Thiazolidine Derivatives Ameliorate High Glucose-induced Insulin Resistance via the Normalization of Protein-tyrosine Phosphatase Activities*

(Received for publication, May 23, 1994, and in revised form, December 12, 1994)

Hiroshi Maegawa*, Rie Ide, Masaaki Hasegawa, Satoshi Ugi, Katsuya Egawa, Masanori Iwanishi, Ryuichi Kikkawa, Yukio Shigeta, and Atsunori Kashiwagi

From the Third Department of Medicine, Shiga University of Medical Science, Seta, Ohtsu, Shiga, 520-21, Japan

The mechanisms for the insulin resistance induced by hyperglycemia were investigated by studying the effect of high glucose concentration (HG) and its modulation by thiazolidine derivatives, on insulin signaling using Rat 1 fibroblasts expressing human insulin receptors (HIRc). Incubating HIRc cells in 27 mM d-glucose for 4 days impaired the insulin-stimulated phosphorylation of pp185 and receptor β-subunits. Both protein kinase C activities and phosphatase activity of insulin resistance were unchanged; however, cytosolic protein-tyrosine phosphatase (PTPase) activity increased within 1 h prior to the impairment of insulin kinase in HG cells (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). Increased PTPase activity was consistent with a 2-fold increase in the amount of PTP1B, and anti-PP1B antibody inhibited this increment of cytosolic PTPase activity in HG cells. Co-incubating cells with pioglitazone prevented these abnormalities in cytosolic PTPase, the PTP1B content and the impaired phosphorylation of pp185 and receptor β subunits in HG cells. Finally, HG cells had impaired insulin-stimulated α-aminoisobutyric acid uptake, which was ameliorated by exposure to thiazolidine derivatives. In conclusion, exposing cells to high glucose levels desensitizes insulin receptor function, and thiazolidine derivatives can reverse this process via the normalization of cytosolic PTPase, but not of protein kinase C.

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). Therefore, the enhancement of insulin sensitivity in patients with NIDDM is one treatment modality. The thiazolidine derivatives, pioglitazone and troglitazone, have been tested for use as oral anti-diabetic drugs (5–8). We reported that pioglitazone may increase insulin sensitivity by activating the tyrosine kinase of skeletal muscle insulin receptors isolated from not only insulin-resistant Wistar fatty rats, but also from rats given a high-fat diet (9–10).

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 (11). The in vitro studies of Müller et al. (12) have shown that insulin receptor kinase activity in rat adipocytes is modulated by incubating the cells in high glucose medium. Similarly, we also reported (13) that in NIDDM patients without hyperinsulinemia, there is a reverse relationship between fasting plasma glucose levels and the insulin receptor kinase activities of the skeletal muscles. Thus, hyperglycemia per se may induce in vivo insulin resistance by desensitizing insulin receptors in insulin-sensitive tissues. To study how high glucose affects insulin receptor function in cell cultures, we used Rat 1 fibroblasts that overexpress human insulin receptors (HIRc). We found that high glucose impaired the insulin receptor kinase activity and that pioglitazone, a thiazolidine derivative, normalized the insulin receptor kinase level in culture (14).

Kellerer et al. (15) have also reported that the thiazolidine derivative, troglitazone, can normalize the insulin receptor dysfunction induced by incubating cells in high glucose medium. They have shown that protein kinase C (PKC) activity is increased in HIRc cells exposed to high glucose and that troglitazone can normalize the PKC activation. Their results suggest that the effect of troglitazone on insulin receptor kinase activity is mediated by the normalization of PKC activity. However, in a preliminary study, we did not find significant PKC activation in HIRc cells exposed to high glucose, although pioglitazone significantly improved the receptor function (14). Therefore, the mode of action of thiazolidine derivatives needs further clarification. Protein-tyrosine phosphatase (PTPase) is considered to be an important modulator in the desensitization of insulin receptor function (16). Thus, in this study, we investigated whether the activities of PKC and PTPase were altered in HIRc cells cultured in high glucose, whether they led to a dysfunction in insulin receptors, and if so, whether thiazolidine derivatives prevented these processes in high glucose-induced insulin resistance.

7724
EXPERIMENTAL PROCEDURES

Materials—Purified porcine insulin was a gift from Novo-Nordisk Pharmar (Copenhagen, Denmark) and Eli Lilly Co. (Indianapolis, IL). Porcine insulin \( ^{125}\)I-labeled at Tyr\(^{A4} \) (\( ^{125}\)I-Tyr\(^{A4}\)insulin; 2200 Ci/ mmol), \( ^{1}\)H]Hamilamicositic acid (AIB) and \( ^{1}\)H]Hiborphol dibutyrate (FDBu) were obtained from DuPont NEN (Boston, MA). \( ^{32}\)P]orthophosphate and \( ^{32}\)P]ATP were purchased from Amersham Corp. Protein A and wheat germ agglutinin (WGA) agors were purchased from Pharmacia Biotech Inc. Aprotinin, phenylmethylsulfonyl fluoride, and bacitracin were purchased from Sigma. Anti-insulin receptor antisera (aIR) was obtained from a Type B insulin-resistant patient. Polyclonal anti-phosphotyrosine antisera (aPY) was a gift from Dr. H. Fujio (Osaka University). A monoclonal phosphotyrosine antibody, aPT20, was produced in this laboratory. (Costa Mesa Biodev.) A nonradioactive peptide kinase assay kit was purchased from MBL (Nagoya, Japan), and anti-protein-tyrosine phosphatase-1B (PTP1B) antibody was purchased from UBI (New York, NY). All other reagents were of analytical grade from Nakarai Chemicals (Kyoto, Japan).

Cell Culture—Rat 1 fibroblasts that expressed HIIRs, provided by Dr. J. M. Olefsky (University of California, San Diego), were recloned to obtain cells expressing about one-tenth of the number of receptors as the original HIIR cells (17). These HIIR cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Subconfluent HIIR cells were cultured for up to 4 days with various combinations of glucose (5.5 mM normal glucose (NG); 27 mM high glucose (HG)) or insulin. The medium was changed every other day. There was no significant difference in the cellular protein content (230–250 \( \mu \)g in each 6 multwell dish) among NG and HG cells with or without pitglostatine.

Insulin Binding Assay—Insulin binding to cells and purified receptors has been described (18). In brief, \( ^{125}\)I-insulin binding to cells was measured in Eagle's medium containing 1% bovine serum albumin and 20 mM HEPES (pH 7.6) at 4°C for 16 h. Insulin receptors were obtained from HIIR cells cultured in NG and HG media and partially purified using a WGA column. Insulin binding to solubilized receptors was assessed by the polyethylene glycol precipitation, and the binding capacity was determined by Scatchard plot. Inulin binding to cells at a concentration of 8.3 pm in all three groups was similar (11.7–12.5%), indicating that these culture conditions had no effect upon insulin binding.

Western Blots of Phosphotyrosine of pp185 and Insulin Receptors—After exposing NG and HG cells to 167 mM insulin for 10 min at 37°C, they were solubilized and immunoprecipitated using a monoclonal phosphotyrosine antibody (aPY20) in the presence of phosphatase inhibitors. Bound proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to an Immobilon membrane (Millipore) using standard procedures. Immunoblotting was performed using horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies, or by enhanced chemiluminescence (ECL kit, Amersham Corp.) according to the manufacturer’s specifications (19).

Tyrosine Kinase Activity of WGA-purified Insulin Receptors—Tyrosine kinase activities of insulin receptors (300 fmol of insulin binding capacity; 10 min at 37°C) were measured at 1 mg/ml of polyglycerol, and 50 \( \mu \)M ATP (\( ^{32}\)P]ATP; 3 \( \mu \)Ci/\( \mu \)l at 4°C for 30 min and then assayed using filter paper as described (14).

Labeling Living Cells with Orthophosphate—Cells were incubated with phosphate-free medium for 2 h and then with 1 ml of \( ^{32}\)P]orthophosphate for 2 h. After the cells were stimulated with 167 mM insulin for 10 min, they were solubilized and immunoprecipitated with either aIR or aPY. Bound proteins were analyzed by SDS-PAGE as described (20). Twice as much protein from HG cells was resolved by SDS-PAGE to adjust the amount of phosphatase of insulin receptors.

Partial Purification and Measurement of PKC Activity—PKC samples from HIIR cells were sonicated, and the proteolytic and partially purified by DEAE-cellusose chromatography. The protein yield in these purified fractions was 30–40% and 50–60%, respectively, and was not different among NG and HG cells with or without pitglostatine. Fifty-four micrograms of each fraction was used in this assay. PKC activities were determined using a nonradioisotope protein kinase assay kit according to the manufacturer’s protocol. This system is based on an enzyme-linked immunosorbent assay that utilizes a synthetic peptide of glibliable acidic protein. A monoclonal antibody recognizes the phosphorylated G1 peptide (21). PKC activities of both fractions (54 \( \mu \)g) were measured by incubating with microsomal fraction obtained with G1 peptide at 25°C for 5 min in 25 mM Tris-HCl (pH 7.0) containing 2 mM MgCl\(_2\), 0.1 mM ATP, 0.8 mM CaCl\(_2\), and 50 \( \mu \)M phosphatidylserine. Specific PKC activities were determined by subtracting the nonspecific activity measured in the presence of 200 \( \mu \)M EGTA.

Binding of \( \text{H}^{32}\)P]BDPb to NG and HG Cells—PKC activity was also determined by measuring the specific high-affinity binding of \( \text{H}^{32}\)P]BDPb to intact cells according to the methods of Guzman et al. (22), which are considered to be a precise index of PKC activation (23). NG and HG cells were incubated in HEPES buffer containing 5 \( \mu \)M \( \text{H}^{32}\)P]BDPb for 15 min at 22°C. Nonspecific binding was determined in the presence of 1 \( \mu \)M tetradecanoyl phorbol acetate (TPA).

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, 0.5 mM EGTA, 1 mM dithiobiotetrol, 0.2 mM phenylmethylsulfonyl fluoride, and 1000 IU aprotinin) using a polytron (setting 7 for 20-20 s periods) and then centrifuged at 500 \( \times \) g for 10 min at 4°C. The supernatant was ultracentrifuged at 100,000 \( \times \) g for 60 min at 4°C. The final supernatant was designated as the particulate 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 Beug et al. (24). Partially purified insulin receptors were autophosphorylated in the presence of 100 \( \mu \)M ATP (\( ^{32}\)P]ATP; 500 \( \mu \)Ci/tube), after overnight stimulation with insulin. A Bio-Gel P6 spin column (Bio-Rad, Richmond, CA) was used to remove free labeled phosphopeptide. Autophosphorylated insulin receptors (100 fmol) were incubated with cysteolic (20 \( \mu \)g of protein) or particulate (10 \( \mu \)g of protein) fractions at 30°C for 10 and 20 min, respectively. The reaction was terminated with 0.5 ml of chilled stop solution containing 4 mM EDTA, 100 mM NaF, 5 mM Na\(_2\)VO\(_4\), 1000 IU aprotinin, 2 mM phenylmethylsulfonyl fluoride, in 10 mM HEPES buffer according to the procedure of Hashimoto et al. (25). Insulin receptors, then immunoprecipitated with aIR, resolved by SDS-PAGE, and then the amount of radioactivity in the bands corresponding to the \( \beta \)-subunits was determined by Cerenkov counting.

Measurement of PTPase Activity by an Immunoenzymatic Assay—PTPase activities were also determined using an enzyme-linked assay system that involved the phosphorylated insulin receptor (150 fmol/well) and a monoclonal phosphotyrosine antibody (aPY20) according to the method of Peraldi et al. (26). The dephosphorylation reaction was initiated by adding 20 \( \mu \)g of protein from the cystolic and particulate fractions obtained from NG and HG cells. After a 30-min incubation at 22°C, PTPase activities were measured using aPY20 and peroxidase-conjugated anti-rabbit antibody. Receptor dephosphorylation was calculated as the ratio of the A\(_{405}\) of the dephosphorylated receptor measured in the presence of phosphatases over that of the control receptors (incubated in the absence of phosphatases). PTPase activities measured in this manner were time- and dose-dependent within the range between 15 and 45 min and between 10 and 50 \( \mu \)g 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 obtained from NG and HG cells. After a 30-min incubation at 22°C, PTPase activities were measured using aPY20 and peroxidase-conjugated anti-rabbit antibody. The immunoprecipitated proteins from both fractions were resolved by SDS-PAGE and transferred to an Immobilon membrane (Millipore) using standard procedures. Immunoblotting proceeded using anti-PTP1B antibody, and proteins were visualized by means of an anti-rabbit secondary, using enhanced chemiluminescence (ECL kit, Amersham Corp.), according to the manufacturer’s specifications. The uptake of A\(_{23188}\)—AIB uptake was determined as described (27). Cells were incubated with Earle’s balanced salt solution containing 25 mM HEPES, 1.0 mM glucose (HG) and 1.5 mM glucose (NG) for 3 h, and then the medium was then replaced with the same buffer containing various concentrations of insulin (0–167 mM). After an incubation for 3 h, the uptake of \( ^{3}\)H]AIB (8 \( \mu \)M, 0.5 \( \mu \)Ci/tube) by the cells for 12 min was determined.

Statistical Analysis—Data are mean ± S.E. as indicated. \( p \) values were determined by Scheffe’s multiple comparison test, and \( p < 0.05 \) was considered statistically significant.

RESULTS

High Glucose-induced Insulin Receptor Dysfunction in Vivo—To investigate whether high glucose can attenuate insulin receptor kinase in HIIR cells, we Western blotted the protein of cells cultured with NG and HG media using aPY20. As shown in Fig. 1, the insulin-stimulated phosphorylation of both pp185 and insulin receptor \( \beta \)-subunits was reduced in the cells exposed to high glucose for 4 days, and incising these cells
with 0.1 μM pioglitazone increased the level of phosphorylation in HG cells in vivo. This was in accordance with our study, which showed that partially purified insulin receptors from HG cells had decreased autophosphorylation and tyrosine kinase activities and that pioglitazone improved the defective receptor kinase in HIRc cells (14).

Effects of High Glucose Culture on Protein Kinase C Activity—To investigate the molecular mechanism of high glucose-induced receptor kinase dysfunction, we tested whether PKC activation in HG cells interfered with insulin receptor kinase. After the cells were cultured with high glucose for 4 days, the PKC activities of both cytosolic and membrane fractions from HG cells purified by DEAE cellulose chromatography did not differ from those of NG cells as shown in Table I. Similarly, PDBu binding to the HG cells was 15% greater than that of NG cells after 30 min-high glucose incubation, but it was not statistically significant, as shown in Table II. Similarly, there was no change in PDBu binding to the cells exposed to high glucose for 4 days. Furthermore, 0.1 μM pioglitazone had no effects on either PKC activities or PDBu binding to those cells. Orthophosphate labeling also showed that the serine and threonine phosphorylation of insulin receptors in the basal state did not differ between NG and HG cells (data not shown). To assess in vivo PKC activity in HG cells, we studied the effect of H7, a potent PKC inhibitor on receptor kinase activity in HG cells. We found that basal kinase activities toward Glu/Tyr polymers were 10.6 ± 0.6, 5.1 ± 0.5, and 5.0 ± 0.2 pmol phosphate/pmol receptor/30 min in NG and HG cells with or without 1 μM H7, respectively, and the maximally insulin-stimulated kinase activities were 22.3 ± 1.8, 10.3 ± 0.6, and 11.3 ± 1.7 pmol phosphate/pmol receptor/30 min in NG and HG cells with or without 1 μM H7, respectively. These results indicated that co-incubating cells with H7, a potent PKC inhibitor for 4 days, failed to prevent the impairment of insulin receptor kinase in HG cells.

Effects of High Glucose Culture on Protein-tyrosine Phosphatase Activity—We next tested whether PTPase activity was altered in HG cells, since PTPase is thought to be an important regulator of insulin action (16). After the cells were cultured in high glucose for 4 days, the PTPase activities in both the cytosolic and particulate fractions were measured using 32p phosphorylated insulin receptors as a substrate. As shown in Fig. 2A, the cytosolic PTPase activities in HG cells increased by 2-fold (p < 0.01) when compared with NG cells. We also measured PTPase activities using an immunoenzymatic assay system using phosphorylated insulin receptors and αPY20. We reported that these assays correlated well (28). As summarized in Fig. 2B, the cytosolic PTPase activities in HG cells were significantly elevated 2-fold (p < 0.01) compared with those in NG cells. The particulate PTPase activities in HG cells also increased, but the increase was less than that of the cytosolic fraction. Thus, we confirmed that the cytosolic PTPase activity in HG cells is increased by two methods. Co-incubating cells with 0.1 μM pioglitazone inhibited the activation of the cytosolic PTPase (Fig. 2, A and B). This effect of pioglitazone upon PTPase activity was observed within 24 h (data not shown). However, co-incubating cells with 1 μM H7, a potent PKC inhibitor, failed to decrease cytosolic PTPase activity in HG cells (Fig. 2A). Furthermore, incubating cells with 100 nM TPA for 30 min had no effect on cytosolic PTPase activity (data not shown). As shown in Fig. 3, cytosolic PTPase activities in HG cells significantly increased at 1 and 6 h of culture compared with those in NG cells (p < 0.05), and the increase was 44 and 68%, respectively, of the maximal effect obtained after culture for 4 days. On the other hand, the increase in particulate PTPase activities was not significant after a 1- or 6-h incubation, and it reached only 48.7% above the basal level after culture in high glucose for 4 days as shown in Fig. 3.

PTP1B is considered to be involved in insulin action. As shown in Fig. 4, the amount of PTP1B in the cytosolic fraction of HG cells was significantly elevated, which was consistent with the increased PTPase activity in the cytosolic fraction in HG cells. On the other hand, the change in the PTP1B content in the particulate fraction was not significant between NG and HG cells, even though PTP1B was dominantly localized in the
Fig. 2. Protein-tyrosine phosphatase (PTPase) activities in NG and HG cells. A, PTPase activities were detected using 32P phosphorylated insulin receptors in NG and HG cells. Autoradiogram of dephosphorylated insulin receptors incubated with the cytosolic fraction obtained from NG and HG cells. No treatment (lanes 1 and 9), NG (lanes 2 and 8), HG (lanes 3–7), 0.1 μM pioglitazone (AD; lanes 4 and 6), 1 μM H7 (lane 5). B, PTPase activities determined by an immunoenzymatic assay in NG and HG cells. PTPase activities were determined using an immunoenzymatic assay system that included a phosphorylated insulin receptor (150 fmol) and a monoclonal phosphotyrosine antibody (αPY20). PTPase activities time- and dose-dependently increased within the range between 15 and 45 min and between 10 and 50 μg of protein, respectively (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 A600 of dephosphorylated receptors (measured by incubating insulin receptors in the presence of phosphatases) over that of control receptors (incubated in the absence of phosphatases). Each value is presented as the means of five separate experiments in quadruplicate (± S.E.). *p < 0.01 versus PTPase activity in the other groups, and **p < 0.01 versus PTPase activity compared with the normal glucose group by Scheffe's multiple comparison test.

Fig. 3. Time course of changes in PTPase activities in HG cells. Cytosolic and particulate PTPase activities were measured after the indicated time periods. *p < 0.05; **p < 0.01 versus cytosolic PTPase activity in NG cells (time 0); #p < 0.05 versus particulate PTPase activity in NG cells (time 0). Each point is presented as the mean ± S.E. of three to five experiments. Inset shows time course within 6 h.

We studied the effects of anti-PTP1B antibody on PTPase activities. As shown in Fig. 5, adding anti-PTP1B (125 μg/tube) to the assay system efficiently inhibited the increment of cytosolic PTPase activity in HG cells. We observed a similar tendency in the particulate fraction, but it was not statistically significant.

Thiazolidine Derivatives Improve High Glucose-induced Insulin Resistance—Finally, to test whether high glucose actually induced insulin resistance, we measured the insulin-stimulated AIB uptake in HIRc cells. Both the basal and maximally insulin-stimulated uptake of AIB was significantly decreased in HG cells. As shown in Fig. 6, the dose-response curve for insulin-stimulated AIB uptake in HG cells shifted to the right compared with NG cells (ED50: 24.6 ± 8.1 nM for HG, 0.02 ± 1.5 nM for NG cells, p < 0.01, respectively) even though insulin binding to the NG and HG cells did not differ. Incubating the cells with the thiazolidine derivative, pioglitazone, significantly increased the basal and maximal insulin-stimulated uptake of AIB and normalized the insulin dose-response curve (ED50: 2.1 ± 0.5 nM). Troglitazone, another thiazolidine derivative also increased the sensitivity of insulin-stimulated AIB uptake (ED50: 2.0 ± 0.6 nM) as shown in Fig. 6.

DISCUSSION

We studied hyperglycemia-induced insulin resistance by measuring the in vitro autophosphorylation and the tyrosine kinase activities of WGA-purified insulin receptors obtained from cells cultured in HG for 4 days. We found that the relatively chronic exposure of HIRc cells to high glucose led to impaired autophosphorylation and tyrosine kinase activity. Furthermore, the thiazolidine derivative, pioglitazone, com-
Fig. 4. Western blotting of PTP1B in the cytosolic and particulate fractions from NG and HG cells. A, samples from NG and HG cells were separated into cytosolic and particulate fractions, and PTP1B from both fractions (40 and 20 μg of cytosolic and particulate fractions, respectively) was immunoprecipitated using anti-PTP1B antibody. The immunoprecipitated proteins were resolved by SDS-PAGE and transferred to an Immobilon membrane (Millipore) by standard procedures. Immunoblotting was carried out using anti-PTP1B antibody, and proteins were visualized by means of enhanced chemiluminescence (ECL kit, Amersham Corp.) using a anti-rabbit antiserum. B, PTP1B content from each fraction was quantified by densiometric scanning. □, NG cells; ■, HG cells; △, HG cells with 0.1 μM pioglitazone; □, NG cells with 0.1 μM pioglitazone. Each column is presented as mean ± S.E. of three to five experiments. Statistically significance was determined by unpaired Student’s t test.

Fig. 5. Effects of αPTP1B antibody in PTPase activities in HG cells. PTPase activities were measured in the presence of either 125 μg of PTP1B antibody or preimmune IgG using the immunoenzymic assay. Each column is presented as the mean ± S.E. of three to four separate experiments. *p < 0.01 versus other high glucose groups, and †, p < 0.01 versus normal glucose groups by Scheffe’s multiple comparison test.

δ, ε, and ζ 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, 31). Furthermore, their results indicate that troglitazone, another thiazolidinedione derivative, prevents the rapid deactivation of insulin receptor kinase in HIRc cells by preventing PKC activation in HG cells (15). However, we could not find increased PKC activities in HG cells nor a pioglitazone effect upon PKC activities using two methods. Furthermore, we did not see any change in PKC activities in the presence of 4.5 μM troglitazone (data not shown). Consistent with these results, an orthophosphate labeling study showed that the serine and threonine residues in the insulin receptor did not increase by exposing cells to high glucose (data not shown). Furthermore, co-incubating the cells with 1 μM H7, a potent PKC inhibitor, failed to prevent the impairment of insulin receptor kinase. Although the possibility that the early transient activation of PKC (at most 15% increase in PDBu binding after 30 min-high glucose culture) has some effect on insulin receptor kinase afterward cannot be discarded, there was no persistent activation of PKC in HG cells.

PTPase is considered to be an important regulator of insulin action. Furthermore, its activation may produce insulin resistance (16), and abnormal regulation of PTPase has been reported in animals and patients resistant to insulin (32–34). In this study, we found that PTPase activity in the cytosolic fraction in HG cells was increased 2-fold compared with that of NG cells using two methods. This activation of cytosolic PTPase occurred within 1 h, and reached a plateau at 72 h. On the other hand, we reported that no changes in receptor kinase occurred within 1 h, and receptor kinase defects were observed only after 24 h (40% of the maximal effect) (14). These results suggested that the activation of PTPase occurs prior to the impairment of insulin receptor kinase. The amount of PTP1B, a PTPase involved in insulin action (16), was significantly increased in the cytosolic fraction in HG cells. Furthermore, the presence of anti-PTP1B antibody in the PTPase assay system significantly inhibited the increased cytosolic PTPase activities in HG cells, but preimmune IgG did not. On the other hand, anti-PTP1B antibody had little effect on PTPase activities in
NG cells. These results suggested that PTP1B does not significantly contribute to PTPase activities in NG cells. Although it is unclear whether other PTPases are also activated in NG cells, it is evident that at least the activity and content of PTP1B was increased in the cells exposed to high glucose. Furthermore, one of the thiazolidine derivatives, pioglitazone, normalized the increased PTPase activities with a significant decrease in level of PTP1B in the cytosolic fractions of HG cells. Therefore, cytosolic PTPase activity may be stimulated in the presence of high glucose, resulting in the decreased autophosphorylation of the insulin receptor and its kinase activity, and these agents may reverse these abnormal insulin receptor functions.

Concerning the regulation of PTPase activity in high glucose, the activation of PKC induced by TPA can stimulate PTPase activity in the soluble fraction of human erythrocytes (35). However, in rat adipocytes, TPA has no effect on PTPase activity. In our study, TPA had no effect on cytosolic PTPase activity in HGRC cells. Furthermore, incubating the cells with 1 μM TPA, a potent PKC inhibitor, failed to normalize the increased PTPase activity in HG cells. Although the regulatory mechanisms for gene expression of PTPases are not completely understood, the activation of PKC by TPA, insulin, and insulin-like growth factor I can promote the gene expression of PTP1B (36, 37). Currently, there is no clear explanation for the mechanism that may be responsible for the stimulation of cytosolic PTPase activity in HG cells. Further investigations are required to clarify the regulation of PTP1B activity including not only de novo synthesis of PTP1B protein but also its activation, which may be another mechanism of glucose-induced insulin resistance.

In our study, the relatively chronic exposure of HGRC cells to high glucose condition led to impaired insulin-stimulated AIB uptake accompanied by a decrease in the level of insulin receptor kinase and the activation of cytosolic PTPase activity. Pioglitazone normalized both the increased PTPase activity and the increase in PTPase content in the cytosolic fractions of HG cells and ameliorated the insulin sensitivity on AIB uptake with normalization of the insulin tyrosine kinase activities. Furthermore, troglitazone, another thiazolidine derivative also ameliorated insulin resistance in HG cells (Fig. 6). These results indicated that high glucose can impair insulin signaling via the activation of PTPase, as well as the insulin stimulation of both autophosphorylation of β-subunits of insulin receptor and phosphorylation of pp185. It is possible that the regulation of PTPase activity is a crucial step in high glucose-induced insulin resistance, and thiazolidine derivatives specifically improve the high glucose-induced desensitization of insulin receptor signaling via the normalization of PTPase activity.

Although further investigation is required to clarify the exact mechanisms of hyperglycemia-induced insulin resistance, these agents may be useful tools with which to clarify the mechanism of the impaired insulin receptor signaling in diabetes mellitus.

Acknowledgments—We thank Dr. J. M. Olefsky for the gift of HGRC cells, Dr. H. Fujio (Osaka University) for the gift of anti-phosphotyrosine antiserum, Dr. H. Ikeda (Takeda Chemical Industries, Ltd., Osaka, Japan) for the gift of pioglitazone, and Dr. H. Horikoshi (Sankyo Co. Ltd., Japan) for the gift of troglitazone and useful discussion.

REFERENCES
19. EBC Western Blotting Protocols, Amersham Corp., Arlington Heights, IL.
Hyperglycemia-induced Insulin Resistance