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Hyperglycemia Impairs the Insulin Signaling Step between PI 3-Kinase and Akt/PKB Activations in ZDF Rat Liver

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Akt/PKB activation is reportedly essential for insulin-induced glucose metabolism in the liver. During the hypoinsulinemic and hyperglycemic phase in the Zucker diabetic fatty (ZDF) rat liver, insulin-induced phosphorylations of the insulin receptor (IR) and insulin receptor substrate (IRS)-1/2 were significantly enhanced. Similarly, phosphatidylinositol (PI) 3-kinase activities associated with IRS-1/2 were markedly increased in ZDF rat liver compared with those in the control lean rat liver. However, interestingly, insulin-induced phosphorylation and kinase activation of Akt/PKB were severely suppressed. The restoration of normoglycemia by sodium-dependent glucose transporter (SGLT) inhibitor to ZDF rats normalized elevated PI 3-kinase activation and phosphorylation of IR and IRS-1/2 to lean control rat levels. In addition, impaired insulin-induced Akt/PKB activation was also normalized. These results suggest that chronic hyperglycemia reduces the efficiency of the activation step from PI 3-kinase to Akt/PKB kinase and that this impairment is the molecular mechanism underlying hyperglycemia-induced insulin resistance in the liver.

Key Words: **Akt/PKB; insulin; hyperglycemia; ZDF rat; T-1095.**

Insulin binds to the IR and transmits intracellular signals downstream, eventually leading to various metabolic as well as mitogenic cellular activities. Although much still remains unknown regarding the insulin signaling pathway, IRS-1 and IRS-2 are known to be the major substrates of activated IR (1–4). Tyrosine phosphorylated IRS-1/2 reportedly associate with several SH2 domain-containing proteins including PI 3-kinase (5–8). The synthesis of lipid products by PI 3-kinase, such as PI (3,4)P_2 or PI (3,4,5)P_3, is reportedly involved in the activation of Akt/PKB, which exists as a homodimer including plestkrin homology (PH) and catalytic domains (9, 10). The catalytic domain of Akt/PKB possesses serine/threonine kinase activity, which is regulated by the phosphorylation of Thr308 and Ser473 by PDK-1 and PDK-2, respectively (11–14).

To date, it has been demonstrated that Akt/PKB activation plays an essential role in insulin-induced glycogen synthesis by inhibiting the activity of glycogen synthetase kinase (GSK)-3 in liver and muscle (15, 16). In the present study, in order to elucidate the effect of hyperglycemia on insulin signaling pathways, ZDF rats, at the hypoinsulinemia and hyperglycemia stage, were used. In addition, to remove the effect of hyperglycemia in ZDF rats, an oral SGLT inhibitor, T-1095, was given by being mixed in food. Based on these experiments, we herein discuss the molecular mechanism underlying hyperglycemia-induced insulin resistance.

MATERIALS and METHODS

Materials. Affinity-purified antibodies against IRS-1 and IRS-2 were prepared as previously described. Anti-phosphotyrosine monoclonal antibody (4G10) and anti-p85α antibodies were purchased from Upstate Biotechnology Institute (Lake Placid, NY). Affinity-purified antibodies against Akt/PKB and phosphorylated Akt (Ser 473) were purchased from New England Labs. Reagents for SDS-PAGE and immunoblotting were obtained from Bio-Rad Laboratories (Richmond, CA), NP-40 from Nacalai Tesque Co. Ltd. (Tokyo, Japan), phosphatidylinositol and streptozotocin from Sigma Co. Ltd.

Abbreviations used: SGLT, sodium-dependent glucose transporter; IR, insulin receptor; IRS, insulin receptor substrate; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B; ZDF, Zucker diabetic fatty.

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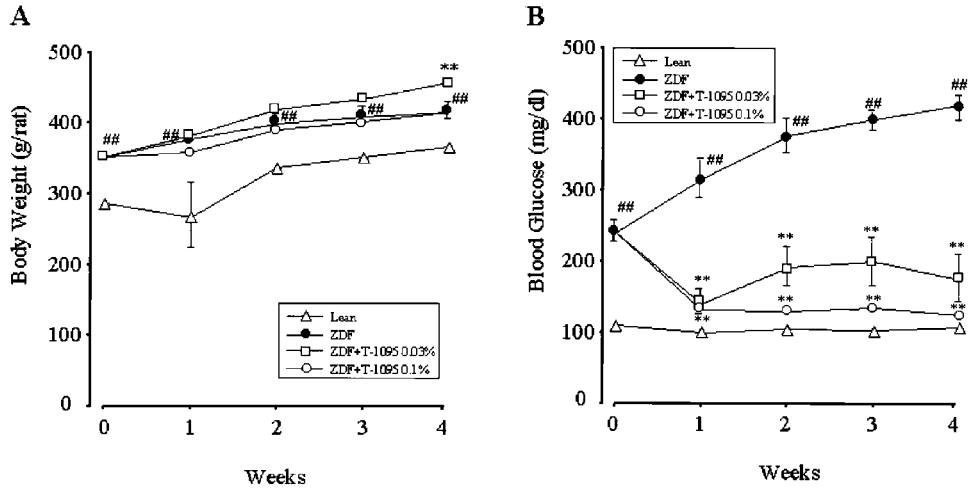

FIG. 1. Effects of T-1095 on body weight (A) and blood glucose levels (B) in ZDF rats ($n = 6$). ZDF rats were given T-1095 for 4 weeks. Each bar represents the mean ± SE. $^{\#\#}P < 0.01$ vs lean rat. $^{*}P < 0.05$, $^{**}P < 0.01$ vs ZDF rat.

(St. Louis, MO), silica gel thin-layer chromatography plates from Merck (Gibbstown, NJ), [γ-^{32}P]ATP from NEN-DuPont (Wilmington, NY), PVDF membrane (W20005, 0.2 μm) from Scleicher & Schuell (Dassel, Germany) and Novolin-U from Novo-Nordisk Co. Ltd. (Copenhagen, Denmark). Protein A–Sepharose 6MB and protein G–Sepharose 4FF were purchased from Pharmacia Co. Ltd. (Uppsala, Sweden). All other reagents were from Wako Chemicals Co. Ltd. (Osaka, Japan).

Animals. ZDF (fa/fa) rats and their normoglycemic lean control (fa/−) counterparts were obtained from Charles River Japan (Tokyo, Japan). Nine-week-old ZDF rats were given food mixed with or without T-1095 (Tanabe Seiyaku Co.Ltd., Osaka, Japan) for four weeks. The food was withdrawn 18 h before the experiments. Under anesthesia with pentobarbital sodium 60 mg/kg i.p., the abdominal cavity was opened and 10 μmol/l insulin in 4 ml of normal saline (0.9% NaCl) was injected into the portal vein. Livers were removed 30 s later, and immediately homogenized in a 6 × vol of homogenization buffer A with a Polytron homogenizer for 30 s. Homogenization buffer A was composed of 1% Triton X-100, 50 mmol/l HEPES (pH 7.0), 100 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride (NaF), 10 mmol/l EDTA-2Na, 50 mmol/l β-glycerophosphate, 10 mmol/l sodium vanadate, 2 mmol/l phenylmethylsulfonyl fluoride (PMSF), and 0.1 mg/ml aprotinin. Both extracts were centrifuged at 15,000 × g at 4°C for 30 min to remove insoluble material, and the supernatants were used as samples for immunoprecipitation and immunoblotting (17).

Immunoprecipitation and immunoblotting. Protocols for this experiment were described previously. In brief, supernatants containing equal amounts of protein were incubated with anti-IRS-1 antibodies (10 μg/ml), anti-IRS-2 antibodies (10 μg/ml), anti-Akt antibodies, 4G10 or anti-p85α antibodies, then incubated with phosphate-buffered saline and boiled in Laemmli sample buffer containing 100 mmol/l dithiothreitol (DTT). Total lysates or immunoprecipitated proteins were subjected to SDS-PAGE (7.5 or 10% Tris acrylamide) and electrotransferred to nitrocellulose membranes for immunoblotting with enhanced chemiluminescence (ECL kit, Amersham Life Science, Buckinghanshire, England). Band intensities were quantified with a Molecular Imager GS-525 using Imaging Screen-CH (Bio-Rad Laboratories, Hercules, CA).

Assay of PI3-kinase activity. After insulin injection into the portal vein, portions of the liver were removed and immediately homogenized in 10 vol of ice-cold solubilization buffer with a Polytron homogenizer for 30 s and then left on ice for 30 min. The solubilization buffer was composed of 50 mmol/l HEPES (pH 7.0), 137 mmol/l NaCl, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 2 mmol/l sodium vanadate, 10 mmol/l sodium pyrophosphate, 10 mmol/l NaF, 2 mmol/l EDTA-2Na, 1% NP-40, 10% glycerol, 2 μg/ml aprotinin, 5 μg/ml leupeptin, and 34 μg/ml PMSF. The homogenates were subjected to centrifugation at 15,000 × g for 30 min at 4°C, and the supernatants were used as samples. IRS-1 and IRS-2 proteins were immunoprecipitated with anti-IRS-1 and anti-IRS-2 antibodies, respectively, followed by protein A–Sepharose 6MB. PI 3-kinase activities in the immunoprecipitates were assayed as previously described (17).

Akt/PKB kinase activity. Samples immunoprecipitated with anti-Akt antibodies were washed three times with lysis buffer and then twice with kinase buffer (50 mmol/l Tris, pH 7.5, 10 mmol/l $MgCl_2$, 1 mmol/l DTT). Beads were resuspended in 50 μl of kinase buffer containing 5 μmol/l ATP, 30 μg/ml GSK-3 protein, 10 mCi/ml [γ-^{32}P]ATP and incubated at 30°C for 30 min (18).

Statistical analysis. The unpaired Student t test was used to compare control and T-1095 treatment results. All analyses were performed on a personal computer (Macintosh) using the statistical software package SuperANOVA (Abacus Concepts, Inc., Berkeley, CA). Data are expressed as means ± SE.

RESULTS

Amelioration of Hyperglycemia by T-1095 Treatment

The body weights of ZDF rats were significantly higher than those of lean rats (Fig. 1A). Although treatment with 0.03% T-1095 for 4 weeks slightly increased body weights of ZDF rats, 0.1% T-1095 did not affect body weight. Blood glucose levels of ZDF rats in the fed state were apparently higher than those of lean rats, and continued to be elevated as the animals grew. T-1095 treatment dose-dependently lowered the elevated blood glucose levels of rats in the fed state (Fig. 1B). This hypoglycemic effect was observed at 1 week and continued for up to 4 weeks after the beginning of treatment. T-1095 treatment also lowered the elevated fasting blood glucose levels in ZDF rats (95.8, 132.5, 114.8 and 100.8 mg/dl in lean, ZDF, 0.03% and 0.1%

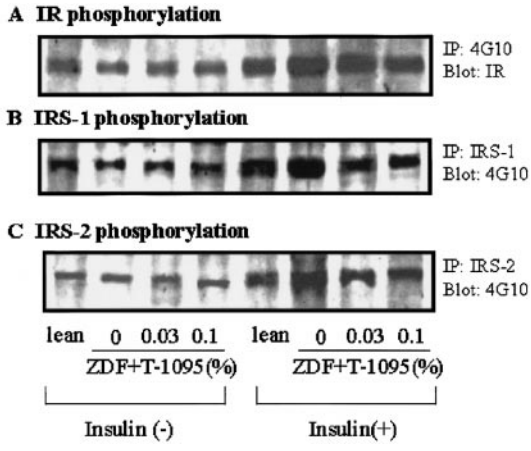

FIG. 2. Immunoblotting of phosphorylated IR, IRS-1, and IRS-2 in ZDF rat liver ($n = 6$). Thirty seconds after insulin injection, the liver was excised, homogenized , and immunoprecipitated with anti-IRS-1, anti-IRS-2, and 4G10 antibodies. Immunoprecipitates were blotted with the corresponding antibodies.

T-1095-treated ZDF rats, respectively, after 4 weeks of treatment). Therefore, not only fed but also fasting high blood glucose levels in ZDF rats were improved by T-1095 treatment, and were restored to nearly normal levels in with 0.1% T-1095.

Insulin Signaling From IR to PI 3-Kinase in ZDF Rat Liver

Next, we investigated the signaling pathways downstream from IR. Basal tyrosine phosphorylation levels of liver IR (Fig. 2A), IRS-1 (Fig. 2B) and IRS-2 (Fig. 2C) did not differ between lean and ZDF rats. There were no changes in the amounts of IR, IRS-1, IRS-2 and p85α proteins (data not shown). The levels of insulin-induced phosphorylation of IR, IRS-1 and IRS-2 in ZDF rats were apparently higher than those in lean rats.

Then, we examined PI 3-kinase activities associated with IRS-1 and IRS-2 in the liver under basal and insulin-stimulated conditions (Fig. 3). Although the basal PI 3-kinase activities associated with IRS-1/2 did not differ between ZDF and lean rats, those in the insulin-stimulated condition were markedly higher in ZDF than in lean rats.

With T-1095 treatment, enhanced phosphorylations of IR (Fig. 2A), IRS-1 (Fig. 2B) and IRS-2 (Fig. 2C), and the associated increases in PI 3-kinase activities (Fig. 4), in ZDF rats were all restored to the levels in lean control rats.

Akt Kinase Activity and Phosphorylation in ZDF Rat Liver

Basal phosphorylation and activity levels of Akt/PKB did not differ between ZDF and lean rats (Fig. 4B and 4C), although Akt/PKB protein amounts were slightly lower in ZDF than in lean rats (Fig. 4A). Akt/PKB phosphorylation under insulin-stimulated conditions was significantly decreased in ZDF rats as compared with lean rats, and this was observed as a reduced mobility shift of Akt/PKB (Fig. 4A) and the bands obtained by immunoblotting using a specific antibody recognizing the phosphorylated serine in Akt/PKB (Fig. 4B). As reflected by reduced serine phosphorylation, insulin-induced activation of Akt/PKB kinase activity was revealed to be significantly lower in ZDF rats.

On the other hand, in the livers of ZDF rats treated with T-1095, impaired insulin-induced phosphorylation (Fig. 4A and 4B) and kinase activation (Fig. 4C) of

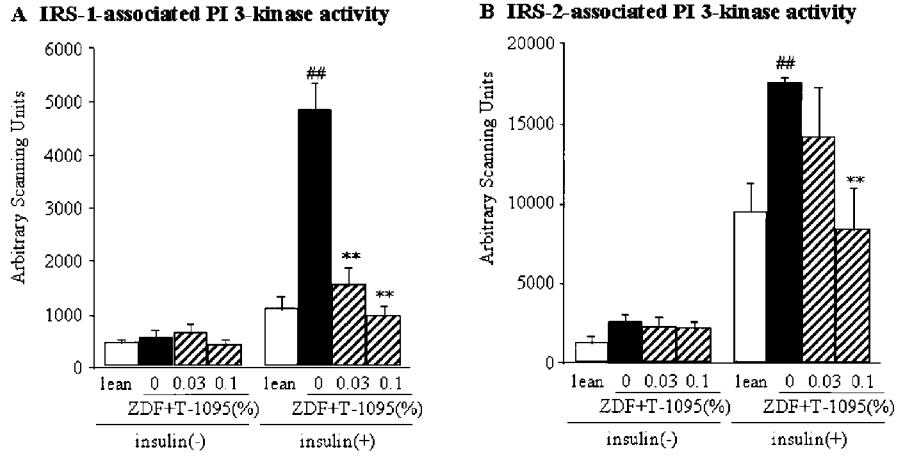

FIG. 3. PI 3-kinase activities associated with IRS-1 (A) and IRS-2 (B) in the ZDF rat liver ($n = 6$). Thirty seconds after insulin injection, the liver was excised, homogenized, and immunoprecipitated with anti-IRS-1 and anti-IRS-2 antibodies. PI 3-kinase activities in immunoprecipitates were assayed as described under Materials and Methods. Each bar represents the mean ± SE. $^{\#\#}P < 0.01$ vs lean rat. $^{*}P < 0.05$, $^{**}P < 0.01$ vs ZDF rat.

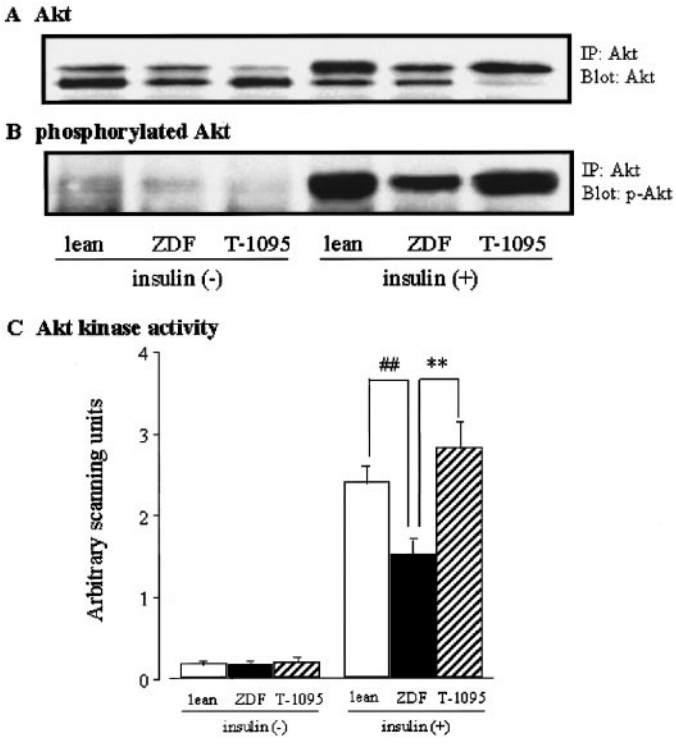

FIG. 4. Immunobloting of Akt/PKB (A) and phosphorylated Akt/PKB (B) and Akt kinase activity (C) in ZDF rat liver ($n = 3$). Rats were anesthetized and 10 μM insulin was injected into the portal vein. Thirty seconds later, the liver was excised, homogenized, and immunoprecipitated with anti-Akt/PKB antibody. Immunoprecipitates were either blotted with anti-Akt/PKB antibodies or assayed for Akt kinase activities. Each bar represents the mean ± SE. $^{\#\#}P < 0.01$ vs lean rat. $^{*}P < 0.05$, $^{**}P < 0.01$ vs ZDF rat.

Akt/PKB were revealed to be restored to nearly the levels in control lean rats.

DISCUSSION

The Zucker diabetic fatty (ZDF) rat, an obese and diabetic animal model, exhibits hyperinsulinemia and mild hyperglycemia at 6 weeks of age, and eventually becomes hypoinsulinemic and hyperglycemic at 10 or 11 weeks of age (19–21). In the present study, we used 9–13 week old ZDF rats showing hypoinsulinemia and marked hyperglycemia (22, 23). T-1095 inhibits SGLT activity located on renal proximal tubules resulting in increased excretion of glucose into urine (24–26). We have observed that hyperglycemia was ameliorated by oral administration of T-1095, and the elevated fasting glucose level was also normalized, indicating the amelioration of insulin resistance in ZDF rat liver (26). Thus, we investigated which insulin signaling step is impaired in the hyperglycemic state, and also examined whether or not this impaired signaling step is normalized by T-1095 treatment.

Although the molecular events in the insulin signaling pathway have not been fully elucidated yet, it is likely that Akt/PKB kinase activity plays a crucial role in the hepatic insulin action, at least in terms of glycogen synthesis (15, 27). Thus, we focused on the pathway leading to the activation of Akt/PKB kinase from IR. Firstly, we found that insulin-induced phosphorylation levels of IR, IRS-1 and IRS-2 were apparently elevated in ZDF rats. In good accordance with these observations, PI 3-kinase activities associated with IRS-1 and IRS-2 were also enhanced. However, although it is believed that the activation of Akt/PKB is located downstream from PI 3-kinase activation, the activation of Akt/PKB was markedly impaired in ZDF rats. Thus, there is clearly a discrepancy between PI 3-kinase activation and Akt/PKB activation in ZDF rat liver. Interestingly, in response to restoring normoglycemia with T-1095, the impaired insulin-induced Akt/PKB phosphorylation response and kinase activities and enhanced PI 3-kinase activation in ZDF rats recovered to normal levels. These results strongly suggest that chronic hyperglycemia is involved in this abnormality, interrupting efficient Akt/PKB activation via PI 3-kinase activation.

Recently, Kurowski *et al.* reported that Akt/PKB activation is impaired by incubation in high glucose containing medium, despite having no effect on the activation of PI 3-kinase, using stripped muscle of normal rats *in vitro* (28). Our results obtained using *in vivo* system also support the hypothesis that hyperglycemia-induced insulin resistance may be attributable to the impaired insulin signaling step between PI 3-kinase and Akt/PKB activations. The activation of Akt/PKB is reportedly induced by the phosphorylation of Thr308 and Ser473 by PDK-1 and PDK-2, respectively (11–14). Although we cannot pinpoint the step impaired by hyperglycemia, we can raise several possibilities. Firstly, alteration of the expression levels of PDK-1/2 or some modification of PDK-1/2 affecting its kinase activity is possible. Another possibility is that some PI 3-phosphatase activity may be enhanced under hyperglycemic conditions, and the cellular contents of PI (3,4)P_2 and PI (3,4,5)P_3 may be decreased despite enhanced PI 3-kinase activity. In addition, we can speculate as to many mechanisms which cause a discrepancy between the degrees of PI 3-kinase and Akt/PKB activations. We believe that clarification of this issue may lead to an understanding of the precise molecular mechanism underlying hyperglycemia-induced insulin resistance.

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