Insulin receptor dephosphorylation by phosphotyrosine phosphatases obtained from insulin-resistant obese mice

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Summary To study the possible involvement of phosphotyrosine phosphatases in insulin resistance, the ability of cytosolic and membrane preparations to dephosphorylate insulin receptors was examined in lean and goldthioglucose-treated insulin-resistant and obese mice. Preparations were obtained from liver, heart, diaphragm and hindleg muscle and their phosphotyrosine phosphatase activities were measured using an immunoenzymatic assay with phosphorylated insulin receptors as substrate. Liver cytosolic and particulate phosphotyrosine phosphatases were more potent than preparations from other tissues and were able to almost completely dephosphorylate the insulin receptor in a dose- and time-dependent manner. No change was observed in cytosolic and membrane-associated phosphotyrosine phosphatases in liver, diaphragm, and heart of obese mice compared with lean mice. In contrast, cytosolic, but not membrane-associated, phosphotyrosine phosphatase activity was decreased in hindleg muscles of obese mice. These results suggest that the regulation of phosphotyrosine phosphatases is tissue-specific. In addition, alterations in total phosphotyrosine phosphatase activity do not appear to play an important role in insulin resistance in all tissues of obese mice, although specific changes cannot be excluded. [Diabetologia (1994) 37: 56–60]

Key words Insulin resistance, experimental obesity, phosphotyrosine phosphatase, muscle, liver.

Insulin resistance linked to obesity results from a series of alterations at the receptor level and at post-receptor steps of insulin action. One of those defects is the decreased receptor kinase activity which occurs in muscle, which has been substantiated in animal models [1] and in human syndromes [2]. The intrinsic tyrosine kinase activity of the receptor depends on its state of phosphorylation resulting from a balance between tyrosine phosphorylation and dephosphorylation. The function of protein tyrosine phosphatases (PTP-ases) is thought to counterbalance the action of protein tyrosine kinases allowing the regulation of the phosphotyrosine content of proteins involved in signal transduc-

tion, such as the insulin receptor [3]. This view is supported by the observation that microinjection of PTP-ases into oocytes inhibits some of the actions of insulin [4]. PTP-ases have been purified and cloned from a variety of tissues and cell lines [5–9]. Two main classes of PTP-ases have been identified thus far: the first includes enzymes with a receptor-like structure, which are associated to membranes; the second consists of soluble enzymes [10, 11]. Since PTP-ases counteract tyrosine kinase activities, the defect in insulin receptor tyrosine kinase activity found in insulin resistance linked to obesity could result from alterations in the PTP-ase activity. To determine a possible involvement of PTP-ases in the obese hyperinsulinaemic syndrome, we prepared particulate and cytosolic PTP-ases from different insulin-responsive tissues of lean and obese mice and tested their ability to dephosphorylate the insulin receptor.

Table 1. Characteristics of the animals

	Lean mice	Obese mice
Weight (g)	45.6 ± 0.8	66.5 ± 1.4
Plasma glucose (mmol/l)	11.1 ± 0.3	13.3 ± 0.4
Plasma insulin (nmol/l)	0.28 ± 0.03	2.27 ± 0.48
Tissue wet weight (g)		
Liver	2.12 ± 0.16	3.54 ± 0.30
Diaphragm	0.102 ± 0.010	0.132 ± 0.005
Heart	0.214 ± 0.008	0.252 ± 0.008

Fed lean and obese mice were used at 20–30 weeks of age. Blood samples were used to measure glucose and insulin levels. Thereafter, liver, diaphragm, heart and hindleg muscles were removed for cytosolic and membrane preparations, as described in Materials and methods.
Values are presented as mean ± SEM of 8–10 animals.
$p < 0.01$ or less for all comparisons lean vs obese mice

Materials and methods

Materials

HEPES, ATP, Triton X-100, N-acetyl-D-glucosamine, dithiothreitol, protease inhibitors were from Sigma (St Louis, Mo., USA). The Bradford protein assay was from Bio-Rad Laboratories (Richmond, Calif., USA). All other reagents were of the highest purity available. Sheep antiphosphotyrosine antibody was produced in our laboratory and γ-globulins (4 µg protein/ml) purified by affinity chromatography were used in all experiments. Peroxidase-conjugated rabbit antisheep antibody was purchased from Dako (Glostrup, Denmark). Plastic ware for the immunoassay was from Nunc (Copenhagen, Denmark).

Animals

Male Swiss albino mice were provided laboratory chow (Usine d'Alimentation Rationelle, Villemoisson, Epinay/Orge, France) ad libitum, and maintained at 22 °C on a 12-h light/dark cycle until the time of death. Mice were rendered obese by goldthioglucose injection at 3 weeks of age and were used at 6 months of age, when obesity had reached a plateau [12, 13]. Mice were anaesthetized with pentobarbitone sodium (50 mg/kg i.p.), and blood samples were withdrawn from the inferior vena cava. Plasma samples were used for the determination of glycaemia and insulinaemia as previously described [12, 13]. Animals were then killed by cervical dislocation, and tissues (liver, diaphragm, heart, and hindleg muscles) were rapidly dissected for membrane and cytosolic preparations.

Membrane-associated and cytosolic phosphatase preparations

Membrane-associated and cytosolic phosphatase preparations were obtained as previously described [14, 15]. Tissues were homogeneized in 250 mmol/l sucrose, 25 mmol/l Hepes, 15 mmol/l β-mercaptoethanol, 1 mmol/l phenylmethylsulphonylfluoride and 100 U/ml Trasylol, pH 7.2. Following the first centrifugation (600 g, 20 min) to eliminate the fat layer, homogenates were centrifuged at 12,000 g for 20 min. NaCl (0.1 mol/l) and MgCl$_2$ (0.2 mmol/l) were added to the supernatants which were centrifuged at 100,000 g for 30 min. The supernatants obtained after

this last step were considered to be the cytosolic fractions. The remaining pellets, corresponding to microsomal fractions, were solubilized with 1 % Triton X-100 in 25 mmol/l Hepes buffer and centrifuged at 100,000 g for 30 min. The final supernatants were used to measure membrane-associated phosphatase activity. Protein content of the preparations was determined by the Bio-Rad assay with bovine serum albumin as standard.

Measurement of tyrosine phosphatase activity by immunoenzymatic assay

The measurements of PTP-ase activity were performed using an immunoenzymatic method with phosphorylated insulin receptors as substrate [14]. Briefly, human insulin receptors, partially purified from a cell line transfected with insulin receptor cDNA and expressing 10^6 receptors per cell [16], were autophosphorylated and coated in a microtitre plate (250 ng protein/well). Dephosphorylation was initiated in the plate by the addition of extracts containing phosphatases. Following incubation (30 min at 22 °C) and washes, antiphosphotyrosine and peroxidase-conjugated rabbit anti-sheep antibodies were successively added [14]. Finally, orthophenylenediamine dihydrochloride was used as a chromogene, and optical density (OD) readings were performed at 405 nm on an ELISA minireader (Dynatec MR700; Saint Cloud, France). PTP-ase activity was expressed as a percentage of receptor dephosphorylation, based on the OD ratios, where 0 % corresponds to receptor incubation without PTP-ase.

Statistical analysis

Comparisons were performed using Student's t-test.

Results

Following goldthioglucose injection, mice were markedly obese, hyperinsulinaemic and moderately hyperglycaemic (Table 1) and were thus insulin-resistant. Obesity was accompanied by hepatomegaly, and increase in the weight of the heart and diaphragm muscles. We first assessed the particulate and cytosolic PTP-ase activity of liver from lean and obese mice [14, 17]. Insulin receptor dephosphorylation by hepatic microsomal fraction was dependent upon the incubation time and protein concentration (Fig. 1) both in lean and obese preparations. Particulate fractions were more potent than cytosolic preparations. There was no difference in hepatic particulate and cytosolic activities between lean and obese mice, at any protein concentration or incubation time.

The following muscle preparations were studied: heart, diaphragm, and hindleg muscles. All muscle preparations were able to dephosphorylate the insulin receptor, but to different extents (Table 2). Muscle preparations were less potent than liver extracts. In all tissues the PTP-ase activity was higher in microsomal than in cytosolic preparations. In particulate fractions from muscles (hindleg, heart and diaphragm), no significant difference was found in PTP-ase activities be-
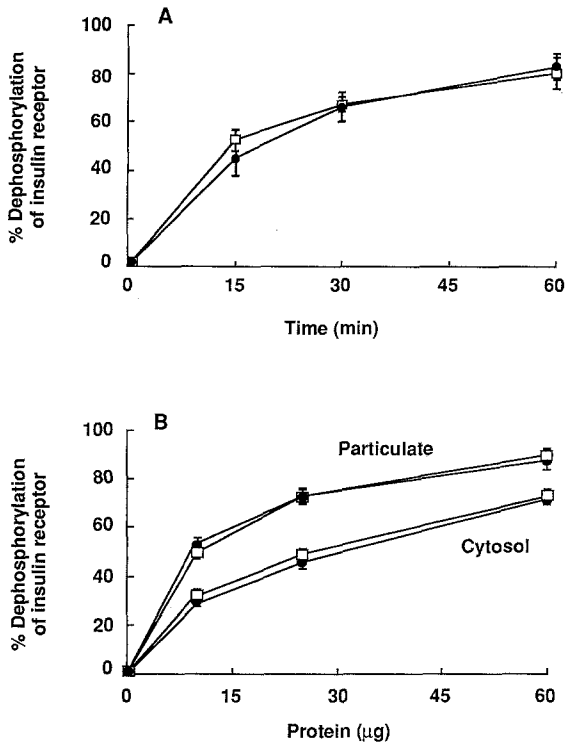
Fig. 1. (**A, B**) Time course and dose-response of insulin receptor dephosphorylation by hepatic phosphotyrosine phosphatases (PTP-ase). PTP-ase preparations were obtained from liver of lean (□) and obese (●) mice as described in Materials and methods. PTP-ase activity was assessed using an immunoenzymatic assay with antibody to phosphotyrosine as a probe. Partially purified insulin receptors were prephosphorylated, adsorbed on microtitre plates. The immobilized receptors were then incubated with 25 μg of microsomal proteins for various lengths of time (**A**) or with increasing amounts of cytosolic or particulate proteins for 30 min (**B**). Results are expressed as a percent of receptor dephosphorylation. Autophosphorylated receptors incubated in the absence of PTP-ases correspond to 0% dephosphorylation. Values are the mean ± SEM obtained with six individual preparations
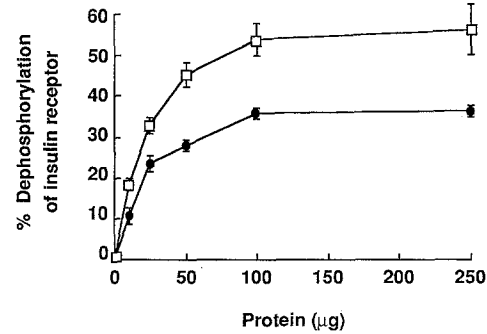

Fig. 2. Insulin receptor dephosphorylation by cytosolic phosphotyrosine phosphatases (PTP-ases) from hindleg muscles of lean (□) and obese mice (●). PTP-ase cytosolic preparations were prepared from hindleg muscles as described in Materials and methods. Dephosphorylation reactions were performed as described in Figure 1 with increasing amounts (0 to 250 μg) of cytosolic PTP-ases. Values are the mean ± SEM obtained with four individual preparations

Table 2. Insulin receptor dephosphorylation by cytosolic and membrane fractions from different tissues of lean and obese mice

| | Insulin receptor dephosphorylation | | | |
| | Membrane-associated | | Cytosolic | |
Mice	Lean	Obese	Lean	Obese
Muscle				
Hindleg	67.9 ± 1.0	65.8 ± 2.8	42.6 ± 4.4	27.9 ± 4.1[a]
Heart	51.9 ± 1.6	56.6 ± 2.7	43.1 ± 1.7	47.7 ± 1.7
Diaphragm	65.1 ± 3.6	59.4 ± 4.9	39.5 ± 3.0	38.1 ± 2.3
Liver	90 ± 3	88 ± 4	73.0 ± 1.4	71.7 ± 1.8

Particulate and cytosolic fractions were prepared from lean and obese mice and the phosphotyrosine phosphatase activity was measured towards the phosphorylated insulin receptor with 50 μg of protein as described in Materials and methods. Values are expressed as percent of insulin receptor dephosphorylation and are presented as mean ± SEM of results obtained with four to six individual preparations assayed in triplicate.

[a] $p < 0.01$ lean vs obese mice; $p = $ NS for all other comparisons

tween lean and obese mice (Table 2). By contrast, the ability of phosphotyrosine phosphatases to dephosphorylate insulin receptors was decreased in the cytosolic fraction obtained from hindleg muscles of obese mice compared to lean animals, whereas no difference was observed in cytosolic fractions from heart or diaphragm (Table 2). This decrease in PTP-ases from obese hindleg muscles was restricted to the cytosolic fraction, since no difference was observed at any of the protein concentrations used from the particulate fraction (data not shown). As shown in Figure 2, the dephosphorylation of insulin receptors by cytosolic PTP-ases from lean and obese animals was dependent upon the amount of protein, and was observed at all protein concentrations.

Discussion

Insulin resistance is commonly associated with obesity both in humans and rodents. The present study was conducted in mice rendered obese following injection of goldthioglucose, which induces hyperphagia, hyperinsulinaemia and insulin resistance [12]. This is associated with altered muscle glucose uptake, both in basal and insulin-stimulated conditions [12, 13]. Previous studies have shown that this is a result of multiple alterations both at the receptor and postreceptor levels. One alteration is the defective insulin receptor autophosphorylation and tyrosine kinase activity described in muscle from obese mice [1] and in obese non-insulin-dependent diabetic patients [2]. The state of receptor autophosphorylation results from a balance between tyrosine phosphorylation and dephosphorylation. This led us to study PTP-ase activities in particulate and cytosolic fractions from different tissues of lean and obese mice, using prephosphorylated insulin receptors and an immunoenzymatic assay with antibodies to

phosphotyrosine [14, 17]. Dephosphorylation of insulin receptors measured by this assay was dependent upon the protein concentration and incubation time, and excludes a possible interference with serine-threonine phosphatases. Furthermore, a natural substrate, the insulin receptor, was used rather than artificial proteins or peptides [3].

Preparations from all insulin-responsive tissues investigated, possessed PTP-ase activities which were higher in microsomes than in cytosol, as reported in rats [17–20] and humans [21]. Liver extracts were the most potent, resulting in 90% and 73% insulin receptor dephosphorylation for microsomes and cytosol, respectively. In contrast, in hindleg muscle, a maximum of 55% dephosphorylation was obtained with 100 μg of cytosolic proteins. Different tyrosine residues within the insulin receptor undergo phosphorylation during insulin stimulation. Our observation that muscle PTP-ase preparations are unable to completely dephosphorylate the phosphorylated insulin receptor in contrast to liver PTP-ase preparations could suggest that some "specific" PTP-ases present in liver are absent or inactive in muscle.

In hindleg muscles of obese mice, cytosolic PTP-ase activity was decreased at all protein concentrations compared to the preparations from lean animals. Thus, in insulin-resistant obese mice, a defect in the ability to dephosphorylate the insulin receptor was observed, but only in hindleg muscles and was restricted to the cytosolic fraction. No difference was observed in diaphragm, heart and liver. These results suggest that PTP-ases can be regulated in a tissue-specific manner. It was originally thought that an increase in PTP-ase activity might cause a decrease in tyrosine kinase activity of insulin receptor and thus contribute to insulin resistance. However, a reduced PTP-ase activity may also lead to altered insulin action if the dephosphorylation of some phosphotyrosine residues are obligatory steps in the insulin signalling. Conflicting results have been found in the literature using different models. For example, hepatic PTP-ase activity measured with a synthetic peptide was decreased in db/db or ob/ob mice [20], but was unchanged in our study in experimentally-induced obese mice with insulin receptor used as substrate. A diminution of PTP-ase activity was associated with a decreased insulin receptor autophosphorylation in livers of streptozotocin-diabetic animals in some studies [17], but not in others [18, 22]. A slight increase in particulate PTP-ase activity has been observed in muscle from insulin-resistant humans [21], while we observed a decrease in cytosolic PTP-ases from mouse hindleg muscle. Finally, in the alloxan diabetic rat, divergent results were obtained depending on the substrate used for assay [18, 23]. Whether this discrepancy is the result of the insulin-resistant models with various blood glucose or insulin levels, the preparation of the subcellular fractions, or more likely the different substrates used for measuring the PTP-ase activity is not known. Clarification of these problems will require better knowledge of how the PTP-ases specifically act on the insulin receptor.

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