Inhibitory effect of hyperglycemia on insulin-induced Akt/protein kinase B activation in skeletal muscle

AKIRA OKU,1,2* MASAO NAWANO,1,2* KIICHIRO UETA,1 TAKUYA FUJITA,1 ITSURO UMEBAYASHI,1 KENJI ARAKAWA,1 TOMOMI KANO-ISHIHARA,1 AKIRA SAITO,1 MOTONOBU ANAI,2 MAKOTO FUNAKI,3 MASAYOSHI KIKUCHI,3 YOSHITOMO OKA,4 AND TOMOICHIRO ASANO2

1Discovery Research Laboratory, Tanabe Seiyaku Co. Ltd., Saitama 335-8505; 2Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113-8655; 3Institute for Adult Disease, Asahi Life Foundation, Tokyo 116; and 4Third Department of Internal Medicine, Faculty of Medicine, University of Yamaguchi, Yamaguchi 755, Japan

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Oku, Akira, Masao Nawano, Kiichiro Ueta, Takuya Fujita, Itsuro Umebayashi, Kenji Arakawa, Tomomi Kano-Ishihara, Akira Saito, Motonobu Anai, Makoto Funaki, Masayoshi Kikuchi, Yoshitomo Oka, and Tomoichiro Asano. Inhibitory effect of hyperglycemia on insulin-induced Akt/protein kinase B activation in skeletal muscle. Am J Physiol Endocrinol Metab 280: E816–E824, 2001.—To determine the molecular mechanism underlying hyperglycemia-induced insulin resistance in skeletal muscles, postreceptor insulin-signaling events were assessed in skeletal muscles of neonatally streptozotocin-treated diabetic rats. In isolated soleus muscle of the diabetic rats, insulin-stimulated 2-deoxyglucose uptake, glucose oxidation, and lactate release were all significantly decreased compared with normal rats. Similarly, insulin-induced phosphorylation and activation of Akt/protein kinase B (PKB) and GLUT-4 translocation were severely impaired. However, the upstream signal, including phosphorylation of the insulin receptor (IR) and insulin receptor substrate (IRS)-1 and -2 and activity of phosphatidylinositol (PI) 3-kinase associated with IRS-1/2, was enhanced. The amelioration of hyperglycemia by T-1095, a Na+–glucose transporter inhibitor, normalized Akt/PKB phosphorylation and activity. In addition, the enhanced PI 3-kinase activation and phosphorylation of IR and IRS-1 and -2 were reduced to normal levels. These results suggest that sustained hyperglycemia impairs the insulin-signaling steps between PI 3-kinase and Akt/PKB, and that impaired Akt/PKB activity underlies hyperglycemia-induced insulin resistance in skeletal muscle.

Akt/protein kinase B; insulin resistance; streptozotocin; T-1095

Hyperglycemia is believed to contribute to the development of peripheral insulin resistance associated with both type 1 and type 2 diabetes (42). Many reports support the significant correlation between blood glucose level and peripheral insulin resistance (14, 19). For example, peripheral insulin resistance in diabetic animals is reversed by phlorizin (11, 23, 43) or a Na+-glucose cotransporter (SGLT) inhibitor (38, 41), which improved blood glucose levels by inhibiting the reabsorption of glucose in renal proximal tubules. In addition, the insulin resistance in patients of type 1 and type 2 diabetes is reversed by a strict blood-glucose control (55, 56). These findings strongly suggest that the chronic hyperglycemia, per se, can impair insulin action in peripheral tissues of both humans and experimental animals.

Although much of the intracellular signaling pathway of insulin is still unknown, the cascade leading to cellular metabolic responses includes insulin receptor autophosphorylation (33), tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and IRS-2 (6, 54), and activation of phosphatidylinositol (PI) 3-kinase associated with IRS proteins (13, 22, 24, 37). One of the downstream targets of PI 3-kinase is a serine/threonine kinase, Akt/protein kinase B (PKB) (4, 12, 28, 44), which is presumably activated through phosphorylation of Thr308 and Ser473 by phosphatidylinositol 3,4,5-triphosphate-dependent protein kinase (PDK)-1 and PDK-2, respectively (3, 5, 7, 27). It has been demonstrated that Akt/PKB activation plays an essential role in insulin-induced glucose transport, GLUT-4 translocation, and glycogen synthesis (20, 29, 34, 35, 48, 50). A recent study using dominant negative mutants of Akt/PKB expressed in L6 myoblasts suggested that Akt/PKB is necessary for insulin-induced GLUT-4 translocation (53). However, overexpression of an inhibitory Akt/PKB mutant in 3T3-L1 adipocytes did not alter the stimulation of glucose transport and GLUT-4 translocation by insulin (25). Therefore, whether Akt/PKB is directly coupled to glucose transport remains a topic of debate.

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We have reported that hyperglycemia impairs insulin sensitivity (38) and insulin-stimulated phosphorylation/activation of Akt/PKB (39) in the liver of Zucker diabetic fatty (ZDF) rats. In addition, Kurowski et al. (32) reported that insulin-induced Akt/PKB activation is reduced in skeletal muscles incubated in medium containing glucose at high concentration, without alteration of PI-3 kinase activity. Furthermore, Krook et al. (31) and Song et al. (45) reported that insulin-induced Akt/PKB activation is decreased in skeletal muscles of the diabetic Goto-Kakizaki (GK) rat (31, 45). These results suggest that the reduced activation of Akt/PKB is a candidate for defects leading to insulin resistance caused by hyperglycemia in both liver and skeletal muscles.

In the above reports (31, 45), the investigators used phlorizin to normalize blood glucose levels in GK rats and to explore the possibility that the reduction of Akt/PKB activation is caused by alterations in the metabolic milieu. Phlorizin treatment in GK rats improved the reduced Akt/PKB activation in skeletal muscle but normalized the blood glucose levels only in the fasted and not in the fed state (31). Because the rats had free access to food in their study, hyperglycemia is presumably not improved during most of the experimental period. In addition, because the authors did not show the data of phlorizin-treated normal (normoglycemic) rats, the possibility exists that phlorizin directly affects the Akt/PKB activity. Therefore, it is still not clear whether the impairment of Akt/PKB activation in skeletal muscle is attributed to hyperglycemia in vivo.

To gain further insight into the molecular mechanism underlying the hyperglycemia-induced insulin resistance in skeletal muscles, we investigated the intracellular insulin-signal cascade, including the insulin receptor, IRS proteins, PI-3 kinase, and Akt/PKB in neonatally streptozotocin-treated diabetic (nSTZ) rats. In addition, we treated nSTZ rats with an orally active SGLT inhibitor, T-1095 (40, 49), to remove the effect of hyperglycemia.

MATERIALS AND METHODS

Materials. Affinity-purified antibodies against IRS-1 and IRS-2 were prepared as previously described (2). Anti-phosphotyrosine (4G10), anti-GLUT-4, and anti-phospho GSK3β antibodies were purchased from Upstate Biotechnology Institute (Lake Placid, NY). Affinity-purified antibodies against Akt/PKB and phosphorylated Akt/PKB (Ser 473) were purchased from New England Biolabs (Beverly, MA). Anti-insulin receptor (β-subunit) antibody was purchased from Transduction Laboratories (Lexington, KY). We obtained reagents for SDS-PAGE and immunoblotting from Bio-Rad Laboratories (Richmond, CA), NP-40 from Nacalai Tesque (Tokyo, Japan), phosphatidylinositol and silica gel thin-layer chromatography plates from Merck (Gibbstown, NJ), [γ-32P]ATP from NEN-Du Pont (Wilmington, DE), polyvinylidene difluoride (PVDF) membrane from Schleicher & Schuell (Dassel, Germany), and human insulin (Humulin R40) from Eli Lilly (Indianapolis, IN). Protein A-Sepharose 4FF, protein G-Sepharose 4PF, and peroxidase-linked secondary antibodies were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). All other reagents were from Wako Pure Chemicals (Osaka, Japan).

Animals. Male Sprague-Dawley rats (Japan SLC, Shizuka, Japan) were injected with streptozotocin (Sigma, St. Louis, MO) intraperitoneally (60 mg/kg in 50 mmol/l citrate buffer, pH 4.5) 6 days after birth (nSTZ rats). Normal (non-diabetic) groups received the buffer only. All animals were weaned 21 days after birth and were housed in stainless wire cages and given normal laboratory chow (CE-2, CLEA Japan, Tokyo, Japan) and water ad libitum. Six-week-old normal and nSTZ rats were given food mixed with or without 0.1% (wt/wt) T-1095 (Tanabe Seiyaku, Osaka, Japan) for 4 wk. Blood glucose level was determined with commercially available kits based on the glucose oxidase method (New Blood Sugar Test, Boehringer Mannheim, Mannheim, Germany). Plasma insulin levels were assayed using an ELISA kit (Seikagaku Tokyo, Tokyo, Japan) with rat insulin as the standard. All animal experiments had approval of the animal ethics committee in Tanabe Seiyaku.

2-Deoxyglucose uptake, lactate release, and glucose oxidation in soleus muscle. After 4 wk of treatment with or without T-1095, soleus muscles from animals anesthetized with penobarbital sodium (50 mg/kg ip) were isolated and split into two equal longitudinal portions. Isolated muscles were preincubated for 60 min at 35°C in 2 ml of oxygenated Krebs-Henseleit buffer (KRB) supplemented with 8 mmol/l glucose, 32 mmol/l mannitol, and 0.1% bovine serum albumin (BSA; Sigma, radioimmunoassay grade).

2-Deoxyglucose (2-DG) uptake and lactate release were measured by a modification of the method described previously by Hansen et al. (21). The muscles were insulin-stimulated for 30 min at 35°C in 2 ml of the KRB incubation medium. After the incubation, 50 μl of the medium were sampled for a lactate assay. Then muscles were rinsed for 12 min at 29°C in 2 ml of KRB containing 40 mmol/l mannitol, 0.1% BSA, and insulin (if it was present in the previous medium) and incubated for 20 min at 29°C in 1.5 ml of KRB containing 8 mmol/l 2-deoxy-D-[1,2-3H]glucose (2.25 μCi/ml; American Radiolabeled Chemicals, St. Louis, MO), 30 mmol/l [U-14C]mannitol (0.3 μCi/ml; American Radiolabeled Chemicals), 2 mmol/l pyruvate, 0.1% BSA, and insulin (if it was contained in the previous incubation). The muscles were blotted on a paper towel, dissolved in 1 ml of Soluene 350 (Packard, Meriden, CT), and counted for radioactivity. The extracellular space and intracellular 2-DG uptake were determined as described previously by Wallberg-Henriksson and Holloszy (52). Lactate concentration in the medium was determined by an enzymatic assay kit (Determiner LA, Kyowa Medics, Tokyo, Japan).

The aerobic glycolysis, i.e., glucose oxidation, was measured by a modification of the method described previously by Espinal et al. (17). After the preincubation, muscles were incubated for 60 min at 35°C in 2 ml of KRB containing 8 mmol/l [U-14C]glucose (0.4 μCi/ml), 32 mmol/l mannitol, and 0.1% BSA. The reaction vial was connected to a trapping vial containing 0.5 ml of 20% HClO4 and filter paper (no. 3, Whatman, Clifton, NJ) moistened by 0.3 ml CO2-trapping solution (phenethylamine-methanol, 1:1), and incubated at 37°C for 1 h. The radioactivity in the trapping vials was counted by a liquid scintillation counter (Tri Carb 4640, Packard).

The reaction vials were gassed continuously with 95% O2-5% CO2 (except the second incubation of the CO2 release experiment) and shaken 170–180 times per minute. GLUT-4 translocation in skeletal muscle. After 4 wk of treatment with or without T-1095, GLUT-4 translocation in skeletal muscle was measured by the method described pre-
Physiological parameters of animals. Physiological parameters of rats after 4 wk of treatment with or without T-1095 were summarized in Table 1. Diabetic nSTZ rats exhibited severe hyperglycemia and hypoinsulinemia. T-1095 improved not only fasted but also fed blood glucose levels in nSTZ rats. The fasted plasma insulin level of the T-1095-treated group was not significantly different from that of the corresponding control group in both normal and diabetic animals. The body weight of nSTZ rats was significantly lower than that of normal control rats. T-1095 decreased the body weight in normal rats, probably because of the urinary glucose loss, but it did not affect nSTZ rats, as we reported previously (41). Although the food intake of rats was not measured in this study, we estimate that intake of T-1095 in diabetic rats was ~1.5-fold higher than that of normal rats because of their hyperphagia (41).

<table>
<thead>
<tr>
<th>T-1095</th>
<th>Body Weight, g</th>
<th>Fed Blood Glucose, mg/dl</th>
<th>Fasted Blood Glucose, mg/dl</th>
<th>Fasted Insulin, ng/ml</th>
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<tr>
<td>Normal</td>
<td></td>
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<tr>
<td>−</td>
<td>345.3 ± 8.0</td>
<td>94 ± 1</td>
<td>68 ± 1</td>
<td>0.62 ± 0.06</td>
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<tr>
<td>+</td>
<td>312.0 ± 7.6*</td>
<td>94 ± 4</td>
<td>64 ± 3</td>
<td>0.63 ± 0.06</td>
</tr>
<tr>
<td>nSTZ</td>
<td></td>
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<tr>
<td>−</td>
<td>270.2 ± 9.6*</td>
<td>397 ± 11*</td>
<td>157 ± 13*</td>
<td>0.20 ± 0.01*</td>
</tr>
<tr>
<td>+</td>
<td>271.6 ± 8.8*</td>
<td>187 ± 19*†</td>
<td>76 ± 5*†</td>
<td>0.19 ± 0.02*</td>
</tr>
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</table>

Values are means ± SE; n = 6–12. nSTZ, diabetic rats neonatally treated with streptozocin. T-1095 (0.1% wt/wt) was given as food admixture. *P < 0.01 vs. normal control; †P < 0.01 vs. nSTZ control.
2-DG uptake, lactate release, and glucose oxidation in soleus muscles. Insulin stimulated 2-DG uptake (Fig. 1A), lactate release (Fig. 1B), and glucose oxidation (Fig. 1C) in isolated rat soleus muscle from normal rats and rats neonatally treated with streptozotocin (nSTZ) with or without T-1095 treatment. Results are expressed as means ± SE for 4 muscle strips per group. *P < 0.05, **P < 0.01.

**GLUT-4 translocation in skeletal muscle.** Insulin increased and decreased contents of GLUT-4 protein in PM and LDM fractions, respectively (Fig. 2, A and B). This translocation of GLUT-4 from PM to LDM fractions was markedly blunted in diabetic rats (Fig. 2, A and B). T-1095 treatment apparently ameliorated the impairment of translocation (Fig. 2, A and B).

**Analysis of insulin-stimulated intracellular signaling steps.** To study which step of the insulin signal in skeletal muscles is impaired in nSTZ rats, we next determined 1) phosphorylation of the insulin receptor and IRS-1 and -2, 2) the amount (p85α regulatory subunit) and activities of PI-3 kinase associated with IRS-1 and -2, and 3) phosphorylation (Ser 473) and activity of Akt/PKB in skeletal muscles.

The protein expression of the insulin receptor (β-subunit) was not different among the groups (data not shown). Insulin-stimulated tyrosine phosphorylation of the insulin receptor was increased by 39% in nSTZ vs. normal rats (Fig. 3). T-1095 treatment normalized the enhanced phosphorylation in nSTZ rats but did not affect normal rats (Fig. 3).

There were no significant differences in expression of IRS-1 and IRS-2 proteins in skeletal muscles among the groups (data not shown). Insulin-stimulated phosphorylation of IRS-1 and IRS-2 was enhanced by 76 and 89%, respectively, in nSTZ vs. normal rats (Figs. 4A and 5A). Although the IRS-1/2 phosphorylation in normal rats was not influenced by the treatment of T-1095, the increased phosphorylation in nSTZ rats was reduced to normal levels by T-1095 (Figs. 4A and 5A).
The amount of PI-3 kinase (p85α regulatory subunit) associated with IRS-1/2 in the insulin-stimulated state was increased in nSTZ compared with normal rats (Figs. 4B and 5B). After the treatment with T-1095, the increased amount and activity of IRS-associated PI-3 kinase were reduced to normal levels (Figs. 4, B and C, and 5, B and C).

The protein expression level of Akt/PKB was not different among the groups (Fig. 6A). The insulin-stimulated phosphorylation (Ser473) of Akt/PKB was markedly decreased by 76% in nSTZ vs. normal rats (Fig. 6A). In addition, the insulin-stimulated Akt/PKB activity was also reduced by 82% of normal control animals in nSTZ rats (Fig. 6B). T-1095 treatment improved both phosphorylation and activation of Akt/PKB by insulin to normal levels (Fig. 6, A and B).

**DISCUSSION**

In this study, we used an orally active SGLT inhibitor, T-1095, to improve hyperglycemia in nSTZ rats. This compound, a derivative of phlorizin, is a potent and selective inhibitor of SGLT but, unlike phlorizin, is effectively absorbed from the gut after oral administration. We have previously reported that T-1095 increases the excretion of excess plasma glucose and thus reduces hyperglycemia in various diabetic animal models (38–41, 49). Likewise, the blood glucose level was corrected by the chronic treatment with T-1095 in both fed and fasted states in nSTZ rats.

The present study demonstrated that insulin-stimulated 2-DG uptake, glucose oxidation, and lactate release were suppressed in the isolated soleus muscles of diabetic rats. In addition, insulin-stimulated GLUT-4 translocation from LDM to PM fractions was markedly impaired in nSTZ rats. These results clearly indicate that the skeletal muscle of nSTZ rats acquired insulin

![Fig. 3. Tyrosine phosphorylation of insulin receptor (IR; β-subunit) in basal and insulin-stimulated skeletal muscle of normal and nSTZ rats with (+) or without (-) T-1095 treatment. Protein immunoprecipitated with anti-phosphorylated tyrosine antibody (4G10) was subjected to SDS-PAGE followed by immunoblotting with anti-IR (β-subunit). A representative autoradiograph (top) and quantified data (bottom) of tyrosine phosphorylation of IR (β-subunit). N.D., not detected. Results are expressed as means ± SE for 3 animals. **P < 0.01.](image)

![Fig. 4. Tyrosine phosphorylation of IR substrate-1 (IRS-1, A) and amount (p85α, B) and activity (C) of phosphatidylinositol (PI) 3-kinase associated with IRS-1 in basal and insulin-stimulated skeletal muscle of normal and nSTZ rats with or without T-1095 treatment. Protein immunoprecipitated with anti-IRS-1 was subjected to SDS-PAGE followed by immunoblotting with anti-phosphorylated tyrosine (4G10) (A) or anti-p85α (B) or determination of PI 3-kinase activity (C). A: representative autoradiograph (top) and quantified data (bottom) of tyrosine phosphorylation of IRS-1. B: representative autoradiograph (top) and quantified data (bottom) of p85α associated with IRS-1. C: PI 3-kinase activity associated with IRS-1. Results are expressed as means ± SE for 3 animals. *P < 0.05, **P < 0.01.](image)
resistance. Hansen et al. (21) indicated that, under the same conditions as we employed in our isolated muscle preparations, 2-DG uptake accurately reflects glucose transport activity. Therefore, the impaired 2-DG uptake in the insulin-stimulated state of nSTZ rats would be attributable to the defect of GLUT-4 translocation.

T-1095 improved not only the blood glucose levels but also insulin sensitivity in skeletal muscles in dia-

Fig. 5. Tyrosine phosphorylation of IRS-2 (A) and amount (p85α) (B) and activity (C) of PI 3-kinase associated with IRS-2 in basal and insulin-stimulated skeletal muscle of normal and nSTZ rats with or without T-1095 treatment. Protein immunoprecipitated with anti-IRS-2 was subjected to SDS-PAGE followed by immunoblotting with anti-phosphorylated tyrosine (4G10) (A) or anti-p85α (B) or determination of PI 3-kinase activity (C). A: representative autoradiograph (top) and quantified data (bottom) of tyrosine phosphorylation of IRS-2. B: representative autoradiograph (top) and quantified data (bottom) of p85α associated with IRS-2. C: PI 3-kinase activity associated with IRS-2. Results are expressed as means ± SE for 3 animals. *P < 0.05, **P < 0.01.

Fig. 6. Protein expression, phosphorylation (Ser473) (A), and activity (B) of Akt/protein kinase B (PKB) in basal and insulin-stimulated skeletal muscle of normal and nSTZ rats with or without T-1095 treatment. The total lysate was subjected to SDS-PAGE followed by immunoblotting with anti-Akt/PKB or anti-phosphorylated (Ser473) Akt/PKB (A). An equal amount of muscle protein was immunoprecipitated with anti-Akt/PKB followed by determination of Akt/PKB activity (B). A: representative autoradiographs of Akt/PKB (top) and phosphorylated Akt/PKB (middle), and quantified data of Akt/PKB phosphorylation (bottom). B: Akt/PKB activity in skeletal muscle. Results are expressed as means ± SE for 3 animals. *P < 0.05, **P < 0.01.
betic rats, without affecting those of normal rats. In addition, our previous study using the glucose clamp technique showed that T-1095 ameliorates the insulin resistance in whole body and skeletal muscles of nSTZ rats despite no effect on the insulin sensitivity of normal rats (41). These results strongly suggest that the insulin resistance of skeletal muscle in this diabetic model is induced by hyperglycemia, and that T-1095 restores insulin sensitivity via normalizing blood glucose levels.

In diabetic nSTZ rats, insulin-induced Akt/PKB phosphorylation and activation were severely impaired compared with normal rats. The amelioration of hyperglycemia by T-1095 was accompanied by improvement of both the phosphorylation and the activation of Akt/PKB despite the lack of effect of T-1095 on those of normal rats. In addition, Kurowski et al. (32) reported that high concentration (25 mM) of glucose in the medium diminishes both phosphorylation and activation of Akt/PKB in skeletal muscles. These results strongly suggest that the impairment of Akt/PKB activation in skeletal muscle is attributable to hyperglycemia. On the other hand, many reports support a crucial role for Akt/PKB in insulin action, including the increase of glucose uptake and GLUT-4 translocation (20, 29, 48, 50, 53). Therefore, we consider the reduction of Akt/PKB activity to be closely related to the defects of glucose transport and GLUT-4 translocation in skeletal muscle induced by hyperglycemia.

Substantial evidence has accumulated to suggest that Akt/PKB is a major target of PI-3 kinase-generated signals. For example, inhibitors of PI-3 kinase (12, 18, 28) or dominant-negative mutants of the p85 regulatory subunit (12) of PI-3 kinase inhibit insulin-stimulated Akt/PKB activity, whereas overexpression of a constitutively active PI-3 kinase activates Akt/PKB (15, 26). In this study, the upstream signal events of Akt/PKB, which include insulin receptor phosphorylation, IRS-1/2 phosphorylation, and amount and activity of PI-3 kinase associated with IRS-1/2, were not impaired but rather increased in diabetic rats, despite the reduced phosphorylation and activity of downstream Akt/PKB. Recently, Kurowski et al. (32) reported that high concentration of glucose inhibits insulin activation of Akt/PKB, but not PI-3 kinase, in rat skeletal muscles in vitro (32). Song et al. (45) also found impairment of Akt/PKB activation by insulin, in the face of apparently normal activation of PI-3 kinase, in extensor digitorum longus muscle of diabetic insulin-resistant GK rats. In addition, we (39) recently reported that insulin-stimulated Akt/PKB activity was reduced, whereas PI 3-kinase activity was rather enhanced, in the liver of ZDF rats. These results suggest that chronic hyperglycemia reduces the efficiency of the activation step from PI-3 kinase to Akt/PKB. However, we can now only speculate as to how hyperglycemia impairs Akt/PKB activation. Possible mechanisms include PDK-1 and PDK-2 inhibition, activation of PI 3-phosphatase that leads to a decrease of the cellular contents of lipid products by PI 3-kinase, a defect in intracellular PI 3-kinase translocation, and activation of phosphatase, which may modify the Akt/PKB activity.

In our preliminary study, insulin-stimulated phosphorylation of the insulin receptor (IR) and IRS-1/2 and activities of PI 3-kinase associated with IRS-1/2 were enhanced in the liver of nSTZ rats (unpublished observation). The present study has also shown that the insulin-signaling pathway from IR to PI 3-kinase is significantly enhanced in skeletal muscle of the diabetic rats despite the apparent insulin resistance. Because these alterations were reversed when the hyperglycemia was corrected by T-1095, the blood glucose levels themselves seem to be related to these changes. It remains to be determined whether hyperglycemia itself enhances the activation of these signaling molecules, or whether these phenomena are reactions to compensate for the impaired downstream Akt/PKB activation.

Previous studies have suggested that, similar to Akt/PKB, atypical protein kinase C (PKC) isoforms (λ and ζ) may also act as downstream effectors of PI-3 kinase to participate in insulin stimulation of glucose transport (8–10, 30, 36, 46, 47, 51). At present, we cannot rule out the possibility that hyperglycemia leads to defective activation of atypical PKCs, which may also contribute to the impaired GLUT-4 translocation and glucose uptake in nSTZ rats. The effect of hyperglycemia on activities of atypical PKCs waits to be determined in further investigations.

In conclusion, our results suggest that chronic hyperglycemia exerts a harmful effect on the efficiency of the insulin-signaling step from PI-3 kinase to Akt/PKB, and that this impairment is the molecular mechanism underlying hyperglycemia-induced insulin resistance in skeletal muscle.

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