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High Glucose-Induced Abnormal Epidermal Growth Factor Signaling[1]

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We have reported that high glucose conditions (27 mM for 4 days) induces activation of protein tyrosine phosphatases (PTPases) which are associated with impaired insulin signaling in Rat 1 fibroblasts expressing human insulin receptors [Maegawa, H. *et al.* (1995) *J. Biol. Chem.* 270, 7724-7730]. In this study, we found increased mRNA-levels of a non-receptor type PTPase, protein tyrosine phosphatase 1B (PTP1B), and receptor type PTPases, leukocyte common antigen-related phosphatase (LAR), and LAR-related phosphatase (LRP), under high glucose conditions. In accordance with these results, LAR content was significantly increased, whereas LRP content was not increased. Cytosolic PTP1B content was increased, but membrane-associated PTP1B content showed no detectable change. Pioglitazone, a thiazolidinedione, normalized increased cytosolic PTPase activity through reduction of cytosolic PTP1B content, but it had no effect on mRNA levels of these PTPases. Under the high glucose condition, we also found that epidermal growth factor (EGF)-stimulated signaling, including tyrosine-phosphorylation of EGF receptor and phosphatidylinositol 3′-kinase activities, was attenuated. Nevertheless, pioglitazone failed to restore the attenuated EGF-signaling. These results indicate that the high glucose conditions cause dysfunction of EGF receptor. However, the increased cytosolic PTP1B content is not involved in the abnormal regulation of EGF-signaling, in contrast to insulin-signaling.

Key words: EGF, high glucose, insulin, pioglitazone, PTPase.

Non-insulin-dependent diabetes mellitus (NIDDM) is characterized by insulin resistance in insulin-sensitive peripheral tissues, particularly in the skeletal muscles (*1*). It is well established that hyperglycemia leads to insulin resistance, termed glucose toxicity, in patients with diabetes mellitus (*2, 3*). Several studies on insulin receptor function of skeletal muscles in NIDDM have revealed a reduction of the kinase activity of insulin receptors, which may be partially responsible for the decreased action of insulin in patients with NIDDM (*4-7*). Therefore, the enhancement of insulin sensitivity in patients with NIDDM is a possible treatment modality. The thiazolidinediones, pioglitazone, and troglitazone have been tested as new oral anti-diabetic drugs (*8-11*).

Recently, we have reported that insulin receptor kinase activity is impaired under high glucose conditions through activation of protein tyrosine phosphatase (PTPase) (*12, 13*). Thiazolidinedione, a potentiator of insulin signaling, ameliorates the impaired insulin receptor function through inhibition of PTPase activity (*13*). However, there is no evidence that such a high glucose effect is specifically expressed in insulin receptor signaling. Furthermore, PTPases are believed to play an important role in growth inhibition (*14*). For instance, if thiazolidinediones inhibit PTPase activity, it is possible that they may potentiate the signaling of a growth factor such as EGF. Questions arise, therefore, as to whether impaired signal transduction under high glucose conditions is specific for insulin or common to EGF-receptor signaling, and how thiazolidinediones affect PTPase activity.

Thus, we investigated the effect of high glucose conditions on EGF signaling using Rat 1 fibroblasts overexpressing human insulin receptor. We found that exposing cells to high glucose conditions desensitized EGF receptor function as well as insulin receptor function. However, pioglitazone failed to restore EGF receptor function. Thus, different

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Abbreviations: EGF, epidermal growth factor; PTP1B, protein-tyrosine phosphatase 1B; LAR, leukocyte common antigen-related phosphatase; LRP, LAR related phosphatase; IRS-1, insulin receptor substrate-1; MAP kinase, mitogen-activated protein kinase; SH, Src homology; Shc, Src homology/collagen; GST, glutathione-S-transferase; ECL, enhanced chemiluminescence; DMEM, Dulbecco's modified essential medium; BSA, bovine serum albumin; PI 3′-kinase, phosphatidylinositol 3′-kinase; MBP, myelin basic protein; PKC, protein kinase C; PMSF, phenylmethylsulfonylfluoride; GDR, glucose disposal rate.

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mechanisms may be involved in regulation of the phosphorylation status of insulin receptor and EGF receptor under high glucose conditions.

MATERIALS AND METHODS

Materials—Purified porcine insulin was a gift from Eli Lilly (Indianapolis, IL). [γ-^{32}P]ATP, [α-^{32}P]dCTP, and [^{3}H]thymidine were purchased from New England Nuclear (Boston, MA). Protein G-Sepharose, glutathione-Sepharose 4B, and pGEX-3X vector were purchased from Pharmacia PL Biochemical (Uppsala, Sweden). Aprotinin, phenylmethylsulfonyl fluoride (PMSF), bisbenzimide Hoechst 33258 fluorochrome, and phosphatidylinositol were purchased from Sigma (St. Louis, MO). Monoclonal phosphotyrosine antibody (PY20) was purchased from ICN (Costa Mesa, CA). Anti-protein tyrosine phosphatase-1B (PTP1B) antibody was purchased from UBI (New York, NY). Polyclonal antibodies against leukocyte common antigen-related phosphatase (LAR), LAR-related phosphatase (LRP), and Src-homology/collagen (Shc) were from Transduction Laboratories (Lexington, KT). A polyvinylidenedifluoride (PVDF) membrane (Immobilon-P) was from Millipore (Bedford, MA). All other reagents were of analytical grade from either Nacalai Chemicals (Kyoto) or Wako Pure Chemicals (Osaka). Rat PTP1B cDNA was kindly provided by J. Kusari (Tulane University, New Orleans). Rat cDNA of LAR and LRP were provided by Dr. Tahir S. Pillay (University of California, San Diego).

Cell Culture—Rat 1 fibroblasts expressing human insulin receptors (HIRc), provided by Dr. J.M. Olefsky (University of California, San Diego), were recloned to obtain cells expressing about one-tenth the number of receptors of the original HIRc cells (*15*). These HIRc cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Sub-confluent HIRc cells ($\sim 5 \times 10^6$ cells) were cultured in 100-mm dishes for up to 4 days with either 5.5 mM glucose (NG) or 27 mM glucose (HG). The medium was changed every other day. To study the effect of pioglitazone on EGF-signaling, we incubated cells in NG or HG conditions in the presence or absence of 0.1 μM pioglitazone. No significant difference in cellular protein content (230-250 μg in each of 6-well dish) was found between NG and HG cells or between cells cultured with or without 0.1 μM pioglitazone.

Northern Blot Analysis—Northern blot analysis was performed as described previously (*16*). Total RNA isolated from HIRc cells by the acid guanidinium-phenol-chloroform (AGPC) method was electrophoresed through 1% agarose gels containing formaldehyde and transferred onto Nytran membranes (NY13, Schleicher and Schuell, Dassel, Germany). The rat PTP1B-, LAR-, or LRP-cDNA was labeled with [α-^{32}P]dCTP, then hybridized at 65°C overnight in a buffer containing 0.5 M Na_2HPO_4, 1% BSA, 1 mM EDTA, and 7% SDS. After washing and autoradiography, the intensity of the bands corresponding to PTPases was quantified by use of a phosphoimager (Molecular Imager, Bio Rad, Hercules, CA).

Immunoblotting—Immunoblotting was performed as described previously (*16*). After incubation in the presence or absence of 0.1 μM pioglitazone, NG and HG cells were further incubated with the same media in the absence of

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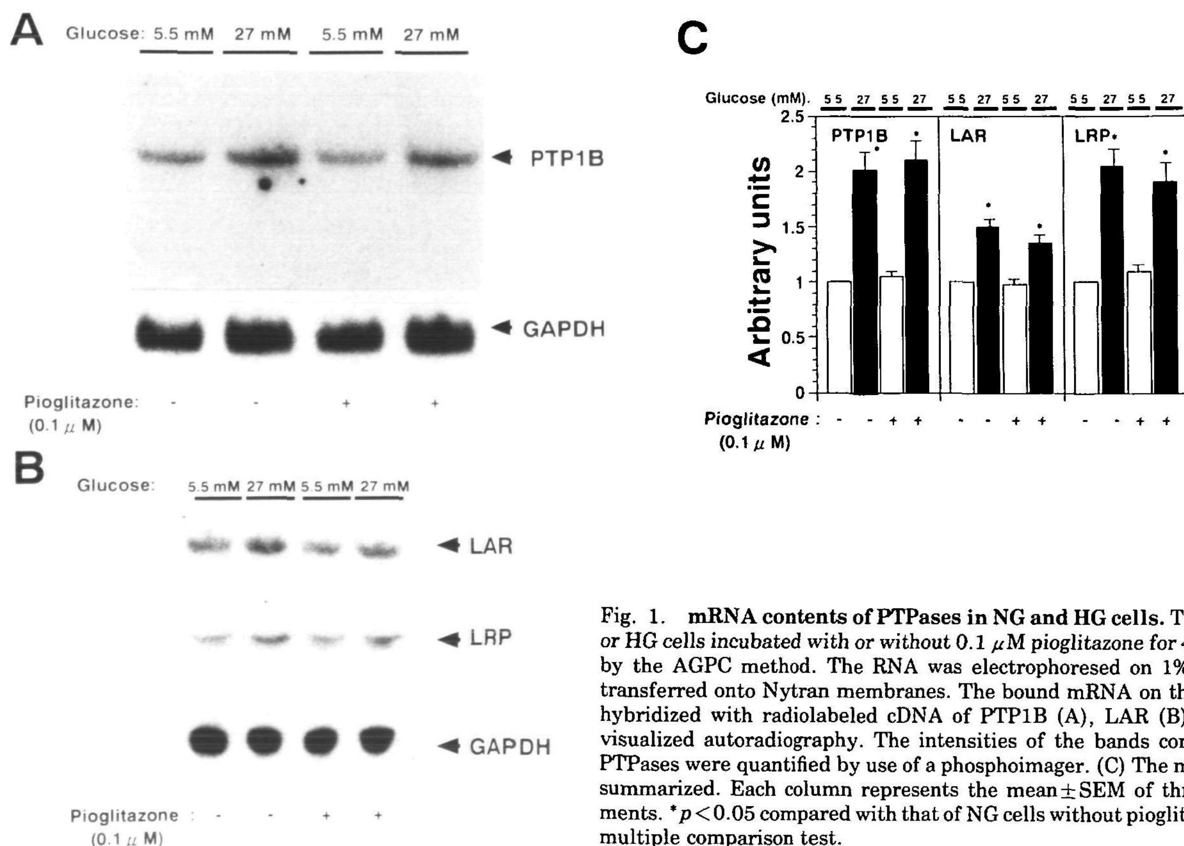

Fig. 1. **mRNA contents of PTPases in NG and HG cells.** Total RNA from NG or HG cells incubated with or without 0.1 μM pioglitazone for 4 days was isolated by the AGPC method. The RNA was electrophoresed on 1% agarose gels and transferred onto Nytran membranes. The bound mRNA on the membranes was hybridized with radiolabeled cDNA of PTP1B (A), LAR (B), or LRP (B) and visualized autoradiography. The intensities of the bands corresponding to the PTPases were quantified by use of a phosphoimager. (C) The mRNA contents are summarized. Each column represents the mean±SEM of three to five experiments. *$p < 0.05$ compared with that of NG cells without pioglitazone by Duncan's multiple comparison test.

fetal calf serum for 24 h, then stimulated with 100 nM EGF for indicated periods. After the stimulation, cells were washed with ice-cold PBS, then lysed in a solubilizing buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 140 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM PMSF, 50 mM NaF, and 50 μM aprotinin at 4°C for 20 min. The cell lysates were centrifuged at $15,000 \times g$ for 20 min. The supernatants were incubated with an indicated specific antibody for 3 h, then with protein G-Sepharose for a further 2 h. The bound proteins in the immunoprecipitates or aliquots from the soluble fractions of the lysates were resolved by SDS-PAGE, transferred onto PVDF membranes by electroblotting, then immunoblotted with indicated specific antibodies. Bound antibodies were detected with horseradish peroxidase-conjugated anti-mouse IgG and visualized with an Enhanced Chemi-Luminescence detection system (ECL, Amersham).

To separate cytosolic and particulate fractions, NG and HG cells were homogenized by use of a polytron in a buffer containing 50 mM HEPES-NaOH (pH 7.0), 5 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 0.2 mM PMSF, and 50 μM aprotinin. Aliquots were centrifuged at $500 \times g$ for 10 min at 4°C, and the supernatants were ultracentrifuged at $100,000 \times g$ for 60 min at 4°C. The final supernatants were designated as the cytosolic fraction. The pellets were solubilized in a buffer containing 50 mM HEPES-NaOH (pH 7.0), 5 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 0.2 mM PMSF, 50 μM aprotinin, and 0.5% Triton X-100 and designated as the particulate fraction as described previously (*13*).

Measurement of Phosphatidylinositol (PI) 3'-Kinase Activity—PI 3'-kinase activity immunoprecipitated with PY20 antibody was measured as described previously (*16*). After exposure to NG or HG conditions in the presence or absence of 0.1 μM pioglitazone, the cells were further incubated with the same media without serum for 24 h, then stimulated with 100 nM EGF for 5 min. The cells were lysed in 20 mM Tris-HCl (pH 7.5) containing 1% NP-40, 10% glycerol, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μM sodium orthovanadate, 1 mM PMSF, 0.1 mg/ml aprotinin, 1 μg/ml leupeptin, and then centrifuged. The supernatant (1.5 mg protein) was incubated with PY-20 antibody for 2 h, then incubated for 1 h with Protein G-Sepharose at 4°C. The immunoprecipitates were washed three times with PBS containing 1% NP-40, 100 μM sodium orthovanadate, three times with 100 mM Tris-HCl (pH 7.5), 500 mM LiCl, 100 μM sodium orthovanadate, and twice with 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 100 μM sodium orthovanadate. The pellets were suspended in 50 μl of 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 100 μM sodium orthovanadate. The reaction was initiated by the addition of 200 μM ATP, 30 μCi of [γ-^{32}P]ATP, 10 mM $MgCl_2$, 10 μg of phosphatidylinositol, allowed to proceed at 30°C for 10 min, and terminated with 20 μl of 8 N HCl. After extraction with chloroform/methanol (1:1), the lower organic phase was applied to a silica gel TLC plate. The plate was developed in methanol/chloroform/ammonia/water (100:70:15:25), dried and visualized by autoradiography. The radioactivity in the PIP was quantified by use of a phosphoimager.

Measurement of Mitogen-Activated Protein (MAP) Kinase Activity—MAP kinase activity was assayed *in vitro* using myelin basic protein (MBP) as a substrate as described previously (*17*). Briefly, after exposure to NG or HG conditions in the presence or absence of 0.1 μM pioglitazone, the cells were further incubated with the same media without serum for 24 h, then stimulated with 100 nM EGF for 10 min. Thereafter, the cells were lysed with 25 mM Tris-HCl (pH 7.4) containing 25 mM NaCl, 80 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin. After brief sonication and centrifugation, 10 μl of the obtained supernatant was assayed for kinase activity. The assay was conducted in a final volume of 40 μl containing 1 μM protein kinase inhibitor, 50 μM ATP, 2 μCi of [γ-^{32}P]ATP, and 20 μg of MBP at 25°C for 15 min. A 25-μl aliquot was spotted onto P81 phosphocellulose paper, a 2.3 cm in diameter (Whatmann, Clifton, NJ). The paper was washed with 180 mM phosphoric acid and rinsed with acetone. Phosphorylation was quantified by Cerenkov counting.

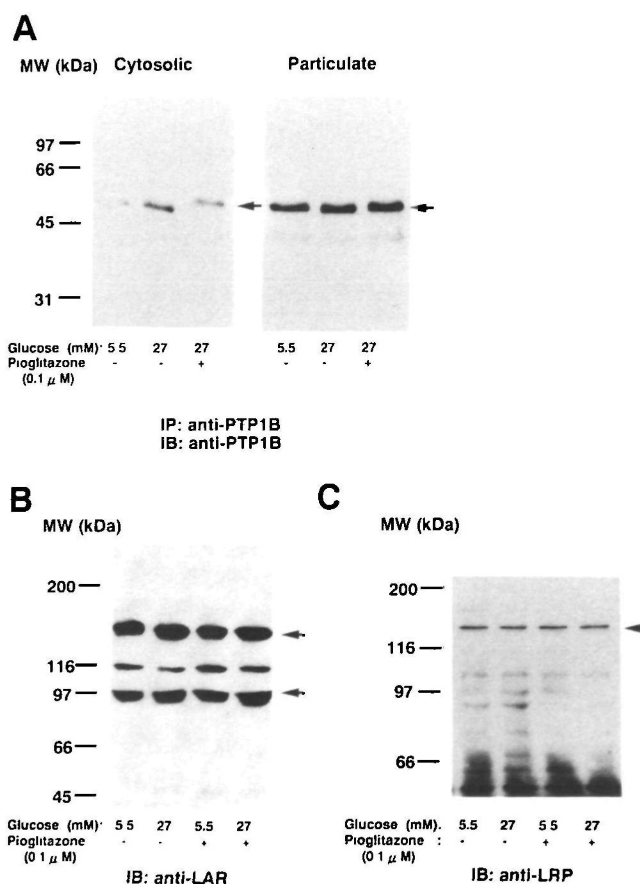

Fig. 2. **Western blotting of PTP1B (A), LAR (B), or LRP (C) in the cells from NG and HG cells.** (A) NG and HG cells were separated into cytosolic and particulate fractions, and PTP1B from both fractions (40 μg cytosolic fraction and 20 μg particulate fraction) was immunoprecipitated using anti-PTP1B antibody. The immunoprecipitated proteins were resolved by SDS-PAGE and transferred to Immobilon P by standard procedures. Immunoblotting was carried out using anti-PTP1B antibody and was visualized with anti-rabbit antiserum by ECL. (B, C) The total cell lysates from NG and HG cells with or without 0.1 μM pioglitazone were subjected to SDS-PAGE and immunoblotted with either anti-LAR or anti-LRP antibody. The bands corresponding PTP1B (A), LAR (B), or LRP (C) are indicated by arrows.

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Measurement of DNA Content—After incubating NG and HG cells in the presence or absence of 0.1 μM pioglitazone for 4 days in 24-well dishes, subconfluent cells in the wells were washed twice with 10% trichloroacetate and once with 95% ethanol. These cells were lysed with 1 N NaOH, then the solution was neutralized with 1 N HCl. DNA contents in these cells were measured fluorometrically using bisbenzimide Hoechst 33258 fluorochrome as described previously (*18*).

Statistics—The data are expressed as means$\pm$SE, unless otherwise stated. Duncan's multiple comparison tests were used to determine the significance of any difference between the two groups. $p<0.05$ was considered significant.

RESULTS

Effect of High Glucose Condition on mRNA Content of PTPases—We previously reported (*13*) that the cytosolic PTPase activity in HG cells increased by ~1.7-fold over that of the NG cells, and that particulate PTPase activity in HG cells was also increased to a lesser extent. Co-incubation with 0.1 μM pioglitazone significantly inhibited the activation of the cytosolic PTPase, but not that of the particulate enzyme.

In this study, to clarify the mechanism of increase of PTPase activity in HG cells, we examined the mRNA contents of PTPases including PTP1B, LAR, and LRP under high glucose conditions, because these PTPases were thought to be negative regulators of insulin- or EGF-signaling (*19-29*). As shown in Fig. 1A, PTP1B mRNA was detectable in NG cells and significantly increased by 2.0$\pm$0.2-fold in HG cells compared with NG cells ($p<0.05$). Though glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels may appear slightly higher in HG cells (Fig. 1A), the difference is not significant; essentially, these levels are not affected by either high glucose conditions or co-incubation with pioglitazone, as shown in Fig. 1B. So, we also calculated PTP1B mRNA levels normalized against GAPDH mRNA. These corrected PTP1B mRNA levels in NG cells, HG cells, NG+pioglitazone cells, and HG+pioglitazone cells were 1.0$\pm$0.0, 1.6$\pm$0.2 ($p<0.05$), 1.0$\pm$0.1, and 1.6$\pm$0.2 ($p<0.05$) arbitrary units (mean$\pm$SE), respectively. Furthermore, as shown in Fig. 1B, phosphoimaging analysis of LAR and LRP mRNA contents were also increased by 1.5$\pm$0.1 ($p<0.05$) and 2.1$\pm$0.2 ($p<0.05$)-fold compared with those in NG cells, respectively. However, neither PTP1B, LAR nor LRP mRNA contents were affected by exposing cells to 0.1 μM pioglitazone.

Effect of Pioglitazone-Treatment on PTPases in Cytosolic and Membrane Fractions—We next reconfirmed our previous data on the sub-cellular distribution of PTP1B (*13*), because PTP1B is reported to be predominantly localized in the endoplasmic reticulum, and translocation of PTP1B to cytoplasm (*30*) under the influence of high glucose effect may activate its PTPase activity. As shown in Fig. 2A, we confirmed that the amount of PTP1B in the cytosolic fraction in HG cells was significantly increased. The elevated PTP1B contents in cytosolic fraction in HG cells were attenuated by co-incubation with 0.1 μM pioglitazone. On the other hand, the amounts of PTP1B in the particulate fraction were similar under these conditions.

Next, we examined the amounts of LAR and LRP, since they are also involved in insulin action (*24-29*). LAR and LRP are localized on plasma membranes and do not translocate. LAR is initially synthesized as an approximately 200-kDa pro-protein, which is processed by protease into a complex of two non-covalently associated subunits: extracellular subunits (E-subunits) and phosphatase subunits (P-subunits). As shown in Fig. 2B, E (approx. 150 kDa) and P (approx. 85 kDa) subunits of LAR were slightly but significantly increased by 25.3$\pm$2.5% (mean$\pm$SE, $p<0.01$) in HG cells by densitometric analysis. Co-incubation with pioglitazone did not affect the amount of LAR. On the other hand, no significant change was found in LRP, as shown in Fig. 2C.

High Glucose Impaired EGF-Signaling—Insulin-stimulated phosphorylation of both IRS-1 and insulin receptor β-subunits was reduced in the cells exposed to high glucose conditions for 4 days as reported previously (*13*). Thus, we examined whether high glucose could also attenuate tyrosine-phosphorylation of EGF receptors in HIRc cells under the same conditions. As shown in Fig. 3, A and B, exposure of cells to high glucose media also led to attenuation of tyrosine-phosphorylation of EGF receptors as shown by the data of immunoprecipitation with either anti-phosphotyrosine antibody or anti-Shc antibody (<20% of NG cells).

However, as shown in Fig. 3B, pioglitazone did not normalize the impaired tyrosine-phosphorylation of EGF receptor. Furthermore, after EGF-stimulation phosphorylation levels of Shc were comparable in NG and HG cells

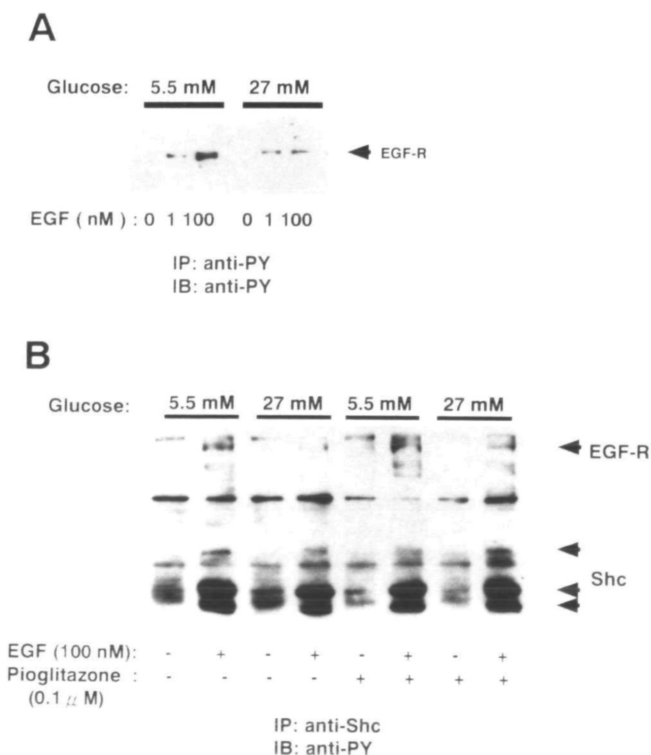

Fig. 3. **High glucose caused impaired EGF-signaling.** Quiescent cells were incubated with or without 1 nM and 100 nM EGF for 10 min at 37°C. Total cell lysates obtained after stimulation were immunoprecipitated with either PY20 (A) or anti-Shc antibody (B). Bound proteins were resolved by SDS-PAGE and transferred to Immobilon-P using the standard procedures. Immunoblotting was carried out using PY20 antibody and visualized with anti-mouse antiserum by ECL. Bands of EGF-R and Shc are indicated by arrows.

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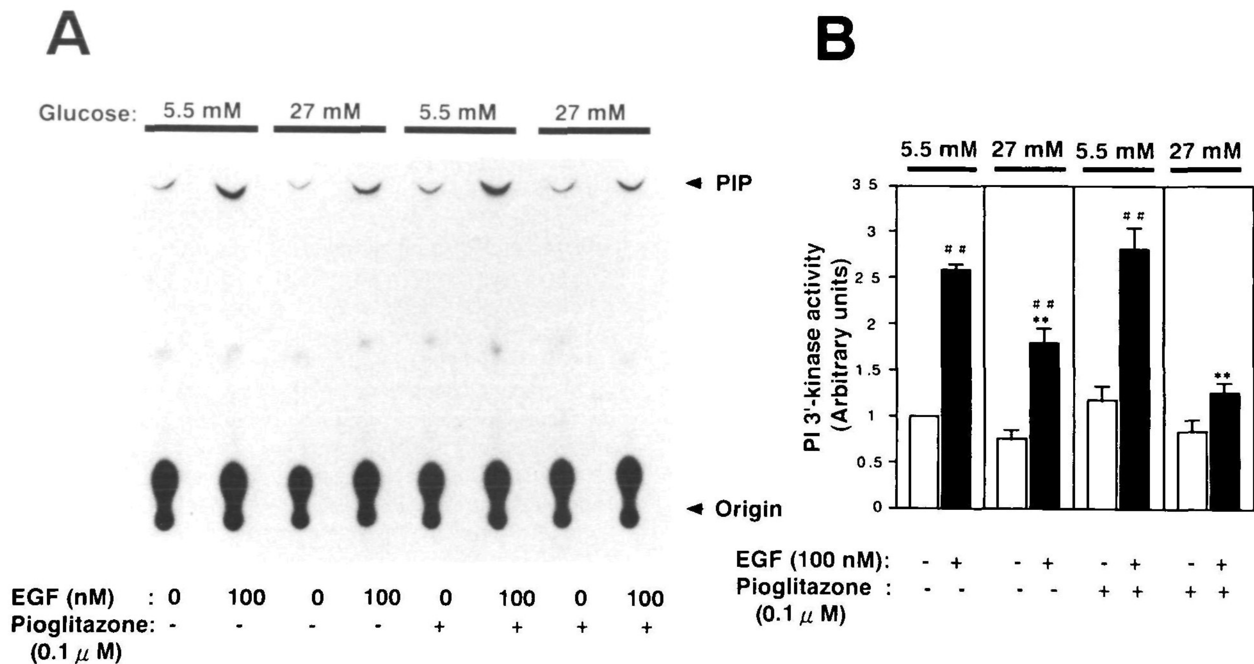

Fig. 4. **Effect of high glucose condition on EGF-stimulated PI 3′-kinase activity.** PI 3′-kinase activity was measured as described in "MATERIALS AND METHODS." Briefly, quiescent cells were stimulated with 100 nM EGF at 37°C for 5 min. Cells were lysed and immunoprecipitated with PY20. The PI 3′-kinase activity in the immunoprecipitates was measured by *in vitro* kinase assay and detected by TLC. [γ-^{32}P]Phosphatidylinositol 3′-phosphate (PIP) and origin are indicated by arrows. (B) Each column is presented as mean±SEM in arbitrary units of four experiments. Statistically significance was determined by Duncan's multiple comparison test. $^{**}p<0.01$ that of NG cells, $^{\#\#}p<0.01$ *vs.* that of the cells without EGF-stimulation.

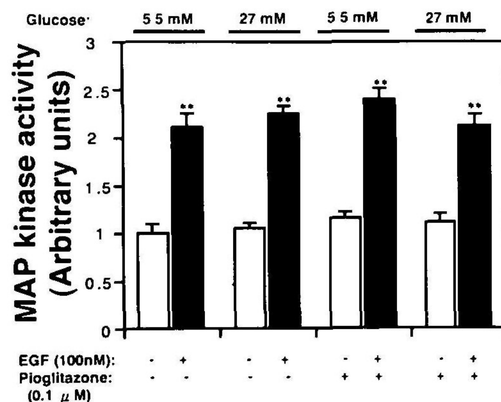

Fig. 5. **Effect of high glucose condition on EGF-stimulated both MAP kinase activity and thymidine incorporation.** MAP kinase activity was measured *in vitro* using myelin basic protein (MBP) as the substrate. NG and HG cells were incubated in the presence or absence of 0.1 μM pioglitazone for 4 days, then in the same media without serum for 24 h, and the quiescent cells were stimulated with 100 nM EGF for 10 min. Thereafter, the cells were homogenized in the buffer by brief sonication, then centrifuged. Resultant supernatant was subjected to MAP kinase assay at 25°C for 15 min. After the termination of reaction, the reaction mixture was spotted onto Whatmann P81 paper. The papers were washed, and the phosphorylation was quantified by Cerenkov counting. Each column is presented as mean±SEM in arbitrary units of three experiments. $^{**}p<0.01$ compared with that of the cells without EGF-stimulation by Duncan's multiple comparison test.

both in the presence and in the absence of pioglitazone, although complex formation of Shc and EGF receptor was expected in parallel with tyrosine-phosphorylation of EGF-receptor.

We next studied the effects of high glucose conditions and pioglitazone-treatment on EGF-stimulated PI 3′-kinase activity. After stimulation of cells with 100 nM EGF for 5 min, the total cell lysates were immunoprecipitated with anti-phosphotyrosine antibody. As shown in Fig. 4, high glucose conditions attenuated EGF-stimulated PI 3′-kinase activity. However, 0.1 μM pioglitazone failed to restore EGF receptor functions.

Effect of High Glucose Conditions on EGF-Stimulated MAP Kinase Activity—To evaluate the effect of high glucose conditions and pioglitazone treatment on the EGF-stimulated MAP kinase pathway, we examined EGF-stimulated MAP kinase activity. As shown in Fig. 5, EGF-stimulated MAP kinase activities were not different between NG cells and HG cells. Furthermore, 0.1 μM pioglitazone itself did not stimulate MAP kinase activity. We also measured DNA content in these cells in order to confirm the data of MAP kinase as a paradigm of mitogenic activity. These DNA contents in the NG cells, HG cells, NG+pioglitazone cells and HG+pioglitazone cells were 2.96±0.14, 2.82±0.15, 2.89±0.18, and 2.85±0.11 μg/well (24-well plate, mean±SE), respectively. There was no significant difference in DNA content among these cells.

DISCUSSION

We have been investigating the mechanism of induction of insulin resistance by hyperglycemia. Previously (*13*), we showed that the insulin-stimulated tyrosine-phosphorylation states of both insulin receptor and IRS-1 were impair-

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ed under high glucose conditions, and insulin-stimulated α-aminoisobutylic acid uptake was impaired in HIRc cells. The effects of high glucose culture were specific to glucose, but they were not specific under high osmolar conditions (*12*). Furthermore, these impaired insulin actions were restored by co-incubation with thiazolidinediones, and the mechanism of improvement was attributed to the inhibition of increased cytosolic PTPase activity, but not of PKC (*13*). In HG cells, the cytosolic PTPase activity was increased by approximately 1.7-fold when compared with the activity in NG cells, and the increased PTPase activity was attenuated by co-incubation with 0.1 μM pioglitazone (*13*). In addition, although PTPase activity in the particulate fraction was also increased by approximately 50% as compared with those of NG cells, pioglitazone did not significantly suppress this activity (*13*). However, it is still unclear whether impaired signal transduction under high glucose conditions is specific for insulin, and how thiazolidinediones affect PTPases.

In this study, we found increased mRNA levels of PTP1B, LAR, and LRP under high glucose conditions (Fig. 1). In accordance with these results, LAR content was increased (Fig. 2B), whereas LRP was not significantly increased (Fig. 2C). Furthermore, cytosolic PTP1B content was increased (Fig. 2A), and pioglitazone inhibited the increased cytosolic PTPase activity. However, treatment with pioglitazone had no effect on the mRNA expression levels. Under high glucose conditions, tyrosine-phosphorylation of EGF receptor (Fig. 3) and EGF-stimulated PI 3′-kinase activities (Fig. 4) were also markedly attenuated. However, pioglitazone also failed to restore the attenuated EGF-signaling.

It may be speculated that cytosolic and particulate PTPases have different roles in regulation of tyrosine-phosphorylation of various signaling molecules (*30*). In the current study (*12, 13*), we found that PTP1B content was increased in the cytosolic fraction in HG cells, and pioglitazone inhibited this increment of PTP1B content and brought about amelioration of impaired insulin action. Furthermore, it has also been reported that PTP1B physically associates with insulin receptors *in vivo* (*23*). Therefore, we believe that intracellular localization of PTPases and physical association with their substrates are critical in modulation of insulin receptor signal. Although the substrate specificity of the PTPase family is less stringent than that of the tyrosine kinase family based on *in vitro* experiments either using purified enzymes (*20*) or cells over-expressing these PTPases, cytosolic PTP1B content was constitutively increased in the cell line overexpressing PTP1B, and intracellular localization of PTP1B has already been changed in these transfected cells (*31*). Thus, we speculate that the insulin receptor may display substrate specificity *in vivo*, although it is still possible that increased activities of both cytosolic and membrane PTPase activities may be involved in dephosphorylation of both insulin and EGF receptors. Treatment with thiazolidinedion did not reverse the dysfunction of EGF receptors under high glucose conditions, while it did ameliorate the dysfunction of insulin receptor through normalization of cytosolic PTPase activity. Taking these results together, we may postulate that the dysfunction of EGF receptor is regulated not by PTP1B but by other PTPases, such as membrane-localized PTPases including LAR, which is not affected by pioglitazone treatment.

Concerning the mechanism of increase of PTPase activity under high glucose conditions, activation of PKC is suggested as a mediator (*32*) and increased mRNA content of the PTPase is also involved (*33*). Consistent with those studies, mRNA levels of PTPases including PTP1B, LAR, and LRP were increased under high glucose conditions in the present study. Thus, it is possible that activated PKC may increase mRNA content under high glucose conditions. However, in our previous study (*13*), TPA had no effect on cytosolic PTPase activity in HIRc cells. Furthermore, incubating the cells with 1 μM H7, a potent PKC inhibitor, did not both subcellular localization of PTP1B or PTPase activity under high glucose conditions (*12*). Thus, further studies are necessary to clarify the exact mechanisms for high glucose-induced activation of PTPases.

Consistent with the status of phosphorylation of Shc, abnormalities in EGF-stimulated MAP kinase activity that occur downstream of Shc were not observed in HG cells. Although the detailed mechanism is still unclear. Gotoh *et al.* have reported that a mutant EGF receptor lacking all major phosphorylation sites induced normal tyrosine-phosphorylation of Shc and retained mitogenic activities including MAP kinase (*34*). Sasaoka *et al.* have also reported the compensation of ErbB2 for impaired tyrosine-phosphorylation of EGF receptors, and EGF-stimulated tyrosine-phosphorylation of Shc and MAP kinase activity were retained (*35*). However, we did not observe the compensated tyrosine-phosphorylated bands corresponding to ErbB2 in an immunoblotting study, as shown in Fig. 3, A or B. Possible effects of thiazolidinediones on growth factor signaling through inhibiting PTPase activity, as shown in insulin receptor signaling, is an important issue affecting the clinical use of this drug. However, we observe no difference in DNA content among these cells. Law *et al.* have also reported that troglitazone, a kind of thiazolidinedione, inhibits vascular smooth muscle cell growth rather than stimulating it (*36*). The mechanism of inhibition of cell growth is unclear. According to our results, PI 3′-kinase activity was attenuated markedly on EGF-stimulation and slightly at the basal condition, and MAP kinase activity did not change. According to our present data, attenuated PI 3′-kinase activity did not affect on DNA content in the cell, but further study seems to be necessary.

Finally, regarding the action mechanism of thiazolidinediones, it has been shown that their effects are mediated through peroxisome proliferator-activated receptors (PPARs) which are present in heterodimers with another nuclear receptor, retinoic acid X receptor (RXR), and transactivated thiazolidinedione-responsive genes are triggered by the binding of thiazolidinediones to PPARs (*37*). Although this theory has been generally accepted, some reports suggest another action mechanism for thiazolidinediones. Lee and Olefsky reported that infusion of troglitazone *in vivo* during hyperinsulinemic euglycemic clamp-study acutely increased glucose disposal rate (GDR) in normal rats (*38*). The effect of thiazolidinedione appeared as early as 30 min after the intravenous administration. Furthermore, we observed that oxazolidine, another potentiator of insulin action, also acutely increased GDR and glycogen synthase activity associated with increased IRS-1 associated PI 3′-kinase activity (*39*). Thus, it is possible that the present rapid effect of thiazolidinedione does not

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need to be mediated through the activation of PPARs by this compound.

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