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High Glucose Condition Activates Protein Tyrosine Phosphatases and Deactivates Insulin Receptor Function in Insulin-Sensitive Rat 1 Fibroblasts

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Summary To investigate the mechanism for the impairment of insulin receptor kinase activity induced by high glucose (HG) in Rat 1 fibroblasts that expressed human insulin receptors (HIRc), we measured protein tyrosine phosphatase (PTPase) activity in HG cells. Incubating HIRc cells for 4 days in 27 mM D-glucose (HG) stimulated cytosolic PTPase activities, but not particulate PTPase activity as determined by two methods using the dephosphorylation of insulin receptors. Furthermore, PTP1B, a major non-transmembrane PTPase in the cytosolic fraction,was increased in HG cells according to Western blots. These results indicate that desensitization of insulin receptor function by a high glucose condition is associated with the activation of PTPase activity.

Non-insulin-dependent diabetes mellitus (NIDDM) is characterized by insulin resistance in insulin-sensitive peripheral tissues, particularly the skeletal muscles (1). Several studies on insulin receptor function of skeletal muscles in NIDDM have revealed a decrease in the kinase activity of insulin receptors, which may be partially responsible for the decreased action of insulin in patients with NIDDM (2-4).

Hyperglycemia *per se* induces insulin resistance in experimental animal models, based upon the finding that the correction of hyperglycemia with phlorizin normalizes *in vivo* insulin sensitivity in diabetic rats (5). The *in vitro* studies of Müller et al.(6) have demonstrated that the insulin receptor kinase activity in rat adipocytes was modulated by incubating the cells in high glucose medium. Similarly we reported (7) that in NIDDM patients without hyperinsulinemia, there was a reverse relationship between fasting plasma glucose levels and the insulin receptor kinase activities of the skeletal muscles. Hyperglycemia *per se* may induce *in vivo* insulin resistance by desensitizing insulin receptors in insulin-sensitive tissues. To study how the high glucose condition affects insulin receptor function in cell culture system, we used Rat 1 fibroblasts that over-expressed human insulin receptors (HIRc). We found that high glucose induced the impairment of insulin receptor kinase activity (8). However, the mechanism of glucose-induced insulin receptor dysfunction remains unclear.

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Protein tyrosine phosphatase (PTPase) is considered to be an important regulator for insulin action and its activation may deactivate insulin receptor kinase activity (9). Furthermore, the abnormal regulation of PTPase has been reported in animals and patients resistant to insulin (10, 11). In this study, therefore, we investigated whether PTPase activity is altered and whether it causes dysfunction in insulin receptors in HIRc cells cultured in high glucose medium.

Materials and Methods

Materials: Purified porcine insulin was a gift from Novo-Nordisk Pharmacy and Eli Lilly and Company. Pork insulin ^{125}I-labeled at A14 (A14^{125}I-insulin; 2200Ci/mmol) was obtained from DuPont New England Nuclear. [γ-^{32}P]ATP was purchased from Amersham International plc. Protein A was obtained from Calbiochem-Behring Co. Wheat germ agglutinin (WGA) agarose was purchased from Pharmacia PL Biochemical. Aprotinin, phenylmethylsulfonyl fluoride (PMSF), and bacitracin were all purchased from Sigma. Anti-insulin receptor antiserum (αIR) was obtained from a Type B insulin-resistant patient. Monoclonal phosphotyrosine antibody (αPY20) was purchased from ICN and anti-protein tyrosine phosphatase-1B (PTP1B) antibody was purchased from UBI. All other reagents were of analytical grade from Nakarai Chemicals.

Cell culture: Rat 1 fibroblasts that expressed human insulin receptors (HIRc), provided by Dr. J.M. Olefsky (University of California, San Diego)(12), were recloned to obtain cells expressing about one-tenth the number of receptors as the original HIRc cells. Recloned HIRc cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Subconfluent HIRc cells were cultured for up to 4 days with various concentrations of D-glucose (5.5 mM: normal glucose, NG; 27 mM: high glucose, HG) or D-raffinose. The medium was changed every other day. These culture conditions did not influence the cell number and content of cellular proteins (data not shown).

Insulin binding assay: Insulin binding to purified receptors has been described (13). In brief, ^{125}I-insulin binding to solubilized receptors was assessed by polyethylene glycol precipitation while insulin binding capacity was determined by Scatchard plots.

Measurement of PTPase activity using ^{32}P-phosphorylated insulin receptors: NG and HG cells were homogenized in buffer A (50 mM HEPES, pH 7.0, 5 mM EDTA, 0.5mM EGTA, 1 mM DTT, 0.2 mM PMSF, and 1000 KIU aprotinin) by polytron (setting 7 for two 20 sec periods), and centrifuged at 500 g for 10 min at 4°C. The resultant supernatant was ultracentrifuged at 100,000 g for 60 min at 4°C. The final supernatant was designated as the cytosolic fraction. The pellet was solubilized in buffer A containing 0.5 % Triton X-100 and designated as the particulate fraction according to the modified method of Begun et al.(14). Partially purified insulin receptors were autophosphorylated in the presence of 100 μM ATP (γ-^{32}P-ATP; 500 μCi/tube), after overnight stimulation with insulin. A Bio Gel P6 spin column was used to remove free labeled ATP. Aliquots of autophosphorylated insulin receptors (100 fmol) were incubated with cytosolic (20 μg protein) or particulate (10 μg protein) fractions at 30°C for 10 and 20 min, respectively. The reaction was terminated by adding 0.5 ml of chilled stop solution containing 4 mM EDTA, 100 mM NaF, 5 mM Na_3VO_4, 1000 KIU aprotinin, 2 mM PMSF, in 50 mM HEPES buffer according to the procedure of Hashimoto et al.(15). Insulin receptors were then immunoprecipitated with αIR and resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the amounts of radioactivity in the band corresponding to the ß-subunits were determined by Cherenkov counting.

Measurement of PTPase activity by an immuno-enzymic assay : PTPase activities were also determined using an enzyme-linked assay system that utilizes a phosphorylated insulin receptor (150fmol/well) and a monoclonal phosphotyrosine antibody (αPY20) according to the method of Peraldi et al. (16). The dephosphorylation reaction was initiated by adding 20 μg of protein from the cytosolic and particulate fractions obtained from NG and HG cells. After a 30 min incubation at 22°C, PTPase activities were measured using αPY20 and peroxidase-conjugated anti-rabbit antibody. Receptor dephosphorylation was calculated as the ratio of A492 of dephosphorylated receptors (measuring by incubating insulin receptors in the presence of phosphatases) over that of the control receptor (incubated in the absence of phosphatases). PTPase activities measured by this assay system were time- and dose-

dependent within the range between 15-45 min and between 10-50 μg of protein (data not shown).

Western blots of PTP1B in the cytosolic and particulate fractions obtained from NG and HG cells: Samples from NG and HG cells were separated into cytosolic and particulate fractions as described above, and PTP1B was immunoprecipitated using anti-PTP1B antibody. Total cellular proteins and the immunoprecipitated proteins from both fractions (20-40 μg protein) were resolved by SDS-PAGE and transferred to an Immobilon membrane (Millipore) using standard procedures. Immunoblotting was proceeded using anti-PTP1B antibody and proteins were visualized by means of an anti-rabbit antiserum, using enhanced chemiluminescence (ECL kit, Amersham) according to the manufacturer's specifications (17).

Results

Cells incubated for four days in high glucose medium (27 mM) showed a significant decrease in both the basal and maximally stimulated tyrosine kinase activities in Rat 1 fibroblasts expressing human insulin receptors (HIRc) as recently reported (8). To investigate the molecular mechanism of high glucose-induced receptor kinase dysfunction, we tested whether PTPase activity would be altered in HG cells, because PTPase is thought to be an important regulator for insulin action (9). After the cells were cultured in high glucose medium for 4 days (HG), the PTPase activities of both cytosolic and particulate fractions from HG cells were measured using ^{32}P phosphorylated insulin receptors as a substrate. As shown in Figure 1, cytosolic PTPase activities in HG cells increased about 2-fold when compared with cells cultured in normal glucose medium (NG). On the other hand, the particulate PTPase activities were comparable among two groups. We also measured PTPase activities by an immuno-enzymic assay system using phosphorylated insulin receptors and αPY20. As summarized in Figure 2, the cytosolic PTPase activity in HG cells was significantly elevated, but the particulate PTPase activity in HG cells was comparable with that in NG cells. These similar results indicated that cytosolic PTPase activity in the cells cultured in high glucose medium for 4 days was elevated through two different methods.

We studied further mechanism for the increase in cytosolic PTPase activity in HG cells by Western blotting, to test whether the PTP1B content is altered. This is because PTP1B is considered as the most likely to be involved in insulin action. As shown in Figure 3, the amount of PTP1B in the cytosolic fraction of HG cells was significantly elevated, in accordance with increased PTPase activity in the cytosolic fraction in HG cells. On the other hand, high glucose condition did not induce any changes in the PTP1B content in the particulate fraction, even though PTP1B was dominantly localized in the particulate fraction.

Discussion

To study hyperglycemia-induced insulin resistance, we measured both the *in vitro* autophosphorylation and tyrosine kinase activities of WGA-purified insulin receptors obtained from cells cultured in high glucose medium for 4 days, and found that relatively chronic exposure of HIRc cells to the high glucose concentration in the medium led to impaired autophosphorylation and tyrosine kinase activity as previously reported (8). This glucose

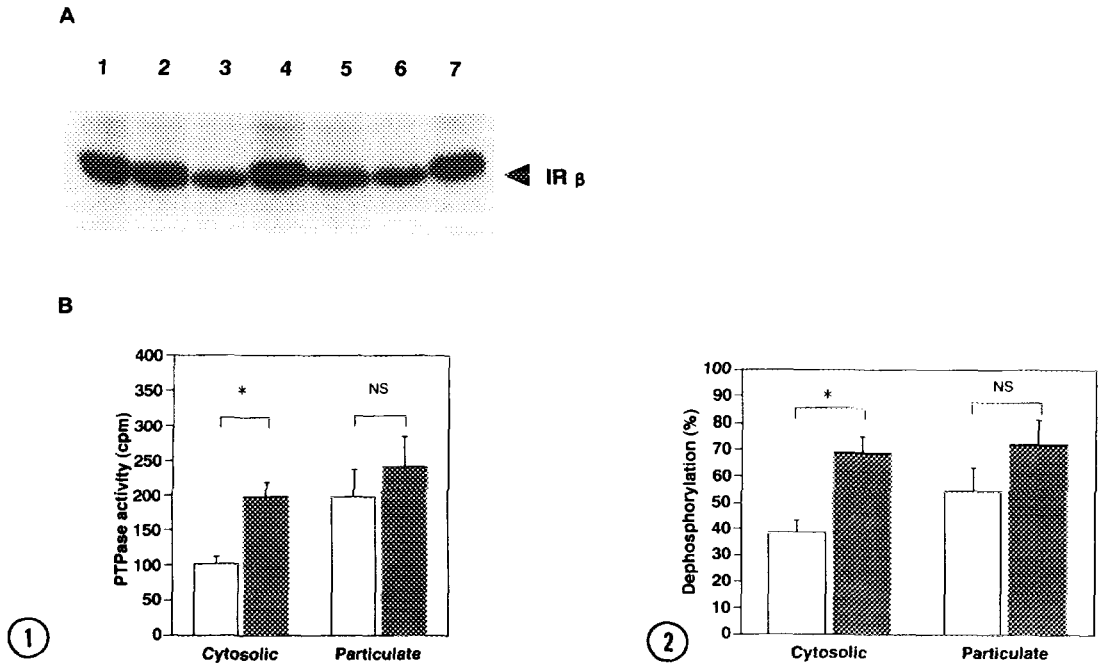

Figure 1.
Protein tyrosine phosphatase (PTPase) activities using ^{32}P-phosphorylated insulin receptors in NG and HG cells.
(A) Autoradiogram of dephosphorylated insulin receptors treated with either cytosolic or particulate fraction obtained from NG and HG cells. No treatment (lanes 1, 4 and 7); normal glucose, NG (lanes 2 and 5); high glucose, HG (lanes 3 and 6); cytosolic fraction (lanes 2 and 3) and particulate fraction (lanes 5 and 6). (B) Cytosolic and particulate PTPase activities from NG and HG cells. Normal glucose ☐ High glucose ▨.
Values are expressed as counts (cpm) and are the means ± SE of six separate experiments. Statistic significance was calculated by Student's t test. * : $p<0.01$ vs. HG cells.

Figure 2.
PTPase Activities determined by an immuno-enzymic assay in NG and HG cells.
PTPase activities were determined using an immuno-enzymic assay system that includes a phosphorylated insulin receptor (150 fmol) and a monoclonal phosphotyrosine antibody (αPY20). PTPase activities measured by this assay system were time- and dose-dependent within the range between 15-45 min and between 10-50 μg of protein (data not shown). Dephosphorylation was initiated by adding 20 μg of protein of the cytosolic and particulate fractions from NG and HG cells. After a 30 min incubation at 22°C, PTPase activities were measured using αPY20 and peroxidase-conjugated anti-rabbit antibody. The receptor dephosphorylation was calculated as the ratio of the A_{492} of dephosphorylated receptors (measuring by incubating insulin receptors in the presence of phosphatases) over that of control receptor (incubated in the absence of phosphatases). Each value is presented as the means of five separate experiments in quadruplicate (± SE) and statistic significance was calculated by Student's t test. * : $p<0.01$ vs. HG cells.

effect depended on both incubation time and the concentration of D-glucose in the media. Insulin receptor kinase activity was not affected by co-incubating cells with 5.5mM D-glucose and 21.5 mM raffinose as a high osmotic control group, suggesting that the effect was specific for D-glucose metabolism.

Concerning the molecular mechanism for dysfunction of insulin receptor kinase in HG cells, it is possible that a short-term, high glucose concentration (within 24 h) can activate

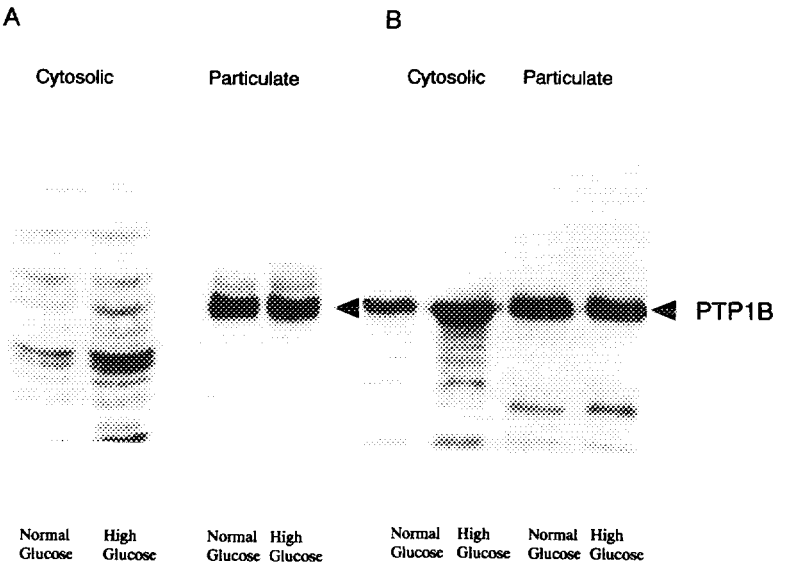

Figure 3.
Western Blotting of PTP1B in the cytosolic and particulate fractions from NG and HG cells. Samples from NG and HG cells were separated to cytosolic and particulate fractions, and PTP1B was immuno-precipitated by anti-PTP1B antibody. Total cellular proteins (cytosolic fraction, 40 μg and particulate fraction, 20 μg)(A) and the immuno-precipitated proteins (B) from both fractions were resolved by SDS-PAGE and transferred to an Immobilon membrane (Millipore) using standard procedures. Immunoblotting was carried out using anti-PTP1B antibody and visualized by anti-rabbit antiserum using enhanced chemiluminescence (ECL kit, Amersham).

protein kinase C (PKC) [18], then impair the tyrosine kinase activity of insulin receptors as found in rat adipocytes [6]. Furthermore Berti et al. [19] have reported that high glucose medium induced insulin receptor dysfunction via the activation of PKC in Rat 1 fibroblasts that overexpress human insulin receptors. They observed glucose effects within 30 min and they were paralleled by the rapid translocation of several PKC isoforms ($\alpha\ \delta\ \varepsilon\ \zeta$) to the plasma membrane within 1 min. This is in accordance with the direct activation of PKC by phorbol ester leading to the serine and threonine phosphorylation of insulin receptors, resulting in the impairment of receptor kinase activity (20, 21). However, we reported that co-incubating cells with the new oral agent, pioglitazone, ameliorates the dysfunction of insulin receptor kinase induced by a high glucose concentration and this agent had no effect on PKC activity [8].

Protein tyrosine phosphatase (PTPase) is considered to be an important regulator of insulin action. Furthermore, its activation may produce insulin resistance (9), and abnormal regulation of PTPase has been reported in animals and patients resistant to insulin (10, 11). In this study, we found that PTPase activity in a cytosolic fraction of HIRc cells exposed to a high

concentration of glucose was increased 2-fold compared to that of NG cells. An immuno-enzymic assay for PTPase activity also demonstrated increased cytosolic PTPase activity in HG cells. Also, the amount of PTP1B, a candidate of PTPase involved in insulin action, was significantly increased in the cytosolic fraction in HG cells. On the other hand, both PTPase activity and PTP1B content in particulate fractions in HG cells were unchanged. Thus, it is possible that cytosolic PTPase activity is stimulated in the presence of high glucose, resulting in decreased autophosphorylation of the insulin receptor and its kinase activity. In the cytosolic fraction obtained from HG cells, increased PTPase activity was comparable to the PTP1B content. However, it is unclear whether increased PTP1B content in cytosolic fraction is due to translocation to the cytosol from the membrane fraction including the endplasmic reticulum or to an increased *de novo* synthesis of PTP1B protein. Furthermore, it is possible that the other types of PTPases are activated, as well as PTP1B.

Concerning the regulation of PTPase activity at high glucose concentration in the medium, the activation of PKC induced by tetradecanoyl phorbol acetate (TPA) can stimulate PTPase activity in the soluble fraction of human erythroleukemia cells (22). However, in rat adipocytes, TPA treatments had no effect on PTPase activity, but accumulation of cAMP inhibited PTPase activity (23). Thus, the regulatory mechanisms for gene expression of PTPases are not completely understood. In the case of PTP1B, activation of PKC by TPA-treatment, insulin and insulin like growth factor 1 can induce PTP1B expression (24, 25). Currently, there is no clear explanation for the mechanism that may be responsible for the stimulation of cytosolic PTPase activity in the cells cultured in medium containing high glucose. Further investigation is required to clarify the regulation of PTPase activity, which may be one mechanism of glucose-induced insulin resistance.

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References

1. DeFronzo, R.A., Bonadonna, R.C and Ferrannini, B.E. (1992) Diabetes Care 15, 318-368.
2. Arner, P., Lithell, H., and Livingston, JN (1987) Diagetologia 30, 437-440.
3. Caro, JF., Shinha, MK., Raju, SM., Ittoop, O., Pories, WJ., Flickinger, EG., Meelheim, D. and Dohm, GL (1987) J Clin Invest 79, 1330-1337.

4. Obermaier-kusser, B., White, MF., Pongratz, D., Su, Z., Ermel, B., C. Mühlbacher, and HU. Häring (1989) J Biol Chem 264, 9497-9504.
5. Rossetti, L., Smith, D., Shulman, GI., Papachristou, D. and DeFronzo, RA. (1987) J Clin Invest 79, 1510-1515.
6. Müller H.K., Kellerer, M., Ermel, B., Muhlhofer, A., Ober maier-Kusser, B., Vogt, B. and Häring, HU (1991) Diabetes 40, 1440-1448.
7. Maegawa, H., Shigeta, Y., Egawa, K. and Kobayashi, M. (1991) Diabetes 40, 815-819.
8. Maegawa, H., Tachikawa-Ide, R., Ugi, S., Iwanishi, M., Egawa, K., Kikkawa, R., Shigeta, Y., and Kashiwagi, A. (1993) Biochem Biophys Res Commun 197, 1078-1082.
9. Goldstein, B.J. (1992) J Cell Biochem 48, 33-42.
10. Meyerovitch, J., Backer, JM. and Kahn CR. (1987) J Clin Invest 84, 976-983.
11. McGuire, MC., Fields, RM., Nyomba, BL., Rat, I., Bogardus, C., Tonks, NK. and Sommercorn, J. (1991) Diabetes 40, 939-42.
12. McClain, DA., Maegawa, H., Lee, J., Dull, TJ., Ullrich, A. and Olefsky, JM. (1987) J Biol Chem 262, 14663-14671.
13. Maegawa, H., Kobayashi, M., Egawa, K., McClain, DA., Olefsky, JM. and Shigeta, Y (1990) . Biochim Biophys Acta 1053, 185-188.
14. Begum, N., Sussman, KE. and Draznin, B. (1991) Diabetes 40, 1620-29.
15. Hashimoto, N., Feener, EP., Zhang, WR. and Goldstein, BJ. (1992) J Biol Chem 267, 13811-4.
16. Peraldi, P., Hauguel-De Mouzon, S., Alengrin, F., and Von Obberghen, E. (1992) Biochem. J. 285, 71-78.
17. ECL western blotting protocols Amersham International plc.
18. Draznin, B. Wayne, J., Leitner, KE., Sussman, NA. and Sherman G. (1988) Biochem Biophys Res Commun 156; 570-575.
19. Berti, L., Mosthaf, L., Kroder, G., Kellerer M., Tippmer, S., Mushack, J., Seffer E., Seedor, K., and Häring H. (1994) J Biol Chem 269, 3381-3386.
20. Takayama, S., White, MF. and Kahn, CR. (1987) J Biol Chem 263, 3440-3347.
21. McClain, DA., Maegawa, H., Thies, RS. and Olefsky, JM. (1990) J Biol Chem 265, 1678-82.
22. Bütler, TM., Ziemiecki, A., and Friis, RR. (1990) Cancer Res 50, 6323-6329.
23. Begum, N., Graham, AL., Sussman, KE. and Draznin, B. (1992) Am J Physiol 262, E142-E149.
24. Hashimoto, N., and Goldstein, BJ. (1992) Biochem Biophys Res Commun 188, 1305-1311.
25. Kenner, K.A., Hill, D.E., Olefsky, J.M. and Kusari, J. (1993) J Biol Chem 268, 25455-25462.