Protein kinase C modulates insulin action in human skeletal muscle

RONALD N. CORTRIGHT,1,2* JOHN L. AZEVEDO, J R.,1,2* QIAN ZHOU,1 MADHUR SINHA,1
WALTER J. PORIES,1 SAMAR I. ITANI,1 AND G. LYNIS DOHM1

1School of Medicine and 2Human Performance Laboratory, East Carolina University, Greenville, North Carolina 27858

Cortright, Ronald N., John L. Azevedo, J r., Qian Zhou, Madhur Sinha, Walter J. Pories, Samar I. Itani, and G. Lynis Dohm. Protein kinase C modulates insulin action in human skeletal muscle. Am. J. Physiol. Endocrinol. Metab. 278: E553–E562, 2000.—There is good evidence from cell lines and rodents that elevated protein kinase C (PKC) overexpression/activity causes insulin resistance. Therefore, the present study determined the effects of PKC activation/inhibition on insulin-mediated glucose transport in incubated human skeletal muscle and primary adipocytes to discern a potential role for PKC in insulin action. Rectus abdominus muscle strips or adipocytes from obese, insulin-resistant, and insulin-sensitive patients were incubated in vitro under basal and insulin (100 nM)-stimulated conditions in the presence of GF 109203X (GF), a PKC inhibitor, or 12-deoxyphorbol 13-phenylacetate 20-acetate (dPPA), a PKC activator. PKC inhibition had no effect on basal glucose transport. GF increased (P < 0.05) insulin-stimulated 2-deoxyglucose (2-DG) transport approximately twofold above basal. GF plus insulin also increased (P < 0.05) insulin receptor tyrosine phosphorylation 48% and phosphatidylinositol 3-kinase (PI 3-kinase) activity ~50% (P < 0.05) vs. insulin treatment alone. Similar results for GF on glucose uptake were observed in human primary adipocytes. Further support for the hypothesis that elevated PKC activity is related to insulin resistance comes from the finding that PKC activation by dPPA was associated with a 40% decrease (P < 0.05) in insulin-stimulated 2-DG transport. Incubation of insulin-sensitive muscles with GF also resulted in enhanced insulin action (~3-fold above basal). These data demonstrate that certain PKC inhibitors augment insulin-mediated glucose uptake and suggest that PKC may modulate insulin action in human skeletal muscle.

insulin resistance; diabetes; non-insulin-dependent diabetes mellitus; glucose transport; muscle

Several studies have suggested that serine/threonine phosphorylation of the insulin receptor decreases the ability of the tyrosine kinase to be activated by insulin (42, 43). Moreover, serine kinases have been reported to be associated with purified insulin receptor preparations (38) and with phosphatidylinositol 3-kinase (PI 3-kinase) (31). This suggests that serine/threonine phosphorylation of the receptor may modulate insulin signal transduction. The kinase responsible for serine/threonine phosphorylation of the insulin receptor is not known with certainty, but cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) have been suggested (32). Earlier, Considine and Caro (12) and Shmueli et al. (40) suggested that elevated PKC activity may impair insulin signal transduction and thus may be causally linked to insulin resistance.

There are three classes of PKC isoforms: 1) the conventional isoenzymes that are activated by phorbol esters and calcium, 2) the novel, calcium-independent isoenzymes, which can be stimulated by phorbol esters, and 3) the atypical isoenzymes, which do not respond to either phorbol esters or calcium. In cells overexpressing several isoforms of PKC, activation by phorbol esters produces a state of insulin resistance with depressed insulin activation of PI 3-kinase (9, 10, 15). In contrast, in primary hepatocytes from rats treated with insulin, phorbol 12-myristate 13-acetate (PMA) had no effect on insulin-mediated receptor autophosphorylation/tyrosine kinase status (39). However, in skeletal muscle, PKC-α, a novel PKC isoform, is higher in white muscle fibers, which are more insulin resistant (18). In addition, PKC-α is increased in white muscle when rats are made insulin resistant by fructose feeding. Recently Condino et al. (13) reported that PKC isoforms-α, -ε, and -ζ were elevated in liver of non-insulin-dependent diabetes mellitus patients and diabetic rats. Although Cooper et al. (14) found that the protein and mRNA levels of PKC-α, -β, and -ε were decreased in skeletal muscle of insulin-resistant obese Zucker rats, Alivign et al. (3) found PKC-α, -β, -ε, and -δ to be elevated in the membranes of muscles from the lean diabetic Goto-Kakizaki rats, with a concomitant decrease in these cytosolic PKC isoforms, suggesting a translocation from the cytosol to membrane. Although this study reported that PKC-α was decreased in cytosolic and membrane fractions from skeletal muscles of diabetic vs. control Wistar rats, muscle from obese Zucker rats was found to contain elevated levels of PKC-α, -β, and -δ in the membrane, with concomitant declines in the cytosolic fraction. This again suggests increased translocation of these isoforms from the cytosol to the membrane in the insulin-resistant state. More recent support for this hypothesis has been gained from the demonstration that BRL-49653, an insulin sensitizer, reverses the...
skeletal muscle insulin resistance observed in fat-fed rats (39). BRL-49653 increased the recovery of novel PKC (nPKC) isoenzymes in cytosolic fractions of soleus muscle of rats, thus reducing the apparent PKC activation. In contrast, increased PKC-c membrane protein content has also been shown to be associated with declines in in vivo glucose uptake in rats made insulin resistant by lipid/heparin infusion (25). The finding that the concentration of the endogenous PKC activator diacylglycerol was elevated in insulin-resistant muscle (8, 40) adds to the contention that PKC may be involved in insulin resistance in skeletal muscle.

The potential role of altered PKC activity on insulin action in human skeletal muscle has not been investigated. Therefore, the present study was performed to determine whether specific inhibitors or activators of PKC would induce concomitant changes in insulin action in human skeletal muscle. It was found that PKC inhibition enhanced, whereas activation of PKC reduced, insulin action in obese or lean subjects, respectively, suggesting that PKC may modulate insulin signal transduction in human muscle. This observation also suggests that certain PKC inhibitors might provide a rational treatment for insulin resistance in type II diabetic patients.

RESEARCH DESIGN AND METHODS

Human subjects. The experimental protocol was approved by the East Carolina University Policy and Review Committee on Human Research, and informed consent was obtained from all patients. Surgery was performed on the patients after an overnight fast. General anesthesia was initiated with a short-acting barbiturate and maintained with fentanyl and a nitrous oxide-oxygen mixture. Effects of PKC inhibitors on glucose transport were performed on biopsies (3 × 2 × 0.5 cm; ~3 g) of rectus abdominus (n = 54 patients) muscle or subcutaneous fat (n = 10; BMI = 27). The study of the effects of PKC activators on glucose transport and PKC activity were made on lean (BMI < 27), insulin-sensitive subjects (BMI < 30) and insulin-resistant subjects (BMI > 30).

Muscle strip incubation. Immediately after removal, the muscle sample was placed in a sealed container with oxygenated Krebs-Henseleit buffer for transport to the laboratory. Muscle strips were incubated in a modified incubation system as previously described (16). Briefly, muscle strips weighing ~25 mg were teased from the biopsy sample and clamped in muscle strips weighing 25 mg were teased from the biopsy sample and clamped in liquid nitrogen-cooled metal tongs. The muscles were homogenized in 20 mM Tris·HCl, 10 mM EDTA, 2 mM EGTA, and 100 µM phenylmethylsulfonyl fluoride (PMSF). Samples were centrifuged at 12,000 rpm, 4°C, for 20 min, and the supernatant was labeled as the particulate (membrane) fraction. The pellet was resuspended with the above homogenization buffer (plus 0.5% Triton), incubated on ice, and homogenized in 20 mM Tris·HCl containing 0.05% (wt/vol) sodium azide, pH 7.5, and 25 µg/ml phosphotidyl-L-serine and a 12-deoxyphorbol 13-phenylacetate 20-acetate (dPPA; 5 µM). Where appropriate, samples were preincubated with insulin (100 nM) for the last 10 min of this first period. Afterward, the muscle strips were transferred to incubation wells under identical conditions with the exception that the incubation media contained 5.0 mM 2-deoxyglucose (2-DOG), 20 mM sorbitol, 2 µCi 2-deoxy-[1,2-3H(N)]glucose to quantify glucose transport, and 0.1 µCi [U-14C]sorbitol as an extracellular space marker. Preincubation and incubation volumes were 4 ml. Samples were continuously gassed with 95% O2-5% CO2, and incubation temperature was maintained at 37°C in a shaking water bath. After incubation, muscle strips were transferred to ice-cold Krebs-Henseleit buffer for two 5-min periods to wash off excess 2-DOG and sorbitol from the samples. After washing, muscle strips were blotted, weighed, and solubilized in 0.5 ml of 32 M hexadecyltrimethyl ammonium bromide and 0.29 M potassium hydroxide in a 1:1 mixture of methanol-H2O. Solubilized muscle strips and incubation media samples (specific activity determination) were counted in a Beckman LS 5000 TD liquid scintillation counter preset to count 14C and 3H channels simultaneously. Because of the number of PKC inhibitors studied and the limited amount of tissue obtainable from human biopsies, muscle samples did not receive all treatments. However, all muscle samples were incubated with GF as a “standard” (thus responses may be compared between treatments).

Glucose transport in isolated human adipocytes. Adipocytes were prepared by the collagenase digestion method, as described earlier (41), from subcutaneous fat biopsies of morbidly obese subjects. Glucose transport in the freshly isolated adipocytes was determined by a modified method of Kashwagal et al. (30). Fat cells (10% lipocrit) were incubated for 1 h at 37°C under 95% O2-5% CO2 atmosphere in 10 mM HEPES-Krebs-Ringer bicarbonate buffer with 3% BSA (pH 7.4). Incubations contained tracer (500 nM) [U-14C]glucose concentration and varied insulin concentrations (0.01–100 nM) and were in the presence or absence of the PKC inhibitor GF (2.0 µM). The cells were then separated from the incubation medium by centrifugation through silicon oil for 2 min at 13,000 rpm with a Beckman microfuge, and 14C radioactivity associated with the cells was counted in a liquid-scintillation counter. Net glucose transport (glucose clearance rate as f1-cell-1-s-1) was expressed as the percent change above basal glucose transport.

PKC enzyme assay. PKC activity was determined by using a commercial PKC enzyme assay kit (Amersham Life Science, Piscataway, NJ). Rectus abdominus skeletal muscle (n = 18) was incubated with insulin alone or insulin plus GF or dPPA for 15 min and then snap-frozen immediately with liquid nitrogen-cooled metal tongs. The muscles were homogenized in 20 mM Tris (pH 7.4), 10 mM EDTA, 2 mM EGTA, 100 mM β-glycerophosphate, 1 mg/ml leupeptin, 0.1 mg/ml aprotinin, 0.1 mg/ml ovalbumin, and 50 µg phenylmethylsulfonyl fluoride (PMSF). Samples were centrifuged at 12,000 rpm, 4°C, for 20 min, and the supernatant was labeled as the cytosolic fraction. The pellet was resuspended with the above homogenization buffer (plus 0.5% Triton), incubated on ice, and centrifuged at 12,000 rpm, 4°C, for 20 min. The supernatant was labeled as the particulate (membrane) fraction. The protein content was determined by use of the Bio-Rad (Her- cules, CA) protein assay, based on the method of Bradford. Equal volumes of calcium buffer (12 mM calcium acetate in a buffer containing 50 mM Tris·HCl and 0.05% (wt/vol) sodium azide, pH 7.5), lipid [0.3 mg/ml L-α-phosphatidyl-L-serine and 24 µg/ml PMA in 50 mM Tris·HCl containing 0.05% (wt/vol) sodium azide, pH 7.5], peptide buffer [900 µM peptide] in 50 mM Tris·HCl containing 0.05% (wt/vol) sodium azide, pH
buffer (0.2 μCi of [32P]ATP added to 1.2 mM ATP in a buffer containing 30 mM HEPES, 72 mM magnesium chloride, pH 7.40) was added to all tubes. Samples were incubated at 37°C for 15 min. The reaction was terminated by the addition of the stop reagent (300 mM orthophosphoric acid, containing cumanosine red), and the phosphorylated peptides were separated with binding paper discs and counted in 5 ml of scintillant with the Beckman LS 6500 multipurpose scintillation counter (Beckman Instruments, Fullerton, CA).

Insulin receptor phosphorylation. For insulin receptor tyrosine phosphorylation determinations, human muscle strips were preincubated as above. However, the incubation period was only 15 min (n = 6). Muscles from the same biopsy were also measured for glucose transport, as described above. For tyrosine phosphorylation measurements, the muscle samples were immediately frozen with metal tongs precooled in liquid nitrogen and stored at -70°C until analyzed.

Approximately 100 mg of frozen muscle were homogenized in 1 ml of buffer containing HEPES (50 mM, pH 7.4), NaF (100 mM), sodium pyrophosphate (10 mM), EDTA (2.5 mM), sodium orthovanadate (2 mM), PMPS (2 μM), leupeptin (1 μM), pepstatin (1 μM), and aprotinin (0.2 μM). Samples were then centrifuged at 150,000 g for 1 h. The pellet was solubilized in 1% Triton X-100 for 1 h and centrifuged, and an aliquot of the supernatant was measured for protein content [bicinchoninic acid (BCA) procedure, Pierce Chemical, Rockford, IL]. Equal amounts of protein were incubated overnight with 1 μg monoclonal anti-insulin receptor antibody (CalBiochem) and 75 μl of protein G agarose beads. The resulting immunoprecipitates were washed 4 times by resuspension in homogenization buffer and were centrifuged. The immunoprecipitates were solubilized in 75 μl of sample buffer with 2.5% DTT by heating at 100°C for 8 min. Proteins were separated by SDS-PAGE on an 8% resolving gel. Samples were then transferred onto Immobilon-P transfer membrane in buffer containing 20 mM Tris-acetate (pH 8.3), 0.1% SDS (wt/vol), and 20% isopropanol (vol/vol) at 25 V for 14 h. Gels were stained with Commassie Blue to assess completeness of transfer. To reduce nonspecific antibody binding, the Immobilon-P transfer membranes were blocked with 5% BSA for 8 h at 4°C in Tris-NaCl buffer (pH 7.8). The membranes were incubated overnight with a polyclonal anti-phosphotyrosine antibody in 5% BSA at 4°C. Afterward, samples were washed 2 times for 5 min in Tris-buffered saline (TBS), 2 times for 10 min in TBS-Tween (0.05%), and 2 times for 5 min in TBS. Bound antibodies were detected by incubation with 13 µl of donkey anti-rabbit antibody linked to horseradish peroxidase (Amersham, Arlington Heights, IL), followed by enhanced chemiluminescence. Band intensity was quantified on a Hewlett-Packard Scanjet 5100C by use of Imagequant software (Molecular Dynamics).

PI 3-kinase activity. Because of the large amount of tissue required for the assay, a subset of samples was assayed for PI 3-kinase activity (n = 8). PI 3-kinase was assayed according to a modified protocol of Chen et al. (8). Skeletal muscle (100–200 mg) was homogenized (1:10, wt/vol) in cold homogenization buffer [HEPES (50 mM, pH 7.4), NaCl (150 mM), EDTA (2 mM), Na3P2O7 (10 mM), Na2VO3 (2 mM), MgCl2 (1 mM), CaCl2 (1 mM), NaF (10 mM), PMSF (2 mM), 5 μg/ml leupeptin, and 10% glycerol] for 30 s on ice, solubilized for 60 min (Nonidet P-40, 1% vol), and centrifuged (207,000 g, 1 h). An aliquot of the supernatant was assayed for protein (BCA method, Pierce Chemical). The supernatant was incubated overnight with monoclonal anti-phosphotyrosine antibody conjugated to Sepharose beads at 4°C. The Sepharose was then gently pelleted (10 s at 14,000 rpm) and washed 3 times with 1% Nonidet P-40 in PBS (pH 7.4), 3 times with 0.5 M LiCl and 100 mM Tris (pH 7.6), and 2 times with 10 mM Tris (pH 7.6), 100 mM NaCl, and 1 mM EDTA. The samples were aspirated, and 50 μl of fresh wash buffer were then added to the protein-Sepharose complex. The reaction mixture consisted of washed Sepharose beads, 40 μM ATP at a final concentration with 20 μCi [γ-32P]ATP, 10.6 mM MgCl2, and 10 μl of L-α-phosphatidylinositol (2 μg/μl) sonicated in 10 mM Tris (pH 7.4) and 1 mM EGTA. After 10 min, the reaction was stopped with 20 μl of 8 N HCl, and labeled lipids were extracted with 200 μl of methanol-chloroform (1:1, vol/vol). The extract was vortexed and briefly centrifuged (10 s at 14,000 rpm). The organic phase was then separated by thin-layer chromatography in a mobile phase of chloroform-methanol-ammonium hydroxide-water (60:47:11:3.2, by vol) and developed, and the reaction products (developed overnight) were visualized on a phosphorimager (Phosphorimager 452E; Molecular Dynamics). Band intensity was quantified using Imagequant software (Molecular Dynamics). The results were normalized to the protein content of the homogenate.

Statistical analysis. One-way repeated-measures ANOVA was used to determine differences between treatments. A Newman-Keuls post hoc test was used to locate differences. α was set at 0.05 for statistical significance. A paired t-test was used to evaluate differences in PI 3-kinase activity between insulin and insulin + GF-stimulated muscle.

RESULTS

Our initial interest in PKC came as a result of the cloning of the θ isoform, which was found to be predominantly expressed in skeletal muscle and which was shown to be increased in muscle of insulin-resistant rats (18). Consequently, we wished to examine whether the impaired glucose transport observed in insulin-resistant human skeletal muscle could be ameliorated in the presence of PKCi inhibitors. Initial experiments utilized the PKC inhibitor GF 109203X (GF). The dose-response curve (Fig. 1) shows that the maximal response with this inhibitor is seen at a concentration of 2 μM. Incubation of muscle strips with GF in the basal state (no insulin added) had no effect on glucose transport rate (Fig. 2). However, in the presence of 100 nM insulin, GF increased (P < 0.05) glucose transport 1.5-fold (Fig. 3).

To determine whether the enhancement of insulin action by GF was PKC specific, PKC activity was determined from muscle tissue (n = 14) incubated in the presence of GF plus insulin (Fig. 4). Cytosolic PKC activity was significantly reduced (P < 0.002) – 50% by GF (in the presence of insulin) vs. insulin treatment alone.

The experiment was also repeated with several other known PKC inhibitors. None of the inhibitors altered basal glucose transport (Fig. 2). Insulin-stimulated glucose transport was not changed by 2 μM concentrations of Calphostin C, Staurosporine, or Gö6976 (Fig. 3). On the other hand, Rottlerin increased insulin responsiveness of glucose transport (P < 0.05). Rottlerin showed increased glucose transport rates at concentrations of ≤50 μM (data not shown); however, for the sake
of consistency, comparisons with Rottlerin (as with all inhibitors) were made for samples incubated at 2 µM only.

GF-V, which is an analog of GF without inhibitory activity for PKC, had no effect on insulin stimulation of glucose transport (Fig. 3). The lack of response to GF-V (n = 5) supports the hypothesis that the insulin-enhancing effect of GF is due to inhibition of PKC and not to a nonspecific effect of the compound.

Because it has been proposed that PKC may be a factor in causing insulin resistance (13, 40), we investigated whether GF would have an effect in only insulin-resistant muscles or whether it would enhance insulin response in insulin-sensitive tissue as well. Muscle samples from 18 patients demonstrated insulin stimulation >1.5-fold (P < 0.05), and these patients were grouped as insulin responsive. The remaining 17 were grouped as insulin resistant. Insulin by itself did not significantly increase glucose transport in the muscles that were insulin resistant, but in the presence of insulin and GF, glucose transport increased about twofold (Fig. 5). The rate of glucose transport in insulin-resistant muscles in the presence of GF plus insulin was approximately the same as the insulin-stimulated rate in insulin-sensitive muscles. GF also enhanced insulin action in insulin-responsive muscles. The absolute increase in glucose transport as a result of GF incubation was approximately the same in insulin-responsive and insulin-resistant muscle (Fig. 5).

To determine whether the enhancement of insulin action by GF-mediated PKC inhibition was specific to skeletal muscle, human primary adipocytes were incubated at varied insulin concentrations in the presence or absence of 2 µM GF (Fig. 6). As observed in skeletal muscle, incubation of human adipocytes from morbidly obese patients with GF significantly (P < 0.05) in-
creased insulin-mediated glucose uptake at insulin concentrations ranging from 0.1 to 100 nM.

To determine whether the observed effect of PKC inhibitors in skeletal muscle was due to increased insulin signaling, we determined insulin receptor tyrosine phosphorylation on a separate set of patients (n = 6) in the presence of insulin and insulin plus GF (Fig. 7). Figure 7A shows a typical Western blot from two subjects; Fig. 7B shows the mean data from six patients. Under basal conditions, with and without GF, insulin receptor tyrosine phosphorylation was barely detectable. Insulin, in the presence of GF, increased insulin receptor tyrosine phosphorylation 48% over insulin treatment alone (P < 0.05). In vitro glucose transport in this set of subjects was impaired (2.8 ± 0.7 vs. 3.6 ± 0.8 nmol·g⁻¹·min⁻¹; basal vs. insulin treated), whereas insulin-stimulated glucose transport in the presence of GF (7.7 ± 0.4 nmol·g⁻¹·min⁻¹) was significantly (P < 0.05) elevated vs. insulin alone. PI 3-kinase activity was also determined in muscle fiber strips that had been incubated in the basal and insulin-stimulated conditions in the presence and absence of GF (n = 8).

Fig. 4. Effect of a PKC activator and inhibitor on cytosolic and membrane PKC activity in human skeletal muscle. Muscle samples were incubated 15 min with insulin alone (100 nM) or insulin plus GF (2 µM; N = 14) or the phorbol ester 12-deoxyphorbol 13-phenylacetate 20-acetate (dPPA, 5 µM; n = 4). Incubation of muscle strips with insulin plus the PKC inhibitor GF resulted in a significant reduction in cytosolic PKC activity vs. insulin treatment alone (39 ± 4.7 vs. 22 ± 2.8 pmol·min⁻¹·g⁻¹). In contrast, incubation with insulin plus the PKC activator dPPA significantly increased the membrane-bound (active form) PKC activity above insulin treatment alone (199 ± 10 vs. 275 ± 18 pmol·min⁻¹·g⁻¹). Values are means ± SE. *Significantly different from insulin treatment (P < 0.002); **significantly different from insulin treatment (P < 0.01).

Fig. 5. Effect of the PKC inhibitor GF (2 µM) on insulin-stimulated glucose transport in insulin-sensitive and insulin-resistant muscles. Values are means ± SE. Muscle samples were subdivided according to their insulin responsiveness into insulin resistant (n = 17) and insulin responsive (n = 18) groups. Muscle samples were preincubated for 60 min followed by a 60-min incubation period with isotope for measurement of glucose transport rates. Muscle samples were incubated in the presence of 100 nM insulin during the last 10 min of the preincubation period and during the entire incubation period with and without GF at a concentration of 2 µM. Inhibitor was present during both preincubation and incubation periods. Unlike letters are significantly different (P < 0.05).

Fig. 6. Effects of PKC inhibitor GF on glucose transport in isolated human adipocytes from morbidly obese patients. Adipocytes were prepared by collagenase digestion method from subcutaneous fat biopsies of control and morbidly obese subjects (n = 5 each). Glucose transport in freshly isolated adipocytes was determined at tracer [U-¹⁴C]glucose concentration (500 nM) in the absence or presence of different insulin concentrations (0.01–100 nM) and with or without 2 µM of the PKC inhibitor GF. Net glucose transport (glucose clearance rate as fl·cell⁻¹·s⁻¹) was expressed as the percent change above basal glucose transport. Values are means ± SE. *Significantly different from insulin treatment (P < 0.05).
Insulin stimulated PI 3-kinase activity about fourfold in the absence of GF and about sixfold in the presence of GF (Fig. 8). More importantly, the presence of GF was associated with significantly (P < 0.05) greater glucose transport than with insulin alone. Taken together, the insulin receptor tyrosine phosphorylation data and PI 3-kinase data support the hypothesis that inhibition of PKC by GF increases insulin signal transduction.

Finally, we conducted similar studies in muscle with the phorbol ester dPPA (PKC activator) to further investigate the regulation of insulin action by PKC. Incubation of muscle strips from insulin-sensitive non-obese patients resulted in an ~40% reduction (P < 0.05) in insulin-mediated glucose transport rate (Fig. 9). As with GF, we also wished to substantiate that the observed effects of dPPA on insulin action were specific to PKC (Fig. 4). Muscle strips (n = 4) incubated with dPPA demonstrated a significant (P < 0.01) elevation in membrane-associated PKC activity vs. insulin treatment alone.

DISCUSSION

We have established that insulin stimulation of glucose transport is impaired in the muscle of obese individuals (16, 20, 23). The inability to increase glucose transport in response to insulin could be due to a defect in the glucose transport effector system (i.e., translocation and/or activation of glucose transporters) or a defect in the insulin-signaling system. In insulin-resistant human muscles, glucose transport is stimulated by hypoxia (4), alkaline conditions (6), and phosphatase inhibitors (6). Because several stimuli can produce a normal signal and stimulate glucose transport, it seems likely that insulin resistance is caused by a defect in insulin signal transduction.

A more direct set of observations that demonstrate a defect in insulin signaling comes from studies in which the insulin receptor or the early steps in the signaling pathway were measured in normal and insulin-resistant muscles. Using semipurified receptors, several groups have demonstrated that insulin stimulation of receptor tyrosine kinase activity is depressed in insulin-resistant muscle (2, 7, 36). Consistent with this observation, Goodyear et al. (24) found that autophosphorylation of the insulin receptor, phosphorylation of the
investigated whether dephosphorylation of serine/threonine was depressed. Conversely, Zhou et al. (46) showed that approximately 50% had a significant increase in insulin resistance as a result of polycystic ovary syndrome. Approximately, insulin receptor function decreased in obese Zucker rats. In vivo insulin-induced tyrosine phosphorylation of the insulin receptor and in vitro insulin-stimulated receptor tyrosine kinase activity in obese rats were reduced (P < 0.05) by 82 and 39%, respectively. Dephosphorylation of the insulin receptor by previous alkaline phosphatase treatment increased insulin-stimulated receptor tyrosine kinase activity in both lean and obese rats, but the increase was three times greater in obese (insulin-resistant) rats. These findings suggest that increased insulin receptor serine phosphorylation decreases its ability to transduce insulin signal.

In addition, studies demonstrated that PI 3-kinase isoforms were upregulated in muscle from obese, insulin-resistant patients. The same study also showed that increased PI 3-kinase activity in muscle of obese Zucker rats. In vivo and in vitro experiments demonstrated that PI 3-kinase activation of GF on insulin-mediated glucose transport was due to enhanced receptor tyrosine kinase activity, as measured by insulin receptor tyrosine phosphorylation in receptors immunoprecipitated from human muscle. Results demonstrated that the amount of insulin-stimulated tyrosine phosphorylation from muscle of obese, insulin-resistant patients was significantly elevated (≈50%) in the presence of GF. Accordingly, additional experiments demonstrated that PI 3-kinase activity was also significantly higher with GF plus insulin compared with insulin treatment alone in insulin-resistant skeletal muscle.

The insulin receptor is not the only locus for serine/threonine phosphorylation that would decrease signal transduction. Tanti et al. (44) found that inhibition of serine/threonine phosphatases with okadaic acid treatment of adipocytes decreased insulin-mediated activation of PI 3-kinase. Further data suggested that serine/threonine phosphorylation of IRS-1 in okadaic acid-treated cells reduced the ability of the insulin receptor to phosphorylate IRS-1 and to bind PI 3-kinase. In agreement with this finding, Hotamisligil et al. (29) recently reported that treatment of murine adipocytes with tumor necrosis factor-α caused serine phosphorylation of IRS-1 and inhibition of the insulin receptor kinase activity. From their data the investigators concluded that serine phosphorylation converts IRS-1 into an inhibitor of the insulin receptor tyrosine kinase.

Although our studies demonstrate that certain PKC inhibitors can attenuate skeletal muscle insulin resistance, they do not directly implicate a specific PKC isoform in mediating this process. For instance, Gö 6976, which inhibits conventional PKCs, failed to alter insulin-stimulated glucose transport. Thus, although previous findings (9, 10) have demonstrated that conventional isoforms, such as PKC-α, can induce insulin resistance,

**Fig. 9. Effects of the phorbol ester 12-deoxyphorbol 13-phenylacetate 20-acetate (dPPA) on glucose transport in insulin responsive (glucose transport rate was 1.6 ± 0.20 vs. 3.8 ± 0.16 nmol/g·min−1; basal and insulin treatment respectively; 2.4 fold above basal) human skeletal muscle. Muscle samples (n = 5) were preincubated for 60 minutes (100 nM insulin was present during the last 10 minutes) followed by 60 minute incubation with isotope for the measurement of 2-deoxyglucose transport rates. dPPA was present at a concentration of 5 μM during the preincubation and incubation periods. Values are expressed as means ± SE. * Significantly greater than basal and insulin plus dPPA (P < 0.05).**
resistance in Chinese hamster ovary cells overexpressing PKC-α, the present study may not implicate specific PKC isoforms with regard to their involvement in insulin resistance occurring in the skeletal muscle of human patients. It would be desirable to suggest that novel PKCs are implicated in insulin resistance in human skeletal muscle, because GF targets novel PKCs, and Rottlerin is reported to specifically inhibit PKC-δ (26, 45). However, at the concentrations used in the present study, GF may be inhibiting conventional as well as novel PKCs. Thus, at the present time, a definite conclusion cannot be drawn with regard to the specific isoform(s) involved in insulin resistance in human skeletal muscle.

We were, however, able to substantiate the hypothesis that PKC modulates insulin action by conducting converse studies in muscle with the phorbol ester dPPA (PKC activator). As the current hypothesis would predict, incubation of muscle strips from insulin-sensitive nonobese patients resulted in a ~40% reduction in insulin-mediated glucose transport rate. Undoubtedly, the role that PKCs play in muscle glucose transport is complex. For example, Hansen et al. (27) observed that when glucose transport in rodent skeletal muscle is normal, dPPA treatment can stimulate muscle glucose transport. However, when other studies involving muscle from insulin-resistant rodents (39) are combined with the findings from the present study in humans, the contention remains that, in situations where a dysregulation in glucose metabolism exists, elevated PKC activity is apparently involved in a decline in insulin-mediated glucose transport.

The inability of Staurosporine and Calphostin C to mimic the effects of GF is perplexing. Staurosporine and Calphostin C would be expected to inhibit both conventional and novel PKCs and therefore to mimic the effects of GF. Although the mechanism responsible for this discrepancy is unclear, possibilities such as differences in cellular penetration/metabolism could explain these differences. Alternatively, GF could inhibit a kinase other than PKC that is not inhibited by Staurosporine and Calphostin C and thus exert its effect on insulin action. This is less likely, however, because (as shown in Fig. 4) we have demonstrated in this study that, as expected, incubation of muscle with GF significantly reduces the cytosolic PKC activity vs. insulin treatment alone. Obviously, further studies will be required to determine the explanation for the differences between inhibitors. However, the potential role for elevated PKC activity in impaired muscle insulin action is supported by our finding that PKC activity is elevated in the membrane fraction of muscle incubated with the PKC activator dPPA (the same condition that elicits a decrease in glucose transport; Fig. 4).

Muller et al. (35) found that hyperglycemia caused rat fat cells to become insulin resistant, due to a decrease in insulin receptor kinase activity. They also observed that membrane-bound PKC activity was increased and insulin resistance was reversed by incubating with PKC inhibitors. These results are similar to our findings in human adipose tissue, where incubation of adipocytes with GF in the presence of varied concentrations of insulin enhanced glucose uptake above insulin alone in tissue from morbidly obese patients. This adds further support to the contention that insulin receptor kinase activity is depressed in insulin-resistant tissue and that insulin action can be stimulated by PKC inhibitors.

The role that PKC plays in insulin signaling and/or insulin resistance has been the focus of much current research (12, 21, 39, 40, 46). Using cells overexpressing isoforms of PKC and/or activation of PKC, a number of investigators have provided direct evidence that increased activity of PKC can cause insulin resistance (1, 5, 9, 10). Most recently, Griffin et al. (25) have demonstrated that rats made insulin resistant by fatty acid/heparin infusion also demonstrate elevated PKC activity (4-fold increase in membrane-bound or active PKC-δ protein). As with the present study, the lipid-induced decrease in insulin-stimulated glucose transport was associated with 1) a blunting of insulin-stimulated IRS-1 tyrosine phosphorylation and 2) a 50% reduction in IRS-1-associated PI 3-kinase activity. Our results demonstrating that PKC inhibitors can enhance, and that activation of PKC by phorbol esters can attenuate, glucose transport support the contention that PKC may modulate insulin signal transduction.

In conclusion, although the exact PKC isoform inhibited by GF and Rottlerin has yet to be elucidated, these findings suggest that either these or related compounds could enhance insulin action and improve glycemic control in patients with impaired glucose tolerance and overt diabetes.

We thank Brian Roberts and Dr. Zoya Streletsova for excellent technical contributions, Bobbie Lu Tripp and Alice Hyatt for nursing support, and all participating surgeons and subjects for their cooperation.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Grant DK-46121. Drs. R. N. Cortright (5 F32 DK-09431–02) and J. L. Azevedo (F32 DK-008830) were supported by NIDDK fellowships.

Present address of J. L. Azevedo: Department of Physical Education and Exercise Science, California State University, Chico, CA 95929–0330.

Address for reprint requests and other correspondence: G. Lynis Dohm, Department of Biochemistry, School of Medicine, East Carolina University, Greenville, NC 27858 (E-mail: dohm@brody.med.ecu.edu).

Received 12 February 1999; accepted in final form 22 October 1999.

REFERENCES


