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Activation of liver and muscle insulin receptor tyrosine kinase activity during in vivo insulin administration in rats

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Summary. We have studied autophosphorylation and tyrosine kinase activity of the insulin receptor purified from liver and muscle of fasted rats before and after infusion of insulin (100 mU/h) during a 2.5 h glucose clamp. Recovery of insulin receptors and insulin binding to the solubilised receptors was unaffected by the glucose clamp. Autophosphorylation of the insulin receptor β subunit was increased in liver receptors prepared from rats at the end of the glucose clamp compared to rats in the basal state both in the absence of insulin in vitro (109% increase, $p < 0.001$) and after in vitro stimulation with 10^{-7} mol/l insulin (clamped vs fasted; 96% increase, $p < 0.001$). Insulin (10^{-7} mol/l) stimulated autophosphorylation was also increased in muscle receptor preparations from clamped rats compared with rats in the basal state (58% increase, $p < 0.05$). In both liver and muscle receptors, the clamp increased the amount of [^{32}P]-phosphate incorporated into the β subunit without changing the sensitivity of the insulin stimulation. HPLC analysis of the tryptic phosphopeptides derived from the β subunit after insulin stimulated autophosphorylation of liver receptors revealed an increase of ^{32}P in all phosphorylation sites without any change in the overall pattern. Tyrosine kinase activity of liver and muscle insulin receptors from clamped rats was also increased approximately twofold ($p < 0.05$) when analysed using a synthetic substrate (poly Glu_4Tyr_1). Our results support the notion that the insulin receptor exists in an active and inactive form, and that elevated plasma insulin concentrations increases the proportion of active receptors.

Key words: Hyperinsulinaemic glucose clamp, skeletal muscle, liver, insulin receptors, tyrosine kinase, insulin resistance, β-subunit C-terminus.

The β subunit of the insulin receptor is a tyrosine kinase [1, 2] which undergoes autophosphorylation immediately after the binding of insulin to the α subunit [3, 4]. Site-directed mutagenesis of the β subunit to alter the autophosphorylation cascade [5, 6], and introduction into intact cells of monoclonal antibodies which inhibit autophosphorylation [7], have revealed a close link between the tyrosine kinase activity of the receptor and insulin action. Thus, tyrosine kinase activity intrinsic to the insulin receptor may be important in some of the actions of insulin [8, 9].

The decrease in insulin receptor tyrosine kinase activity in adipocytes [10], liver [11] and skeletal muscle [12, 13] from patients with Type 2 (non-insulin-dependent) diabetes may be important for the development of insulin resistance or even diabetes itself. However, it remains possible that the defect in tyrosine kinase activity in insulin target tissues is acquired as similar abnormalities can be induced by streptozotocin diabetes in rats [14, 15] and by dietary manipulation [16, 17]. The biochemical mechanisms of these acquired defects are unknown but the hormonal milieu of the tissue prior to sampling could be important.

In contrast to the wealth of information on the regulation of the insulin receptor tyrosine kinase in purified insulin receptor preparations [1–4, 18–21] and in certain cultured cell lines [3–9, 21–23] few studies have addressed the physiological regulation of the insulin receptor tyrosine kinase in the intact animal. This study examines in conscious rats the effects of a physiological increase in plasma insulin concentrations on the tyrosine kinase activity of insulin receptors in the two main insulin target tissues, liver and skeletal muscle. In order to provide a defined insulin stimulus without changes in blood glucose and counter-regulatory hormone concentrations we have used the hyperinsulinaemic glucose clamp technique [24].

Materials and methods

Animals

Male Sprague Dawley rats (Charles River, Wilmington, Mass., USA) were maintained on laboratory chow ad libitum and weighed 300–350 g at the time of the study. Jugular and femoral venous cannulae were implanted under ether anaesthesia 24 h before the study. Following recovery from the anaesthetic rats were housed in individual cages with free access to water but not food.

Euglycaemic clamp studies

On the morning of the study a basal blood sample (200 μl) was taken for determination of blood glucose and plasma insulin concentrations. In six rats a 2.5 h infusion of neutral soluble insulin (Humulin R, Eli Lilly, Indianapolis, Ind., USA) diluted in Haemaccel (Hoechst, Frankfurt am Main, FRG) at a rate of 100 mU/h was started. This was infused through one limb of a double lumen cannula connected to the jugular venous cannula. Blood samples for glucose were taken at 5–10 min intervals from the femoral venous cannula and replaced with 0.15 mol/l sodium chloride in water. Blood glucose concentration was measured by the glucose oxidase method (Yellow Springs Glucose Analyser, Clandon Scientific, Ohio, USA) within 2 min of obtaining the sample. Blood glucose was maintained at 4.0 mmol/l by a variable infusion of 500 g/l glucose in water through the second limb of the double lumen cannula. Blood samples (200 μl) for insulin were taken at 60, 90 and 120 min and replaced with fresh washed rat erythrocytes in 0.15 mol/l sodium chloride in water. A second group of six control rats was infused with Haemaccel alone for 2.5 h. A similar schedule of blood sampling was used in these rats as in the insulin-infused rats. At the end of the study, control and glucose-clamped rats were anaesthetised by i. v. injection of amylobarbitone (0.02 g in 200 μl 0.15 mol/l sodium chloride in water) and liver and quadriceps muscle freeze-clamped, ground in liquid nitrogen and stored at −70 °C prior to solubilisation and purification of insulin receptors.

Insulin receptor solubilisation

Tissue (~1 g) was homogenised at 4 °C (Polytron Kinematica, Lucerne, Switzerland) in 7 ml of 50 mmol/l HEPES pH 7.4 containing 1% volume/volume (v/v) Triton X100, 100 mmol/l sodium fluoride, 10 mmol/l sodium pyrophosphate, 2 mmol/l sodium vanadate, 5 mmol/l EDTA, 2 mmol/l phenylmethylsulfonyl fluoride (PMSF) and 0.1 mg/ml aprotinin. The homogenate was stirred at 4 °C for 1 h and detergent insoluble material sedimented by centrifugation at 50,000 rev/min in a Beckman 70.1 Ti rotor for 60 min at 4 °C. The supernatant was passed four times over an agarose bound wheat germ agglutinin (WGA) column (Vector Laboratories, Burlingame, Calif., USA). The resin was washed with 200 ml (80 bed volumes) of 50 mmol/l HEPES pH 7.4 containing 0.1% Triton X100, 100 mmol/l sodium fluoride, 5 mmol/l EDTA, 10 mmol/l sodium pyrophosphate and 2 mmol/l sodium vanadate. Immediately before elution, the agarose was washed with 20 ml 50 mmol/l HEPES pH 7.4 containing 0.1% v/v Triton X100 and 4 mmol/l EDTA. The glycoproteins were eluted with 5 ml of this solution containing 300 mmol/l N-acetylglucosamine (Sigma, St Louis, Mo., USA). Eluates were divided into 100–200 μl aliquots and stored at −70 °C. The protein concentration was determined using the Bio Rad protein assay [25].

Binding of ^{125}I-insulin to solubilised insulin receptors

Aliquots of WGA eluate were incubated at 4 °C for 16 h with A14-^{125}I-insulin (specific activity, 2000 Ci/mmol), and a range of concentrations of unlabelled human insulin (Eli Lilly) in 200 μl of 50 mmol/l HEPES buffer pH 7.4 containing 0.1% (v/v) Triton X100, 150 mmol/l sodium chloride and 1 g/l bovine serum albumin. Insulin receptor complexes were precipitated in the presence of 1 g/l human gamma globulin with 125 g/l polyethylene glycol (final concentration) at 4 °C [26]. Insulin binding was corrected for non-specific binding determined in the presence of 10^{-5} mol/l insulin. In all experiments comparing tissue from clamped and control rats equal binding activity was used in the assays.

Receptor autophosphorylation

WGA-purified insulin receptor from liver (5 μg protein) and muscle (10 μg protein) were incubated at 22 °C for 30 min in 45 μl of 10 mmol/l HEPES pH 7.4 containing 10 mmol/l manganese chloride, 150 mmol/l sodium chloride, 1 g/l bovine serum albumin in the absence or presence of insulin concentrations ranging from 3×10^{-10} to 1×10^{-7} mol/l. Phosphorylation was initiated by adding 60 μCi γ-^{32}P-ATP to give a final ATP concentration in the assay of 25 μmol/l. The phosphorylation reaction was terminated by adding 500 μl of 50 mmol/l HEPES pH 7.4 containing 0.1% (v/v) Triton X100, 100 mmol/l sodium fluoride, 10 mmol/l sodium pyrophosphate, 5 mmol/l EDTA and 2 mmol/l sodium vanadate at 4 °C.

The phosphorylated insulin receptor was immunoprecipitated from each reaction with antiphosphotyrosine antibody prepared as previously described [27]. Purified IgG (3 μg) was added to the phosphorylated insulin receptor preparations and incubated at 4 °C for 16 h. The antibody was immobilised on Pansorbin (10% v/v, 100 μl) and the precipitate washed sequentially with 50 mmol/l HEPES pH 7.4 containing 1% (v/v) Triton X100 and 1 g/l sodium dodecyl sulfate (SDS) and then with 50 mmol/l HEPES pH 7.4 containing 0.1% (v/v) Triton X100 and 1 g/l SDS. The phosphoproteins were eluted from the Pansorbin with Laemmli sample buffer containing 100 mmol/l dithiothreitol, separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 7.5% resolving gels, and identified by autoradiography of Coomassie blue-stained, dried gels at −70 °C with Kodak x-Omat AR film using a Cronex lightning plus enhancing screen. Radioactivity in gel fragments was measured by Cerenkov counting.

HPLC separation of tryptic phosphopeptides

The phosphorylated insulin receptor β subunit resolved by PAGE was digested with TPCK (N-tosylphenylalanyl chloromethyl ketone)-treated trypsin (Worthington Biochem Corp, Freehold, New Jersey, USA) and the phosphopeptides eluted from gel fragments as previously described [28]. The phosphopeptides which eluted from the gel fragment (about 80%) were separated with a Waters HPLC system equipped with a widepore C18 reverse-phase column (RP318, Bio-Rad, Richmond, Calif., USA). The phosphopeptides were applied to the column in 0.05% (v/v) trifluoroacetic acid and the column was eluted by a gradient of acetonitrile increasing linearly from 5% to 25% over 80 min. Fractions (1 ml) were collected at 1 min intervals and the radioactivity measured with 40% efficiency as Cerenkov radiation using a Beckman scintillation counter. Greater than 95% of the radioactivity in the trypsin digest was routinely recovered from the reverse phase HPLC column.

Tryptic removal of the β-subunit C-terminus

To examine the influence of the C-terminal domain of the insulin receptor β subunit on the tyrosine kinase activities of receptors from fasted and clamped rats, autophosphorylation was also measured after removal of this domain by mild tryptic digestion as previously described [28]. Briefly, wheat germ purified receptor preparations were incubated with TPCK-treated trypsin (5 μg/ml) for 1 min at 22 °C and digestion stopped by the addition of aprotinin to a final concentration of 10 μg/ml. Autophosphorylation was then measured as described above.

Phosphorylation of an exogenous substrate

WGA-purified receptor preparations (5–10 μg protein) were preincubated for 30 min at 22 °C in 10 mmol/l HEPES pH 7.4 containing 0.05 mmol/l ATP, 5 mmol/l manganese chloride, 10 mmol/l magnesium acetate and 10^{-7} mol/l insulin. The reaction (final volume 50 μl) was initiated by the addition of substrate (copolymer of Glu/Tyr, 4:1, 1 mg/ml, Sigma) and 3 μCi of γ-^{32}P-ATP. After 15 min the reaction was stopped by adding 20 μl of 40 mmol/l HEPES pH 7.4 containing 20 mmol/l EDTA, 200 mmol/l sodium fluoride, 40 mmol/l sodium pyrophosphate, 40 mmol/l sodium phosphate, 40 mmol/l ATP and 0.4% (v/v) Triton X100. Aliquots were then ap-

plied to filter paper squares and washed in 100 g/l trichloroacetic acid containing 1 mmol/l sodium pyrophosphate. Papers were washed five times over 6 h, dried and counted using Cerenkov counting. Non-specific binding of γ-^{32}P-ATP to the papers (measured in the absence of poly Glu_4Tyr_1) accounted for 2–8% of radioactivity bound and was subtracted from measured values to give specific binding. All assays were performed in duplicate.

Other analyses

Plasma insulin was measured by RIA [29] using a human or rat standard as appropriate.

Statistical analysis

Results are presented as mean ± SEM. Significant differences between the groups were assessed by Student's paired or unpaired *t*-test as appropriate.

Results

Blood glucose and plasma insulin concentrations

Blood glucose concentrations in 24 h fasted rats before the glucose clamp were 3.8 ± 0.2 mmol/l. This was associated with a fasting plasma insulin concentration of 1.09 ± 0.12 μg/l. During the last 30 min of the 2.5 h clamp, blood glucose concentration was 4.0 ± 0.0 (SD) mmol/l. The coefficient of variation of blood glucose for this period measured for each animal was 2.5 ± 1.0 (SD)%. Steady state plasma insulin concentrations measured over the last 60 min of the clamp were 142 ± 9 mU/l (0.85 ± 0.05 nmol/l, human assay). The glucose infusion rate required to maintain the clamp calculated for the period + 120 to 150 min was 168 ± 8 $\mu mol \cdot min^{-1} \cdot kg^{-1}$.

Insulin binding

Recoveries of WGA-purified glycoprotein (liver: – basal, 4.23 ± 0.27, clamp, 4.58 ± 0.22 mg/g; muscle: – basal, 1.71 ± 0.25, clamp 1.72 ± 0.15 mg/g) and of WGA-purified insulin receptors (liver: – basal, 71 ± 4, clamp 67 ± 3%; muscle: – basal, 71 ± 3, clamp 69 ± 3%) were not affected by the glucose clamp. Equilibrium binding of ^{125}I-insulin to partially purified insulin receptors extracted from liver and quadriceps skeletal muscle was identical in basal and clamped rats (Fig. 1). Apparent affinity of receptors for insulin was estimated from the insulin concentrations necessary for half maximal displacement of tracer. This was not affected by the glucose clamp in either skeletal muscle (fasted, 1.29 ± 0.12 nmol/l, clamp, 1.33 ± 0.15 nmol/l) or liver insulin receptor preparations (fasted, 0.75 ± 0.15, clamp, 0.56 ± 0.06 nmol/l) (Fig. 1).

Table 1. Effect of removal of the β subunit C-terminus on in vitro autophosphorylation of liver and skeletal muscle insulin receptors prepared from rats after a 24 h fast and at the end of a hyperinsulinaemic glucose clamp

	In vitro insulin (10^{-7} mol/l)	β subunit ^{32}P incorporation (cpm)	
		Intact receptors	Truncated receptors
Liver			
Fasting	–	728 ± 98	155 ± 39[d]
	+	5661 ± 340	4124 ± 218[c]
Clamp	–	1578 ± 146[b]	373 ± 159[d]
	+	12023 ± 790[b]	8178 ± 561[b,c]
Muscle			
Fasting	–	47 ± 4	46 ± 5
	+	1201 ± 106	823 ± 79[d]
Clamp	–	56 ± 7	47 ± 5
	+	2202 ± 294[a]	1442 ± 166[a,c]

Mean ± SEM ($n = 6$ in each group) [a,b] $p < 0.01$, $p < 0.001$ compared with receptors from fasted rats. [c,d] $p < 0.01$, $p < 0.001$ compared with receptors with an intact β subunit

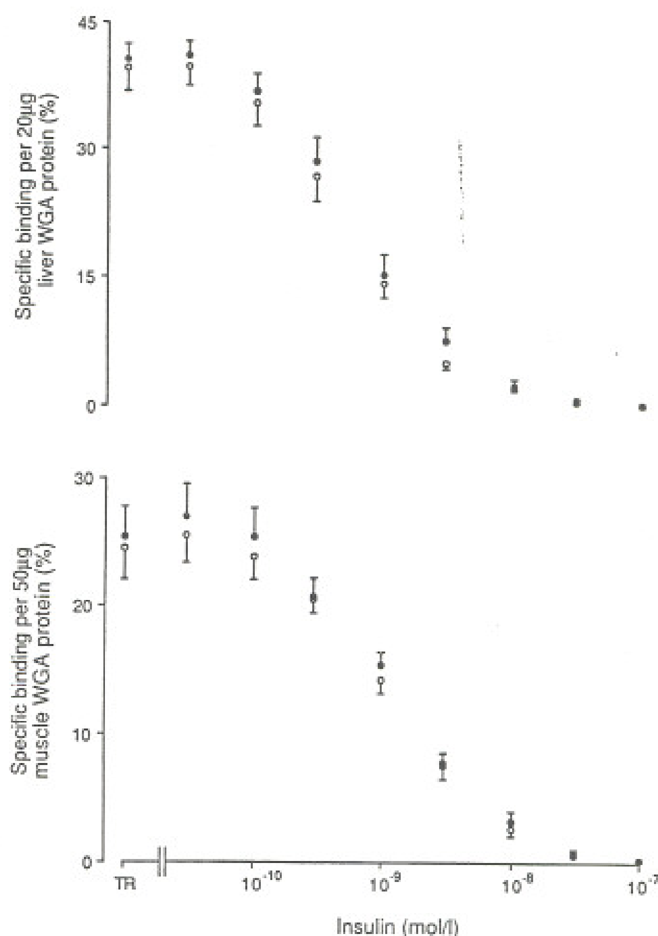

Fig. 1. Specific insulin binding to liver (top) and muscle (bottom) partially purified insulin receptors prepared from rats fasted for 24 h (●) and at the end of a hyperinsulinaemic glucose clamp (○). $n = 6$ in each group. The wheat germ agglutinin (WGA) eluate (muscle, 50 μg protein; liver, 20 μg protein) was incubated with A-14-^{125}I-insulin (~15000 cpm) in 200 μl buffer in the absence (TR) or presence of unlabelled insulin in increasing concentrations. Hormone receptor complexes were precipitated with polyethylene glycol. Non-specific binding obtained in the presence of 10^{-5} mol/l insulin was subtracted from total binding

Insulin stimulated autophosphorylation

Insulin stimulated tyrosyl phosphorylation of a 95 kilodalton (kD) protein in WGA-purified extracts from rat liver

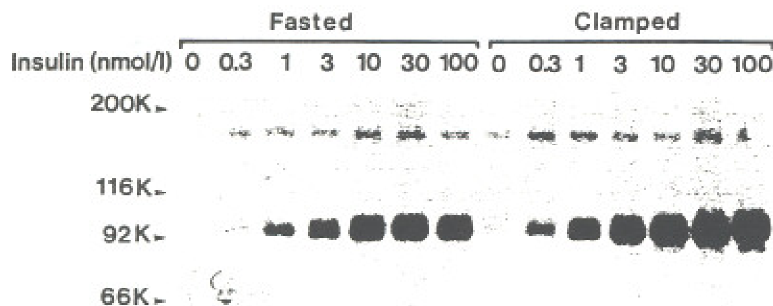

Fig. 2. Insulin dose dependence of autophosphorylation of insulin receptors partially purified from skeletal muscle of a rat fasted for 24 h and a rat at the end of a hyperinsulinaemic glucose clamp. Muscle wheat germ agglutinin-purified insulin receptors (10 µg protein) were incubated at 22 °C for 30 min in the absence or presence of increasing insulin concentrations. Phosphorylation was initiated by adding γ-^{32}P-ATP (25 µmol/l) and after 10 min at 22 °C the phosphorylated receptors were immunoprecipitated with antiphosphotyrosine antibody at 4 °C. Phosphoproteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to autoradiography. K = Kilodalton

and skeletal muscle. Based on its M_r and immunoreactivity with antibody [28] directed against the C-terminus of the insulin receptor β-subunit this phosphoprotein is the β subunit of the insulin receptor. Steady state phosphorylation of the β subunit was attained by 5 min of incubation with γ-^{32}P-ATP (25 µmol/l), manganese chloride (5 µmol/l) and insulin at 22 °C (data not shown). Increasing concentrations of insulin resulted in a dose-dependent stimulation of phosphorylation of the β subunit (Fig. 2).

Intravenous infusion of insulin for 2.5 h in 24 h fasted rats, with maintenance of blood glucose at 4.0 mmol/l, resulted in an activation of the insulin receptor in both liver and skeletal muscle (Fig. 3). Thus, in wheat germ purified liver or muscle receptors from clamped rats, autophosphorylation of the β subunit was increased approximately two-fold and 1.5-fold, respectively compared to receptors prepared from rats in the basal state. In both liver and muscle this stimulation was observed over the whole range of insulin concentrations tested (Fig. 3). In liver, stimulation was also seen in the absence of insulin in vitro (Fig. 3).

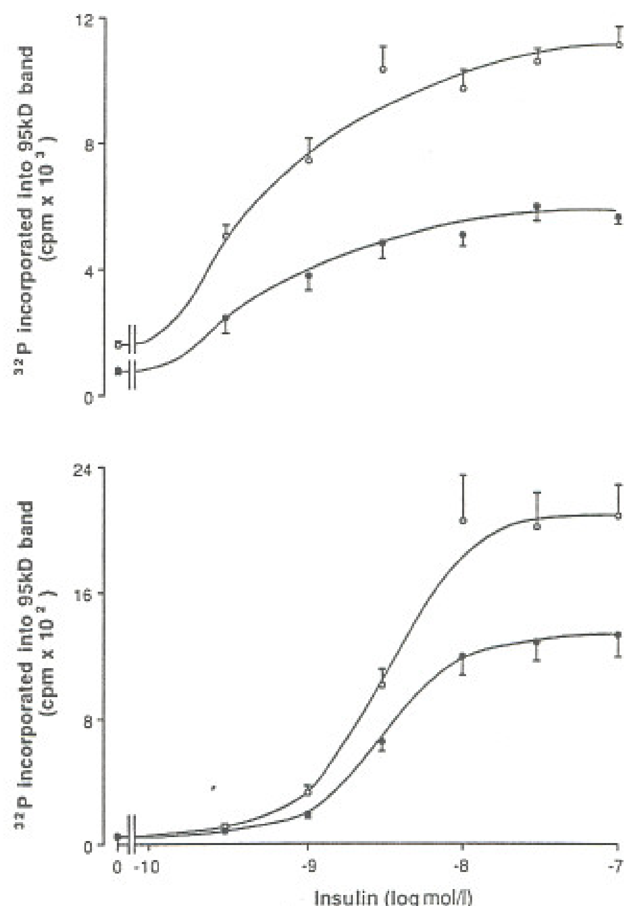

Fig. 3. In vitro autophosphorylation of wheat germ agglutinin (WGA)-purified insulin receptors from liver (top) and muscle (bottom). Receptors were prepared from rats fasted for 24 h (●) and from rats at the end of a hyperinsulinaemic glucose clamp (○). WGA-purified receptors from liver (5 µg protein) and muscle (10 µg protein) were incubated with γ-^{32}P-ATP and varying insulin concentrations at 22 °C as described in Figure 2. Phosphorylated receptors were immunoprecipated with antiphosphotyrosine antibody, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to autoradiography. ^{32}P incorporation into the 95 Kilodalton (kD) subunit of the insulin receptor was determined by Cerenkov counting of excised gel bands. $n = 6$ in each group

Peptide mapping of the β-subunit of the insulin receptor

To assess whether the stimulation of insulin receptor autophosphorylation by hyperinsulinaemia was associated with qualitative differences in the sites of phosphate incorporation into the β subunit during the in vitro assay, the HPLC profiles of tryptic phosphopeptides obtained from the autophosphorylated β subunits from basal and clamped rats were compared. In receptor preparations from both basal and clamped rats, 6 phosphopeptide peaks were resolved (Fig. 4) which have previously been shown to contain phosphotyrosine [18]. Peaks PY1 and PY1a represent triphosphorylated forms of the regulatory domain containing Tyr(P) 1146, Tyr(P) 1150 and Tyr(P) 1151 [18, 19]. Peaks PY2 and PY3 are derived from the C-terminus of the β-subunit and contain Tyr(P) 1316 and Tyr(P) 1322 [18, 19]. Peaks designated PY4 and PY5 are thought to represent diphosphorylated forms of the regulatory region [18], containing Tyr(P) 1146 and either Tyr(P) 1150 or Tyr(P) 1151. The glucose clamp increased the amount of radioactivity associated with each of these peaks without any consistent change in the overall pattern (Fig. 4). Due to low specific activity, it was not possible to obtain comparable profiles of tryptic phosphopeptides from muscle insulin receptors in this study.

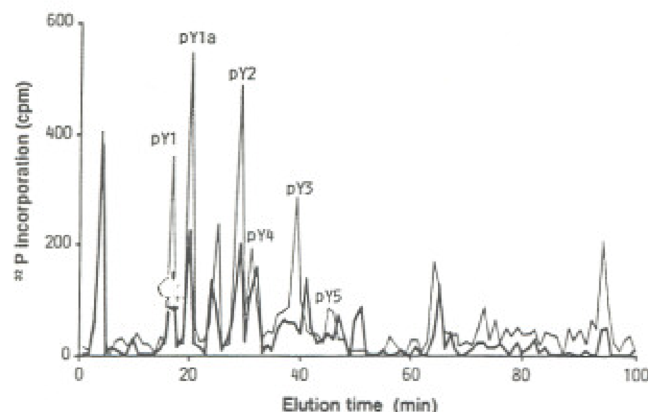

Fig. 4. Reverse phase HPLC profiles of liver insulin receptor β subunit tryptic phosphopeptides after in vitro autophosphorylation for 10 min at 22°C in the presence of 100 nmol/l insulin. For experimental details see legend to Figure 2. The thick line represents the profile for a single fasted rat, the fine line is derived from a clamped rat. Peaks pY1 and pY1a: triphosphorylated peptides containing Tyr (P) 1146, Tyr (P) 1150, Tyr (P) 1151; peaks pY2 and pY3: derived from the C-terminus and contain Tyr (P) 1316 and Tyr (P) 1322; peaks pY4 and pY5: diphosphorylated peptides containing Tyr (P) 1146 and Tyr (P) 1150 or Tyr (P) 1151

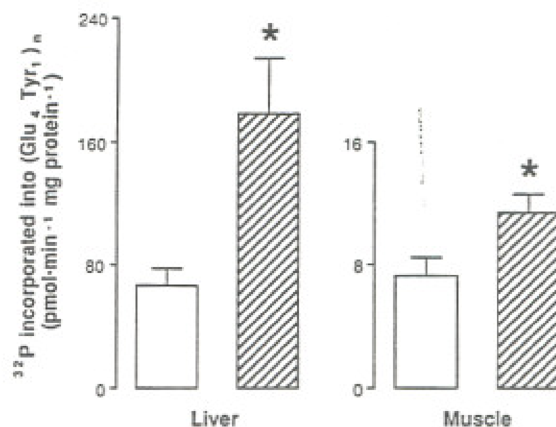

Fig. 6. Tyrosine kinase activity of insulin receptors from liver and skeletal muscle towards an exogenous substrate (poly Glu$_4$ Tyr$_1$). Receptors were prepared from rats fasted for 24 h (□) and from rats at the end of a hyperinsulinaemic glucose clamp (▨). Wheat germ agglutinin-purified receptors (liver. 5 μg protein; muscle, 10 μg protein) were preincubated for 30 min at 22°C with 50 μmol/l ATP and 100 nmol/l insulin. The reaction was initiated by adding 3 μCi γ-^{32}P-ATP and poly Glu$_4$ Tyr$_1$ (final concentration 1 mg/ml). After 15 min the reaction was stopped as described in Materials and methods and incorporation into poly Glu$_4$ Tyr$_1$, determined by Cerenkov counting. Mean ± SEM, $n = 6$ in each group. * $p < 0.05$ compared with fasted rats

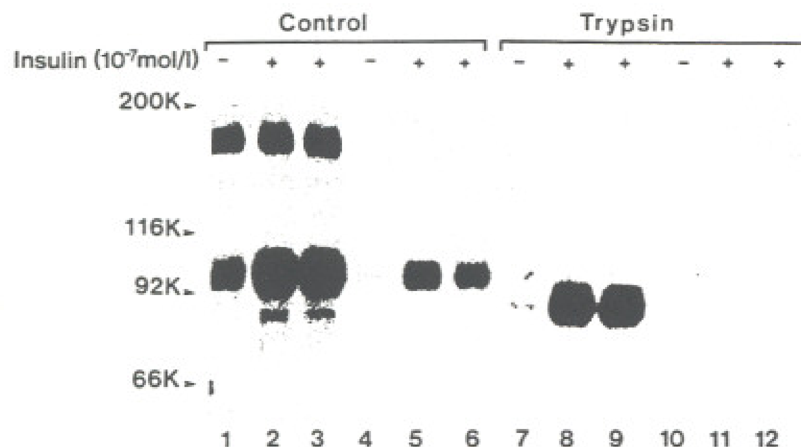

Fig. 5. Autoradiogram showing the effect of mild trypsin digestion (5 μg/ml, 1 min) on the 95 kilodalton (K) β subunit. Control and trypsin treated receptors were allowed to autophosphorylate (10 min at 22°C) in the absence or presence of insulin (100 nmol/l). The autophosphorylated receptors were then immunoprecipitated with either antiphosphotyrosine antibody (lanes 1–3 and 7–9) or with antibody against the C-terminus of the β subunit (lanes 4–6 and 10–12). After reduction samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography as described in Materials and methods.

Effect of removal of the β-subunit C-terminus

Incubation of WGA-purified receptors for 1 min at 22°C with 5 μg/ml trypsin converted the 95 kD β subunit to an 85 kD fragment which was recognised by the antiphosphotyrosine antibody but not by the antibody directed against the C terminus (Fig. 5).

Incorporation of ^{32}P into liver 85 kD β subunits measured in the absence of insulin in vitro was markedly decreased compared with native β subunits (Table 1). This difference was also seen during insulin-stimulated autophosphorylation and was most likely due to removal of C-terminal autophosphorylation sites (Table 1). The 30% decrease was, proportionate in preparations from basal and clamped rats. Thus, the difference in insulin stimulated receptor autophosphorylation between fasted and clamped rats was preserved despite removal of the β-subunit C-terminal region.

Incorporation of ^{32}P into muscle 95 kD β subunits was very low when measured in the absence of insulin and it was not possible to demonstrate any effect of C-terminal removal on this low basal level of autophosphorylation. Removal of the β-subunit C-terminus from muscle receptors resulted in a similar decrease in insulin stimulated ^{32}P incorporation into the 85 kD fragment to that seen in liver (Table 1). Removal of this domain did not change the differences between basal and clamped rats (Table 1).

Phosphotransferase activity of the insulin receptor from basal and clamped rats

To measure full activation of the phosphotransferase activity of the receptor, partially purified receptor preparations were allowed to reach a steady state level of autophosphorylation by preincubation for 30 min with insulin

(10^{-7} mol/l) and unlabelled ATP prior to addition of the synthetic substrate and γ-^{32}P-ATP. Under these conditions ^{32}P incorporation into the copolymer of Glu_4Tyr_1 was linear for 20 min for both muscle and liver insulin receptor preparations. Both liver and skeletal muscle insulin receptor preparations from clamped rats showed increased phosphotransferase activity compared with receptors from rats in the basal state (Fig. 6). Thus, the clamp increased the incorporation of ^{32}P into the copolymer $Glu_4 \cdot Tyr_1$ by 166% in liver ($p < 0.05$) and by 56% in muscle ($p < 0.05$).

Discussion

Decreased insulin receptor tyrosine kinase activity observed in a number of insulin resistant states [10–15, 17, 30] suggests an important role of the kinase in modulating insulin action and a mechanism for insulin resistance. However, insulin resistance, for example, in Type 2 diabetes and obesity is associated with abnormalities of insulin secretion which might be expected to result in altered receptor kinase activity. Caution should thus be exercised in attributing tissue resistance to the decreased receptor kinase activity.

In this study we have investigated the effects on the tyrosine kinase of short-term exposure to a sustained elevation in plasma insulin concentrations as might be expected to occur in relation to meals. Infusion of insulin into 24 h fasted rats, without change in blood glucose concentration, increased autophosphorylation and kinase activity of the insulin receptor purified from liver and skeletal muscle.

It is unlikely that this activation of the insulin receptor tyrosine kinase during the glucose clamp is simply due to continued association of infused insulin with the receptor preparation. Previous studies using ^{125}I-insulin have shown that negligible amounts of insulin can be detected following solubilisation and WGA agarose chromatography [31, 32]. This is supported by the finding that ^{125}I-insulin binding is identical in glycoprotein fractions from insulin infused and fasted rats. Furthermore, the persistence of the difference in tyrosine kinase activity in the in vitro assay between receptors from fasted and clamped rats at maximally stimulating insulin concentrations (approximately 100-fold those attained in vivo) is inconsistent with the above hypothesis.

Enhanced autophosphorylation and tyrosine kinase activity in the in vitro assay in receptors from insulin infused rats cannot be explained by increased in vivo phosphorylation on tyrosines within the regulatory region of the β-subunit as less than 10% of the receptors are expected to be phosphorylated at 1 nmol/l insulin [22]. Moreover, any such increase would be manifest by an apparent decrease in autophosphorylation in the in vitro assay since some of the phosphorylation sites would be occupied by unlabelled phosphate.

In rat hepatocytes [33] and Fao hepatoma cells [34], a portion of occupied insulin receptors are phosphorylated not on tyrosine but on serine residues. These results suggest that phosphorylation of the insulin receptor on serine residues may regulate the tyrosyl kinase in vivo [35]. In our experiments, increased receptor autophosphorylation and kinase activity, and normal but elevated tryptic phosphopeptide maps suggest that the population of insulin receptors capable of autophosphorylation increased during in vivo exposure to elevated insulin concentrations. It is possible that the level of serine phosphorylation is enhanced during fasting but decreases following infusion of insulin with a concomitant increase in the proportion of receptors capable of autophosphorylation on tyrosine residues. Preliminary results suggest that a serine and threonine phosphorylation site may be located in the C-terminal region of the β subunit raising the possibility that this domain exerts a regulatory role on the insulin receptor tyrosine kinase. However, autophosphorylation of liver and skeletal muscle receptors from clamped rats was enhanced despite removal of a 10 kD fragment from the β subunit C-terminus (Fig. 6). Thus, the C-terminus does not play a regulatory role under our experimental conditions. If the differences in receptor autophosphorylation and kinase activity between fasted and clamped rats are indeed due to alterations in serine phosphorylation it would appear that the important site is not located in the C-terminus of the β subunit.

The measurements of autophosphorylation and kinase activity were carried out in partially purified glycoprotein mixtures which contain the insulin receptor and phosphoprotein phosphatases. It is possible that the increased activity of the insulin receptor in tissues from insulin infused rats is due to inhibition of a phosphotyrosine phosphatase or to the activation of a phosphoserine phosphatase. Phosphatase activity in our studies was not specifically evaluated and thus, it is possible that the increased tyrosine phosphorylation which we report is not due to an activated insulin receptor.

The plasma insulin concentrations attained during the clamp (142 ± 9 mU/l) are similar to peak levels after an oral glucose load [36] and thus, one might expect a similar activation of the receptor tyrosine kinase activity to occur in the fed state. Indeed activation of the tyrosine kinase activity of liver insulin receptors has been found after carbohydrate feeding of rats whilst a 72 h fast was associated with decreased enzyme activity [16]. The demonstration that the prevailing plasma insulin concentration results in stable changes in the insulin receptor tyrosine kinase might also account for the decreased kinase activity observed in the streptozotocin diabetic rat [14, 15], a model characterised by insulin deficiency.

In summary, our studies suggest that the prevailing insulin concentration in vivo affects the tyrosine kinase activity of liver and muscle insulin receptors measured in vitro. The regulatory mechanism involved is unknown. It would seem prudent that comparisons of receptor tyrosine kinase activity in tissues from insulin resistant and normal subjects are made after similar in vivo exposure of tissues to insulin.

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