Decreased skeletal muscle phosphotyrosine phosphatase (PTPase) activity towards insulin receptors in insulin-resistant Zucker rats measured by delayed Europium fluorescence

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Summary In order to measure the phosphotyrosine phosphatase (PTPase) activity in small muscle biopsies, a sandwich-immunofluorescence assay was developed using the phosphorylated human insulin receptor as a substrate, a C-terminal insulin receptor antibody as catching antibody and Europium-labelled anti-phosphotyrosine as detecting antibody. Soluble and particulate muscle fractions were prepared from soleus muscle of obese, diabetic (fa/fa) Zucker rats and their lean littermates (Fa/–). In the soluble muscle fractions of the obese (fa/fa) rats PTPase activity was significantly reduced compared to control (Fa/–) rats ($45.2 \pm 2.6\%$ vs $61.3 \pm 4.7\%$, $p < 0.02$). This reduction was completely prevented

by 24 days of metformin treatment which decreased plasma glucose and plasma insulin levels. In particulate muscle fractions, however, no difference in PTPase activity was found among any groups of rats examined. These results show that the alterations in soluble PTPase activity in the insulin-resistant, diabetic Zucker rat vary with the abnormality in glucose homeostasis. [Diabetologia (1996) 39: 142–148]

Key words Insulin receptor, phosphotyrosine phosphatases, insulin resistance, skeletal muscle, Zucker rats, metformin.

Phosphorylation of protein tyrosyl residues plays an important role in intracellular signalling, initiated by insulin and growth factors [1, 2]. The tyrosyl phosphorylation level of a protein in a signal transduction chain reflects a balance between activities of more or less specific phosphotyrosine kinases and phosphatases [1, 3]. The phosphotyrosine phosphatases (PTPases) can be subdivided into two classes: a group of higher molecular weight, transmembrane, receptor-like enzymes and a group of cytosolic enzymes of lower molecular weight [3]. PTPase activity has been

found in various tissues of the organism, including skeletal muscle [4–7], an important target organ for insulin.

Insulin acts by binding to a specific receptor and induces autophosphorylation of tyrosyl residues of the receptor β -subunit, increasing the activity of the insulin receptor tyrosine kinase [8, 9]. In several studies of insulin-resistant animal models and in insulin-resistant human subjects a decreased insulin receptor tyrosine kinase activity in muscle has been found [10–13].

Insulin receptor signalling could be counteracted by PTPases dephosphorylating the insulin receptor, insulin receptor substrates or tyrosine-phosphate intermediates further downstream in the insulin-signalling cascade. Thus, PTPases could control the amplification in the insulin-signalling chain.

PTPase activity in insulin-responsive tissues has been measured with different substrates and different models of insulin resistance, giving conflicting results [4–7, 14–18]. The aim of the present study was, using

Received: 6 June 1995 and in revised form: 14 July 1995

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Abbreviations: PMSF, Phenyl methyl sulphonyl-fluoride; PTPase, phosphotyrosine phosphatase; BHK, baby hamster kidney; RCM-lysozyme, reduced, carboxyamidomethylated, and maleylated lysozyme; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid.

phosphorylated human insulin receptors as the substrate, to measure PTPase activity in insulin-resistant skeletal muscle fractions with an improved technique.

As a model of insulin resistance in skeletal muscle we used the well-characterized adult obese Zucker rat (fa/fa) which displays severe fasting hyperinsulinaemia, moderate hyperglycaemia, glucose intolerance and an elevated plasma insulin response to a glucose challenge [19–21]. In Zucker rats the insulin receptor tyrosine kinase activity as well as autophosphorylation in skeletal muscle have been described as unchanged [22] or decreased [23] compared to lean controls.

To corroborate that changes in PTPase activities in the Zucker rats were related to insulin resistance, we extended the model to include Zucker rats treated with the anti-diabetic drug metformin (N,N-dimethylbiguanide) [24–26].

Materials and methods

Materials. Dithiothreitol, 4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid (Hepes) ATP, Triton X-100, N-acetyl-D-glucosamine, bovine serum albumin, sodium-ortho-vanadate (Na_2VO_4), benzamidine, leupeptin and phenyl methyl sulphonyl-fluoride (PMSF) were from Sigma (St. Louis, Mo., USA). Trasylol was obtained from Bayer (Leverkusen, Germany) and EDTA and magnesium chloride were from Merck (Darmstadt, Germany). Agarose wheat germ lectin was from Pharmacia (Piscataway, N.J., USA) and Dulbecco's modified Eagle's medium (without sodium pyruvate, with 4500 mg/l glucose), fetal calf serum, trypsin, penicillin and glutamine were provided by Gibco BRL, Life Technologies Ltd. (Paisley, Scotland, UK). Bradford protein assay was from Bio-Rad Laboratories GmbH (München, Germany). Europium kit, DELFIA assay-buffer and enhancement-buffer were obtained from Wallac (Turku, Finland). Micro-titre wells were from Nunc (Copenhagen, Denmark). Monoclonal antibody to the β -subunit of the insulin receptor, CT1, was a generous gift from Dr. K. Siddle. Polyclonal anti-phosphotyrosine-antibody from Zymed Laboratories San Francisco Calif., USA) was Europium-labelled as described in the Wallac Europium-labelling kit giving 1.5 mol Eu^{3+} /mol antibody. Phosphatase 1B was purified in Dr. A. Ullrich's laboratory, Martinsried, München, Germany. Baby hamster kidney cells (BHK cells) overexpressing the human insulin receptor were kindly donated by Novo Nordisk (Bagsværd, Denmark).

Preparation of particulate and soluble fractions from soleus muscle. Rat soleus muscles were obtained from Zucker rats (fa/fa) and their lean littermates (Fa/-). The soleus muscles were saved from an earlier study [27] in which 14 obese male Zucker rats and 14 lean littermates from Charles River Laboratories (Cambridge, Mass., USA) were kept on an artificial 12-h day-night cycle at 21 °C and given free access to food. Seven obese Zucker rats and seven lean control rats were chosen randomly for treatment with metformin ingested by means of a gastric tube according to the following scheme: 90 mg/day for 6 days, 180 mg/day for 6 days, 225 mg/day for 6 days and 270 mg/day for 6 days. The remaining 14 rats were treated with vehicle (0.9 % NaCl). The last dose of metformin or vehicle

Table 1. Characteristics of rats used in the study

	Control (Fa/-) rats		Obese (fa/fa) rats	
	- Metformin	+ Metformin	- Metformin	+ Metformin
<i>n</i>	7	6	7	5
Start				
Weight (g)	278 ± 10	275 ± 10	348 ± 18	394 ± 28
End				
Weight (g)	342 ± 11 ^a	329 ± 12	482 ± 10	523 ± 22
Fasting plasma glucose (mmol/l)	6.4 ± 0.3 ^b	6.6 ± 0.3	12.7 ± 1.0 ^c	8.9 ± 1.0
Fasting plasma insulin (pmol/l)	467 ± 100 ^d	267 ± 83	1817 ± 450 ^c	983 ± 17

Data are shown as mean ± SEM

^a $p < 0.001$; ^b $p < 0.002$; ^d $p < 0.01$ vs untreated fa/fa rats;

^c $p < 0.05$ vs fa/fa rats treated with metformin

was given 24 h before the start of the study. After an overnight fast the rats were anaesthetized with pentobarbital sodium (50 mg/kg body weight i. p.) and weighed. Blood samples were taken for analysis of glucose and insulin and soleus muscles were excised and immediately frozen in *n*-hexane and stored at -80 °C until analysis. Three rats died during treatment, probably due to lesions from the gastric tube. In Table 1 the number of rats in each of the four groups, the weight (g) before and after the treatment period, the fasting plasma glucose (mmol/l) and the fasting plasma insulin (pmol/l) levels after the treatment period are given.

Frozen muscle biopsies (16–70 mg) were homogenized for 30 s on ice in 500 µl homogenization buffer (50 mmol/l Hepes, 200 mmol/l NaCl, 4 mmol/l dithiothreitol, 1 mmol/l PMSF, 100 µmol/l benzamidine, 100 µg/ml leupeptin, and 350 kallikrein inhibitor units/ml Trasylol, pH 7.8), using a Polytron homogenizer on maximum setting. The homogenates were centrifuged at 110 000 *g* for 30 min at 4 °C. The supernatants (soluble fractions, expected to contain cytosolic PTPases) were collected, supplemented with Triton X-100 to 0.5 % and frozen immediately in liquid nitrogen. The pellets were homogenized for 30 s on ice in the presence of 0.5 % Triton X-100 in the homogenization buffer. After centrifugation (110 000 *g* for 30 min at 4 °C), the supernatants (particulate fractions, expected to contain membrane-bound PTPases) were retained and frozen in liquid nitrogen. The protein concentration in the samples of soluble and particulate fractions was determined by the Bradford protein assay [28] using BSA as standard. Subsequently, the remaining muscle fractions for PTPase assay were diluted with the homogenization buffer supplemented with 0.5 % Triton to identical protein concentrations, 1.06 mg/ml and 0.37 mg/ml in the soluble and particulate fractions, respectively, prior to analysis of PTPase activity.

Measurement of PTPase activity towards the human insulin receptor by delayed Europium fluorescence. Titre plate wells were coated with a mouse monoclonal antibody (1.1 µg protein/ml, pH 8.0, 50 mmol/l K_2PO_4 , 100 µl/well) against the β -subunit of the human insulin receptor, CT1 [29, 30], for 1 h at room temperature and blocked with a 0.5 % albumin-buffer. Human insulin receptors were prepared from 1 % Triton lysates of BHK cells expressing about 5×10^6 receptors per cell (Finn Wiberg, Novo Nordisk). The insulin receptors in 3 ml lysate (about 50×10^6 cells) were bound to wheat germ agglutinin immobilized on agarose beads (0.32 mg wheat germ agglutinin in 1 ml buffer (50 mmol/l Hepes, 150 mmol/l NaCl, 0.1 % Triton X-100)) by incubation overnight at 4 °C in a rotat-

ing mixer. N-acetyl-D-glucosamine (0.3 mol/l) in buffer (50 mmol/l Hepes, 150 mmol/l NaCl, 0.1 % Triton X-100) was used for elution. Following elution the receptors were phosphorylated for 1 h at room temperature in the presence of 1 mmol/l ATP, 10 mmol/l magnesium chloride and 100 nmol/l insulin. The reaction was stopped by 12 mmol/l EDTA and pH was adjusted to 7.8. The phosphorylated receptor preparation was added to the CT1-coated wells (dilution 1:4, 100 μ l/well) and incubated overnight at room temperature. After six washes (referred to as washing) wells were incubated for 2 h with soluble and particulate muscle fractions (53 μ g protein/well and 18 μ g protein/well, respectively). After washing, Europium-labelled anti-phosphotyrosine antibody was added (0.1 mg/ml, diluted 1:500 in DELFIA assay-buffer, 100 μ l/well) and incubated for 2 h. This was followed by washing of the wells. After addition of enhancement-solution, delayed fluorescence of Europium was measured using a 1234 DELFIA Research Fluorometer (Wallac). The decline in fluorescence, representing dephosphorylation of insulin receptor phosphotyrosyl residues after a 2-h incubation with muscle fractions, was expressed as percentage of the initial fluorescence. The initial level of fluorescence was determined following 2-h incubation of antibody-bound phosphorylated insulin receptor with muscle fractions in the presence of 10 mmol/l Na_3VO_4 , which is considered to be the specific and classical PTPase inhibitor [31]. The decline in fluorescence was thereby corrected for possible non-enzymatic loss of bound phosphotyrosyl caused by, e.g., dissociation of antibody-bound phosphorylated insulin receptors from the wells. In preliminary experiments we investigated the time and concentration dependence of insulin-receptor dephosphorylation by pure PTPase 1B and no further dephosphorylation of insulin receptors was found following incubation for 2 h with 80 ng PTPase 1B/well. The fluorescence remaining after incubation with muscle fraction supplemented with pure PTPase 1B (80 ng/well) was used as 100 % dephosphorylation. This value never exceeded 10 % of the initial fluorescence. The time course of dephosphorylation was nearly linear up to 50 %. The fact that some of the measurements slightly exceeded this value might give rise to an underestimation of the true difference between group mean values. We accepted this probably minor bias since our primary aim was to establish the direction of changes in rate of dephosphorylation between groups, and since estimates based on initial rates of dephosphorylation tended to be severely affected by random error. Measurements were performed in triplicate and median values were used.

Statistical analysis

Data were analysed by means of non-parametric statistical methods. For unpaired data the Mann-Whitney test was used and for paired data the Wilcoxon test was used. Spearman's rho was used for correlation, and *p*-values less than 0.05 were considered significant. Data are presented as mean $\pm$ SEM.

Results

In Figure 1 an autoradiogram of an SDS-gel separation of [γ - ^{32}P]ATP phosphorylated eluate shows the ^{32}P -phosphorylation of the purified insulin receptors. It has been noted that the β -subunit (97 kDa) of the insulin receptor constitutes a major part of the phosphorylated protein. Insulin receptor dephosphoryla-

Fig. 1. Autoradiogram showing ^{32}P -phosphorylation of the wheat-germ agglutinin-purified insulin receptors. Autophosphorylation of insulin receptors (9 μ g protein/ml) was accomplished by the addition of 0.1 μ mol/l insulin, 6.5 μ mol/l ATP, [γ - ^{32}P]ATP (43 Ci/mmol) and 7.4 mmol/l MnCl_2 in 50 mmol/l Hepes, pH 7.6, 1 % Triton X-100, 4 mmol/l EDTA, 400 kallikrein inhibitor units/ml Trasylol, 1 mmol/l PMSF, 8.7 μ mol/l pepstatin, 16.8 μ mol/l leupeptin for 30 min at room temperature. Particulate fraction from normal rat soleus muscle was prepared by homogenization as described in Methods. Twenty microlitres of phosphorylated insulin receptor preparation was incubated with 80 μ l of particulate muscle fractions (0.75 mg protein/ml) without (lane A) and with 5 mmol/l Na_3VO_4 (lane B) for 20 min. Lane C is control (insulin receptors incubated with buffer). Origin of 7.5 % SDS-PAGE resolving gel run under reducing conditions is indicated at the upper left (OR) and molecular masses (kDa) of marker proteins on the left

tion by soluble and particulate fractions of soleus muscle from normal rats was nearly proportional with incubation time and protein concentration in the intervals studied (Fig. 2).

The PTPase activity in the soluble muscle fractions of the obese untreated (fa/fa) rats was significantly reduced compared to the lean untreated (Fa/−) controls (45.2 ± 2.6 % vs 61.3 ± 4.7 %, $p < 0.02$, Fig. 3; the percentage indicates the decline in fluorescence, representing dephosphorylation of insulin receptor phosphotyrosyl residues after a 2-h incubation with muscle fractions).

In the particulate fractions no difference between PTPase activities in obese untreated (fa/fa) rats and lean untreated (Fa/−) rats was found (41.8 ± 5.9 % vs 49.4 ± 4.1 %, NS, Fig. 4).

The reduction in PTPase activity found in the soluble muscle fractions of untreated (fa/fa) rats when compared to the PTPase activity of soluble fractions of untreated (Fa/−) rats was prevented in the soluble muscle fractions of metformin-treated (fa/fa) rats

Fig. 2. Time and concentration dependence of insulin receptor dephosphorylation. Phosphorylated insulin receptors in titre wells were incubated with aliquots of a soluble muscle fraction (125 µg protein/well) for various times (upper panel) or for 2 h with various amounts (µg protein/well) of particulate muscle fraction (lower panel) from normal rat soleus muscle

($62.6 \pm 5.9\%$ vs $45.2 \pm 2.6\%$, $p < 0.05$, metformin-treated fa/fa rats vs untreated fa/fa rats, Fig. 3).

In the rats we studied, protein recovery from soleus muscles was highest in the soluble fractions. The highest specific PTPase activity (defined as per cent dephosphorylation of added phosphorylated insulin receptor per 2 h per mg muscle fraction protein) was found in the particulate fractions (all 25 rats: $44.2 \pm 3.0\%$ vs $20.8 \pm 1.2\%$, $p < 0.001$, data not shown).

No correlation was found between soluble and particulate specific PTPase activities (defined as above) when the individual values of the 25 rats were compared ($r = 0.09$, NS, data not shown). This indicates the absence of interdependence (caused, for instance, by variations in the intensity of homogenization) between recoveries of soluble and particulate activities.

Discussion

Most frequently, PTPase activity has been measured by release of [32 P]P_i from phosphorylated proteins, using phosphotyrosine kinases and [γ - 32 P]ATP for phosphorylation [1]. If the phosphotyrosine kinase preparation used for labelling is contaminated with

Fig. 3. PTPase activity in soluble muscle fractions from Zucker (fa/fa) rats and their lean littermates (Fa/-) with and without metformin (m) treatment. Fractions were adjusted to protein concentrations of 1.06 mg/ml. Mean values are shown; bars denote SEM; number of rats in each group is given in Table 1; * $p < 0.02$, ** $p < 0.05$

Fig. 4. PTPase activity in particulate muscle fractions from Zucker (fa/fa) rats and their lean littermates (Fa/-) with and without metformin (m) treatment. Fractions were adjusted to protein concentrations of 0.37 mg/ml. Mean values are shown; bars denote SEM; number of rats in each group is given in Table 1

phosphoserine/threonine kinases, serine/threonine residues are phosphorylated as well. Such phosphorylation could also occur non-enzymatically, making it difficult or impossible to determine whether subsequently released [32 P]P_i is the result of PTPase or phosphoserine phosphatase activity. In the present study anti-phosphotyrosine antibodies were used for detection of phosphotyrosyl residues and this error source was thereby eliminated.

The 2.1-fold higher specific activity (defined as in the Results section) found in the particulate fraction compared to the soluble fraction is in agreement with results previously published [4] where a 1.5–2.8-fold higher specific PTPase activity was found in particulate fractions compared to soluble fractions of liver, soleus muscle, white adipose tissue and brain using the purified insulin receptor as a substrate. Since some of the measurements were performed at the limit of linearity of our assay, an underestimation of the true differences might occur (see Methods section). This possible underestimation in the assay would not, however, interfere with non-parametric tests for differences between samples.

Recently, several authors have measured PTPase activity by means of different exogenous substrates in different tissues of both insulin-dependent and non-insulin-dependent diabetic animal models and insulin-resistant human subjects. The results do not form a uniform picture of the significance of the direction of changes in PTPase activity in insulin resistance.

Liver PTPase activity has been the most thoroughly studied. It has been reported that the overall PTPase activity was increased in livers of streptozotocin-diabetic rats using a synthetic peptide as a substrate [14]. Gruppiso et al. [15] found a decreased PTPase activity in both soluble and particulate liver fractions in alloxan-diabetic rats only when the insulin receptor was used as a substrate and not when the substrate was RCM-lysozyme (reduced, carboxyamidomethylated, and maleylated lysozyme) or polyglutamic acid:tyrosine 4:1 emphasizing the importance of the substrate for the PTPase activity. A decreased hepatic PTPase activity was found in particulate and soluble fractions of ob/ob mice and in soluble fractions of db/db mice when a synthetic peptide containing the amino acid sequence between residues 1142 and 1153 of the insulin receptor β -subunit was used as a substrate [16]. Begum et al. [5] found an increased cytosolic and membrane-associated PTPase activity in livers of streptozotocin-diabetic rats, using the insulin receptor as a substrate. An increased PTPase activity in a particulate fraction of liver was described in alloxan-diabetic rats only when myelic basic protein was the substrate, whereas no difference was detected with other substrates (RCM-lysozyme and the insulin receptor tyrosine kinase domain) [17]. Hauguel-de Mouzon et al. [4] found a 40–50 % decrease in PTPase activity measured by means of exogenous insulin receptors in a liver particulate fraction from streptozotocin diabetic rats. No difference in either membrane-bound or cytosolic PTPase activity was detected when liver from gold-thioglucose-treated mice was used [6].

Skeletal muscle studies have produced results which are as conflicting as those obtained from liver

studies. McGuire et al. [7] measured particulate and soluble PTPase activity in muscle biopsies from insulin-resistant and insulin-sensitive human subjects with RCM-lysozyme as a substrate. This group was the first to characterize PTPase activity in insulin-resistant patients. In fasting insulin-resistant subjects they found a 33 % increase in PTPase activity in the particulate fractions [7]. These initial findings are at variance with the results of a study on a larger number of subjects where a reduction of 21 % and 22 % was found in particulate skeletal muscle fractions from obese non-diabetic and non-insulin-dependent diabetic patients, respectively. In the latter study, a synthetic peptide (1142–1153) was used as the substrate [18].

In the soluble fraction of hindleg muscle of gold-thioglucose-treated mice Olichon-Berthe et al. [6] found decreased PTPase activity measured by means of exogenous insulin receptors. In the present study of soluble soleus muscle fractions from insulin-resistant Zucker rats we found a decreased PTPase activity, using the human insulin receptor as a substrate. These results agree and in addition our model comprises a group of Zucker rats in which parameters used as indicators of insulin resistance were partially reversed by metformin treatment as was the PTPase activity.

The decrease in PTPase activity in the soluble fractions in these insulin-resistant animal models could be explained as the product of a compensatory mechanism ameliorating insulin resistance (see below). A decreased PTPase activity is expected to increase the tyrosine phosphorylation and thus increase insulin signalling.

It is not known whether a PTPase exists which acts exclusively on the phosphorylated insulin receptor or also on other phosphorylated proteins in the insulin induced signal chain. However, it has been shown that the soluble PTPase 1D (also known as PTP2C, SH-PTP2 and Syp) contains two Src homology 2 (SH2) domains [32], and that these SH2 domains bind to phosphorylated tyrosine residues of IRS-1 (insulin receptor substrate 1) [33, 34]. PTPase 1D rapidly dephosphorylates phosphotyrosyl-IRS-1, and will thereby attenuate signalling from phosphotyrosyl-IRS-1. The insulin signalling cascade is presumed to irradiate from IRS-1 to several chains eliciting different effects. If stimulation of one of these signal chains increased the level of expression of PTPase 1D or other PTPases with activity towards intermediates in the insulin signalling chain, this could constitute a long-term negative feed-back mechanism controlling the early intracellular insulin signal amplification. As an alternative to a lowered expression level of PTPases, a decrease in soluble PTPase activity could be accomplished by association of PTPases with the cytoskeleton. However, two studies on PTPase activity in the cytoskeleton of liver preparations

report no difference between alloxan-diabetic rats and controls [15, 17].

The hypoglycaemic drug, metformin, is used in the treatment of non-insulin-dependent diabetic patients. Several anti-diabetic effects of metformin have been described [24, 35–40] including an increase of glucose uptake in peripheral tissues (fat and muscle) [38–40]. We found that the decreased PTPase activity was abolished in the soluble muscle fractions of the Zucker rats after metformin treatment. If PTPases constitute part of a cellular feedback amplification control as suggested above, the observed increase in PTPase activity following metformin treatment could be the result of a primary, metformin-induced increase in insulin signalling.

In conclusion, we have found a reversibly-decreased PTPase activity in soluble soleus muscle fractions which may represent a futile compensation of the insulin resistance present in skeletal muscle of Zucker rats.

Acknowledgements. We are very grateful to Director A. Ullrich for letting the initial experiments take place in his laboratory at the Max Planck Institute, Martinsried, and for providing materials. The expert technical assistance of Ms. U. Blankensteiner and Ms. J. Harpøth is acknowledged. The study was supported financially by the Danish Diabetes Association and the Clinical Research Institute, Odense University Hospital.

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