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The effect of fasting on the activation *in vivo* of the insulin receptor kinase

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Fasting causes insulin resistance in liver and fat, and increases insulin sensitivity in muscle. We studied the response *in vitro* and *in vivo* to insulin of the insulin receptor tyrosine kinase in muscle and liver from 72 h fasted and control rats. Insulin was injected intraperitoneally together with glucose, and blood and tissue samples were obtained 0, 5, 15 and 30 min later. Basal serum glucose and insulin levels were significantly higher in control than in fasting rats. Serum glucose rose to ~ 300 mg/dl at 5 min and then progressively declined without hypoglycaemia. Receptors were prepared from whole tissue by wheat germ lectin affinity chromatography. ^{125}I-insulin binding to purified receptors was increased by fasting in both muscle (18 %) and liver (50 %). In untreated fasting and control animals, muscle and liver insulin receptor tyrosine kinase activity was stimulated to similar levels by insulin added *in vitro*. With only insulin treatment *in vivo*, muscle receptor tyrosine kinase behaved similarly in fasting and control animals with maximal activation at 15 min post injection. In liver, insulin *in vivo* stimulated receptor tyrosine kinase activity maximally at 5 min post injection in both fasting and control, but in fasting animals the treatment *in vivo* caused a significantly larger and more prolonged activation of the enzymic activity, possibly due to a decrease in the rate of dephosphorylation and deactivation of the β subunits.

INTRODUCTION

Fasting induces a number of metabolic and endocrine changes that appear to be tissue specific. In muscle, fasting causes an increase in sensitivity or responsiveness to insulin [1,2]. In fat, fasting causes a decrease in basal and insulin-stimulated glucose transport and utilization [3–5]. In liver and in isolated hepatocytes, fasting causes insulin resistance characterized by a lack of response to the hormone with regard to glucose production [6], lipid synthesis [7], amino acid uptake [8] and glycogen synthesis [9]. In most tissues studied, insulin binding appears to be increased during fasting [2,5,8–10] but without direct relationship between hormone binding and action, a reason for which post-binding abnormalities are thought to occur. A possible defective site of insulin action in the fasting state would be the insulin receptor tyrosine kinase (for reviews see [11–13]), since its activity is deficient in other states of insulin resistance such as clinical [14,15] or experimental [16] diabetes and obesity [17]. Previous reports have shown an increase [10] or a decrease [18] in receptor phosphorylation in response to insulin added *in vitro* to liver receptors purified from fasted rats. We have studied the response of muscle and liver receptor tyrosine kinase to insulin added *in vitro* or to insulin injected *in vivo* to 72 h fasted and to normally fed control rats.

METHODS

Materials

Porcine monocomponent insulin and [*Tyr*-^{125}I]monoiodoinsulin (376 μCi/μg) were kindly supplied by Eli Lilly & Co. [^{32}P]ATP (300 Ci/mmol) was purchased from Amersham Corp. Wheat germ agarose, obtained from Sigma, was prepared from lectin and CNBr-treated agarose 4B. Hepes, poly(Glu,Tyr) (4:1), phenylmethanesulphonyl fluoride, benzamidine, leupeptin, pepstatin, aprotinin, bacitracin and Triton X-100 were also from Sigma. All other chemicals were of analytical grade.

Animals and treatment

Sprague–Dawley rats with initial weights of 150–180 g were obtained from Charles River Laboratories and housed for 3–4 days on a 12 h light/dark cycle with free access to regular lab chow and water. Food was then removed for 72 h from the animals in the fasting group or for 2 h in the control group. Animals were then injected intraperitoneally with a single solution containing insulin (10 units/kg body weight) and D-glucose (1.5 g/kg body weight). At different times after the injection, the animals were decapitated, blood was collected, and whole livers or whole quadriceps muscles were quickly obtained, frozen with liquid N_2, and stored at −70 °C for up to 4 weeks until processed; 7–10 animals were studied per group for each value reported.

Blood analyses

Serum glucose was analysed with a glucose oxidase/peroxidase method using a kit provided by Sigma. Immunoreactive insulin was measured using a double-antibody radioimmunoassay [19].

Isolation of receptors

Tissue (1 g) was powdered frozen and homogenized (Polytron, 1 min) in 5 ml of a medium containing Hepes

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(50 mM, pH 7.4), NaCl (50 mM), NaF (100 mM), pyrophosphate (10 mM), EDTA (8 mM), vanadate (1 mM), benzamidine (15 mM), phenylmethanesulphonyl fluoride (1 mM), Triton X-100 (1%), leupeptin (2 μM), pepstatin (2 μM), aprotinin (1000 units/ml) and bacitracin (1 mg/ml). The homogenates were centrifuged at 100000 *g* for 90 min at 4 °C using a 50.2 Ti Beckman rotor and the supernatants were diluted 4-fold with a buffer containing Hepes, NaCl, NaF and pyrophosphate in the same concentrations noted for the homogenization buffer. The diluted supernatants were incubated overnight and mixed at 4 °C with 3 ml of wheat germ agglutinin–agarose gel. The gel was then transferred into disposable plastic columns, and these were washed with 10 bed volumes of a buffer containing Hepes (50 mM), NaCl (150 mM), Triton X-100 (0.1%) and phenylmethanesulphonyl fluoride (0.1 mM). Elution was accomplished with 0.3 M-*N*-acetylglucosamine prepared in the same washing buffer. Receptor fractions were immediately divided into aliquots and frozen at −70 °C until further analysis.

Binding studies

Binding of ^{125}I-insulin (100000 c.p.m.) to 50 μl of purified receptors was performed overnight at 4 °C in a total volume of 250 μl in the absence or presence of unlabelled insulin at different concentrations. Receptors were then precipitated with poly(ethylene glycol) and bovine γ-globulin and washed twice before counting, as described previously [21]. Specific binding was calculated after subtraction of the nonspecific value obtained in the presence of 1 μM unlabelled insulin.

Assay of tyrosine kinase

Tyrosine kinase was analysed as previously described [21] with minor modifications. Purified receptors (50 μl) were incubated for 30 min at room temperature in the presence of Hepes (50 mM, pH 7.4), $MgCl_2$ (10 mM), $MnCl_2$ (0.5 mM), [^{32}P]ATP (100 μM, 1.3 mCi/ml) and poly(Glu,Tyr) (4:1) (2.5 mg/ml) in a total volume of 150 μl in the presence or absence of insulin at different concentrations. At the end of the incubation period, 100 μl of mixture was taken and spotted onto a 2.3 cm Whatman 3M filter paper disc which was immediately dropped into 10% trichloroacetic acid containing 10 mM-sodium pyrophosphate. The discs were washed five times with trichloroacetic acid, then with ethanol, and finally counted for radioactivity. Reagent blanks were subtracted. Results were expressed as pmol of phosphotyrosine/fmol of insulin binding.

Statistics

Results are expressed as means ± S.E.M. Control and fasting groups were compared by Student's *t* test.

RESULTS

Blood glucose and insulin levels

As illustrated in Fig. 1(*a*), in non-injected rats (time zero) blood glucose levels were higher in the control (169 ± 8.0 mg/dl) than in the fasting group (106 ± 3.2 mg/dl, $P < 0.01$). After intraperitoneal treatment, glucose levels increased to approx. 300 mg/dl at 5 min in both groups and then decreased to about 160 mg/dl at 15 min and to about 75 mg/dl at 30 min, without significant differences between the two groups

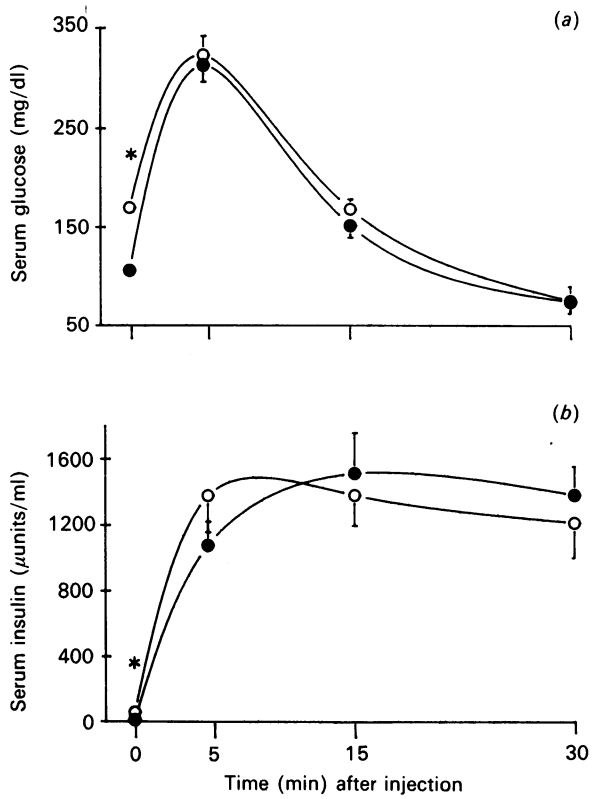

Fig. 1. Serum glucose (*a*) and insulin (*b*) levels

Rats were injected intraperitoneally with insulin (10 units/kg body weight) plus glucose (1.5 g/kg) after either 72 h (fasting, ●) or 2 h (control, ○) of fasting. Animals were decapitated at the time points indicated, and blood was collected and analysed. *Indicates statistical significance of difference between control and fasting (see text for *P* values).

compared. Insulin levels (Fig. 1*b*) at time zero were higher in the control (60 ± 4.5 μunits/ml) than in the fasting group (11.6 ± 1.6 μunits/ml, $P < 0.001$). After the injection, serum insulin rose to about 1400–1500 μunits/ml in both groups and remained at this high level from 5 to 30 min.

^{125}I-insulin binding

Specific ^{125}I-insulin binding to muscle receptors was 7.8 ± 0.3% in control and 9.2 ± 0.5% in fasting ($P < 0.01$). In liver receptors binding was 31 ± 1.1% in control and 46.6 ± 0.7 in fasting ($P < 0.001$). Nonspecific binding was consistently less than 6% of total binding. Scatchard analysis of muscle and liver receptors showed similar hormone binding kinetics for both groups of animals, with no difference in hormone–receptor affinity.

Muscle receptor tyrosine kinase

Basal muscle receptor tyrosine kinase in non-injected animals was 4.3 ± 0.40 pmol/fmol in control and 2.2 ± 0.6 pmol/fmol in fasting ($P < 0.05$). When insulin was added *in vitro* to these receptors (Fig. 2*a*), tyrosine kinase increased in a similar fashion in both the control and fasting groups, with similar dose–response relationships and maximal activities. In both groups of animals receptor tyrosine kinase activity increased after the injection of insulin *in vivo* (Fig. 2*b*), reaching a maximum

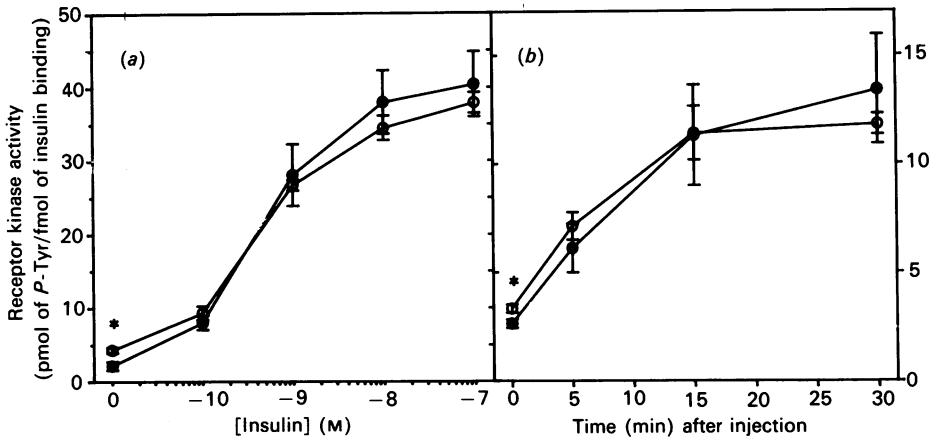

Fig. 2. Muscle receptor tyrosine kinase activity after insulin treatment *in vitro* (*a*) or *in vivo* (*b*)

Fasted (●) or control (○) rats were treated with insulin plus glucose as described in Fig. 1. Quadriceps muscles were rapidly frozen in liquid N_2, receptors obtained by wheat germ agglutinin–agarose affinity chromatography, and tyrosine kinase was assayed using poly(Glu,Tyr) (4:1) as the substrate. Insulin was added *in vitro* in (*a*) but not in (*b*).

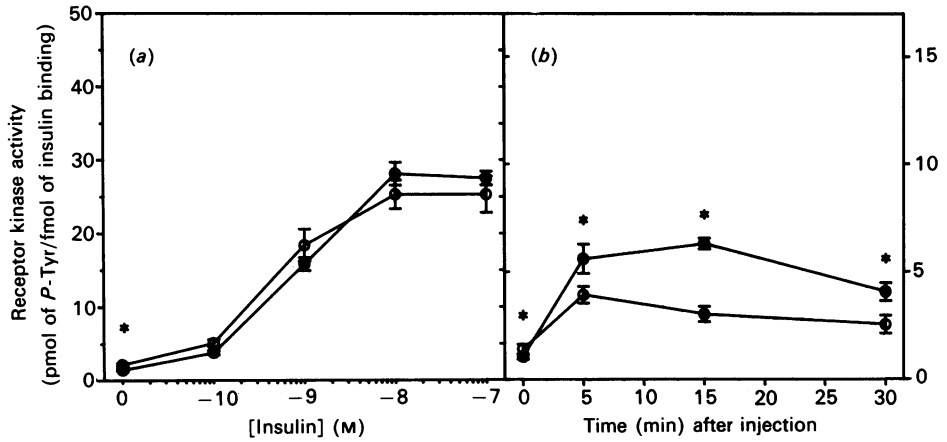

Fig. 3. Liver receptor tyrosine kinase activity after insulin treatment *in vitro* (*a*) or *in vivo* (*b*)

Liver receptor kinase activity was assayed in fasted (●) or control (○) rats as described in Fig. 2. Insulin was added *in vitro* in (*a*) but not in (*b*). *Indicates statistical significance of difference between control and fasting (see text for *P* values).

at 15 min that was maintained at 30 min, when the activities were 11.8 ± 0.50 pmol/fmol for control and 13.4 ± 2.5 pmol/fmol for fasting, with no statistical differences.

Liver receptor tyrosine kinase

Basal activity of liver receptor tyrosine kinase in non-injected animals was 2.16 ± 0.20 pmol/fmol in control and 1.43 ± 0.14 pmol/fmol in fasting ($P < 0.05$). When insulin was added *in vitro* to these receptors, tyrosine kinase activity increased in a similar fashion in the control and fasting groups (Fig. 3*a*) with similar dose–response relationships and maximal activities. In control animals treated with insulin *in vivo* (Fig. 3*b*) the liver enzyme was rapidly activated, reaching a maximal activity of 3.9 ± 0.40 pmol/fmol at 5 min after the injection, and then declining to 3.0 ± 0.36 pmol/fmol at 15 min and 2.6 ± 0.4 pmol/fmol at 30 min. In fasting animals, the treatment *in vivo* caused a significantly larger and more prolonged activation of the liver receptor kinase, with activities of 5.6 ± 0.7 pmol/fmol at 5 min ($P < 0.05$), 6.3 ± 0.26 pmol/fmol at 15 min ($P < 0.001$) and 4.1 ± 0.40 pmol/fmol at 30 min ($P < 0.05$). *P* values refer to differences between control and fasting.

DISCUSSION

Starvation causes a number of metabolic and endocrine changes that may be considered as an adaptative strategy aimed at increasing the chance of survival. After 48–72 h of fasting, insulin responses in fat [3–5] and liver [6–9] appear to decrease, whereas those of muscle appear to increase [1,2]. Thus, in the event of a transient absorption of food with insulin release, sugars and amino acids will probably be directed to muscle where they may be immediately required, while liver continues neoglucogenesis and glucose output to ensure its long-term availability. The molecular events responsible for these

changes in insulin responses during fasting remain largely unknown. We have assessed the response of muscle and liver receptor tyrosine kinase to insulin added *in vitro* as well as to insulin injected *in vivo* to the intact animals. Insulin was rapidly absorbed into the circulation after its intraperitoneal injection, as indicated by the high blood levels detected at the earliest post-injection time studied (5 min) and remained at this high concentration throughout the 30 min of the experiment. The addition of glucose to the insulin treatment prevented the development of hypoglycaemia, which would have triggered undesired compensatory changes in other hormonal systems. The deliberately caused hyperglycaemia should not have interfered with the action of insulin on its receptors, since glucose itself does not affect the activity of the kinase. Our results showed normal muscle receptor tyrosine kinase activation by treatment either *in vitro* or *in vivo* with insulin, thus providing no evidence for any changes in this enzymic activity with the changes in muscle insulin response that appear to occur during fasting. The fact that the basal activity of the receptor kinase in muscle was higher in control than in fasting animals presumably reflects the higher basal blood levels of insulin present in the control animals. Insulin binding by liver receptors was increased in fasting animals by 50% due to an increase in receptor numbers, in agreement with previous reports [8–10]. Our results with liver show normal activation of receptor tyrosine kinase by insulin added *in vitro*, thus differing from those of Freidenberg *et al.* [18], who reported a decrease in this enzymic activity in receptors isolated from liver plasma membranes obtained from 72 h fasted animals. Our discrepancy with this previous work may be related to the fact that we isolated receptors from whole tissue and not only from a plasma membrane fraction. Fasting enhances lysosomal activity in liver [20,21] and causes changes in the sedimentation properties of liver subcellular fractions [20,22], and these factors may have contributed to produce the abnormally low tyrosine kinase activity reported for fasting liver receptors in the previous work already cited. We used a more rapid, whole-tissue extraction of the receptors and added four different protease inhibitors and four phosphatase inhibitors during the extraction procedure, which may have resulted in a receptor preparation that better represents the status *in vivo* in the whole cells. These differences in procedures may partly explain the differences in results. On the other hand, the fact that in our study the activation *in vitro* of liver insulin receptor tyrosine kinase is normal, whereas the activation *in vivo* is abnormally high, suggests that the rate of dephosphorylation (and inactivation) of the liver receptor tyrosine kinase may be decreased in the liver during fasting. Dephosphorylation of the insulin receptor may occur after internalization [23,24] and one might speculate that perhaps a slowing of the process of internalization, dephosphorylation and recycling of the insulin receptor occurs in liver during fasting. This would limit the capacity of the cells to respond to insulin [25] while causing the buildup of internalized, non-deactivated receptors, giving rise to the puzzling paradox of having liver insulin resistance with a seemingly hyper-responsive receptor tyrosine kinase.

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