (Lin *et al.* 1994, Azpiazu *et al.* 1996, Kimball *et al.* 1997), allowing the initiation factor to interact with other subunits of the eIF-4F complex and mediate translation initiation. According to Kimball *et al.* (1996), neither quantity of eIF-4E nor the ability of this protein to bind to the mRNA cap was altered in muscle of alloxan-diabetic rats. Interestingly, diabetes markedly increased the association of PHAS-I with eIF-4E, an effect which was completely reversed within 2 h after treatment with insulin. Even if the above results provide an explanation for diabetes-associated impairment of translation initiation, it is evident that this mechanism does not account for insulin resistance.

Neither basal nor insulin-stimulated p70^{S6K} activity were modified in muscle of diabetic rats. It is actually known that multiple signalling inputs are implicated in the activation of p70^{S6K} (Pullen & Thomas 1997), and that some of them are rapamycin-insensitive (Weng *et al.* 1995,

Dennis *et al.* 1996). In fact, two different protein kinases, 3-phosphoinositide-dependent protein kinase 1 and 2 (PDK 1 and 2) have recently been proposed to phosphorylate Thr³⁸⁹ and Thr²²⁹, the sites essential for full activation of p70^{S6K} (Downward 1998, Pullen *et al.* 1998). However, the finding that insulin-promoted p70^{S6K} is not altered during diabetes, allows the exclusion of the signalling components leading to the activation of this kinase as being responsible for insulin resistance.

Although the mechanism of insulin resistance still remains to be clarified, the presented data provide new insights into the regulation of muscle protein synthesis during diabetes. Based on current results we conclude that: (i) PI-3 kinase is required for insulin-stimulated muscle protein synthesis in diabetic rats; (ii) neither basal nor insulin-stimulated p70^{S6K} activity in skeletal muscle are modified during diabetes and thus this kinase does not account for insulin resistance; (iii) the rapamycin-sensitive pathway makes a relatively greater contribution to the stimulatory effect of insulin on muscle protein synthesis in diabetic rats than in control rats, presumably due to alterations in other insulin-activated intermediates during diabetes.

Further signalling molecules involved in insulin-mediated muscle protein synthesis await determination as they are likely to account for insulin resistance. Keeping in mind that PI-3 kinase is necessary for transmission of the overall stimulatory effect of insulin, these potential elements are probably activated by insulin in a PI-3 kinase-dependent manner. However, current results obtained with LY 294002 under basal conditions suggest the presence of signalling pathway(s), in addition to those occurring via PI-3 kinase (e.g. MAP kinase), also implicated in the regulation of muscle protein synthesis.

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