

Insulin-Stimulated Protein Kinase C λ/ζ Activity Is Reduced in Skeletal Muscle of Humans With Obesity and Type 2 Diabetes

Reversal With Weight Reduction

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In humans with obesity or type 2 diabetes, insulin target tissues are resistant to many actions of insulin. The atypical protein kinase C (PKC) isoforms λ and ζ are downstream of phosphatidylinositol-3 kinase (PI3K) and are required for maximal insulin stimulation of glucose uptake. Phosphoinositide-dependent protein kinase-1 (PDK-1), also downstream of PI3K, mediates activation of atypical PKC isoforms and Akt. To determine whether impaired PKC λ/ζ or PDK-1 activation plays a role in the pathogenesis of insulin resistance, we measured the activities of PKC λ/ζ and PDK-1 in vastus lateralis muscle of lean, obese, and obese/type 2 diabetic humans. Biopsies were taken after an overnight fast and after a 3-h hyperinsulinemic-euglycemic clamp. Obese subjects were also studied after weight loss on a very-low-calorie diet. Insulin-stimulated glucose disposal rate is reduced 26% in obese subjects and 62% in diabetic subjects (both comparisons $P < 0.001$). Insulin-stimulated insulin receptor substrate (IRS)-1 tyrosine phosphorylation and PI3K activity are impaired 40–50% in diabetic subjects compared with lean or obese subjects. Insulin stimulates PKC λ/ζ activity ~2.3-fold in lean subjects; the increment above basal is reduced 57% in obese and 65% in diabetic subjects. PKC λ/ζ protein amount is decreased 46% in diabetic subjects but is normal in obese nondiabetic subjects, indicating impaired insulin action on PKC λ/ζ . Importantly, weight loss in obese subjects normalizes PKC λ/ζ activation and increases IRS-1 phosphorylation and PI3K activity. Insulin also stimulates PDK-1 activity approximately two-fold with no impairment in obese or diabetic subjects. In contrast to our previous data on Akt, reduced insulin-stimulated PKC λ/ζ activity could play a role in the pathogenesis of insulin resistance in muscle of obese and type 2 diabetic subjects. *Diabetes* 52:1935–1942, 2003

Impaired insulin-stimulated glucose transport in muscle and adipose tissue is a major contributor to the pathogenesis of insulin-resistant states such as obesity and type 2 diabetes (1). Insulin stimulates glucose transport by activating a cascade of tyrosyl phosphorylation events, initiated by binding of insulin to its receptor (2,3). The phosphorylated insulin receptor binds to and activates insulin receptor substrates (IRSs), resulting in stimulation of critical downstream pathways such as phosphatidylinositol 3-kinase (PI3K). PI3K is necessary, albeit not sufficient, for insulin stimulation of glucose uptake (4–7). Insulin-stimulated PI3K activity is impaired in skeletal muscle of obese type 2 diabetic subjects (8,9) and nonobese type 2 diabetic subjects (10,11). However, activation of Akt, a serine/threonine kinase downstream of PI3K involved in some of the metabolic actions of insulin (12,13), is normal in these same obese type 2 diabetic subjects (8), although not when muscle from lean type 2 diabetic subjects is incubated with a very high insulin concentration (14). Hence, the pathways downstream of PI3K that are responsible for the impairment in insulin-stimulated glucose metabolism are unclear.

Studies suggest that the atypical protein kinase C (PKC) isoforms λ and ζ are downstream mediators of PI3K and that their activation is required for insulin stimulation of glucose uptake, which involves translocation of the major insulin-responsive glucose transporter GLUT4 from intracellular sites to the cell membrane (15–17). Overexpression of a dominant-negative mutant of PKC λ or PKC ζ abrogates insulin-stimulated glucose transport and GLUT4 translocation in adipose (15,18) and muscle cells (16,19). Overexpression of constitutively active PKC λ in adipocytes (15) or wild-type PKC ζ in muscle in vivo (20) enhances both basal and insulin-stimulated glucose transport. Furthermore, PKC λ and ζ appear to function interchangeably, since overexpression of wild-type PKC λ restores the inhibitory effects of a dominant-negative mutant of PKC ζ on insulin-stimulated GLUT4 translocation and vice versa (21). The possibility that PKC λ/ζ could play an important role in insulin resistance in vivo is supported by studies showing impaired insulin-stimulated PKC λ/ζ activity in skeletal muscle and adipose tissue of nonobese type 2 diabetic Goto-Kakizaki (GK) rats (22,23) and normalization of PKC λ/ζ activity by the insulin-sensi-

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GDR, glucose disposal rate; IRS, insulin receptor substrate; OGTT, oral glucose tolerance test; PDK-1, phosphoinositide-dependent protein kinase-1; PI(3,4,5)P₃, phosphatidylinositol 3,4,5-triphosphate; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; VLCD, very-low-calorie diet.

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TABLE 1
Clinical and metabolic characteristics of lean, obese, and diabetic subjects

| | Lean | Obese | Diabetic |
|--|-----------------|-----------------|------------------|
| Age (years) | 45 \pm 5 | 46 \pm 3 | 50 \pm 2 |
| BMI (kg/m ²) | 24.4 \pm 0.6 | 33.4 \pm 1.4* | 34.7 \pm 1.1* |
| HbA _{1c} (%) | 5.1 \pm 0.2 | 5.2 \pm 0.2 | 10.1 \pm 1.2*† |
| Glucose (mmol/l) | 4.8 \pm 0.1 | 5.1 \pm 0.1 | 9.4 \pm 0.8*† |
| Insulin (pmol/l) | 49 \pm 10 | 86 \pm 21 | 142 \pm 22.8‡ |
| Clamp insulin (pmol/l) | 6,400 \pm 600 | 6,108 \pm 960 | 4,012 \pm 696§ |
| Free fatty acids (mmol/l) | 0.36 \pm 0.06 | 0.53 \pm 0.07 | 0.80 \pm 0.10* |
| GDR (mg \cdot kg ⁻¹ \cdot min ⁻¹) | 13.2 \pm 0.9 | 9.8 \pm 0.4* | 5.0 \pm 0.4*† |

Data are means \pm SD. The study comprised 15 lean (all male), 16 obese (all male), and 21 obese type 2 diabetic (18 male and 3 female) subjects. Glucose, insulin, and free fatty acids were measured after an overnight fast. * P < 0.001 vs. lean; † P < 0.001 vs. obese; ‡ P < 0.01 vs. lean; § P < 0.05 vs. lean; || P < 0.05 vs. obese.

tizing drug rosiglitazone (22,23). Insulin-stimulated PKC α/ζ activity is also decreased in cultured myotubes of obese humans with impaired glucose tolerance (24) and in muscle of obese diabetic monkeys (25). However, there are no data regarding the activation of PKC α/ζ in vivo in humans.

Phosphoinositide-dependent protein kinase-1 (PDK-1) is a serine/threonine kinase, activated by a lipid product of PI3K phosphatidylinositol 3,4,5-triphosphate [PI(3,4,5)P₃] (26–29). PDK-1 directly phosphorylates and activates downstream effectors of PI3K, such as Akt and atypical PKCs, that mediate physiological actions of insulin (27,29–31). PDK-1 appears to be a rate-limiting step since overexpression of wild-type PDK-1 in adipocytes is sufficient to stimulate GLUT4 translocation to the plasma membrane in the absence of insulin (32–34). Overexpression of kinase-inactive PDK-1 inhibits insulin-stimulated GLUT4 translocation (34). These data suggest that PDK-1 plays an important role in the regulation of glucose transport. It is unknown whether insulin activates PDK-1 in skeletal muscle in humans or whether activation of PDK-1 is impaired in insulin-resistant states. Therefore, to determine the mechanism(s) for insulin resistance in obese nondiabetic and type 2 diabetic subjects, we investigated the ability of insulin to stimulate PKC α/ζ and PDK-1 activity in skeletal muscle. Furthermore, we determined whether defects in PKC α/ζ activation were reversed with weight loss therapy in obese humans.

RESEARCH DESIGN AND METHODS

For the initial study, 15 lean and 16 obese nondiabetic subjects and 21 obese subjects with type 2 diabetes participated. Nearly all subjects were male (Table 1) except for the three women in the diabetic group. There was no difference in the insulin action parameters when the three women were excluded from the analyses, so we included them. The experimental protocol was approved by the Committee on Human Investigation of the University of California, San Diego. Informed written consent was obtained after explanation of the protocol. All nondiabetic subjects had normal glucose tolerance (75-g oral glucose load) as defined by fasting glucose <7.0 mmol/l and 2-h glucose <7.8 mmol/l (35). Except for diabetes, the subjects were healthy and on no medications known to influence glucose metabolism. Up until several weeks before the study, the treatment of diabetic subjects was as follows: metformin for two subjects, metformin plus insulin for three, metformin plus sulfonylurea for five, insulin for one, insulin plus sulfonylurea and metformin for one, sulfonylurea for three, exercise and diet for three, and no therapy for three subjects. Hypoglycemic agents were withdrawn at least 2 weeks before studies were performed.

Weight loss protocol. Six obese subjects (BMI 30–45 kg/m²) (calculated as weight in kilograms divided by the height in meters squared) with normal glucose tolerance defined by oral glucose tolerance test (OGTT) criteria, who were weight-stable for at least 3 months before the study, entered the Special Diagnostics and Treatment Unit for baseline characterization: OGTT, hyperinsulinemic-euglycemic clamp, and muscle biopsy. Subjects were then placed on a very-low-calorie diet (VLCD) of 600–800 calories/day for up to 24 weeks or until ~10–15% of initial body weight was lost. Subjects were inpatients during the first 4 weeks of diet therapy and were then monitored at weekly visits. Once the weight loss goal was met, subjects were introduced to a weight-maintenance diet. Metabolic characterization studies were repeated after subjects were weight-stable at their new level for at least 2–3 weeks.

Euglycemic clamp. Subjects were admitted to the Special Diagnostic and Treatment Unit. After an overnight fast, they underwent a 3-h hyperinsulinemic (300 mU \cdot m⁻² \cdot min⁻¹)-euglycemic (5.0–5.5 mmol/l) clamp (8). The glucose disposal rate (GDR) was determined during the last 30 min of the clamp. Percutaneous needle biopsies of vastus lateralis muscle were performed before insulin infusion and at the end of the clamp (8), and muscle tissue was immediately frozen in liquid nitrogen. Plasma glucose and insulin levels were determined before each biopsy.

Tyrosine phosphorylation of the insulin receptor and IRS-1. Muscle lysates were prepared (8), and insulin receptor and IRS-1 phosphorylation were determined as described (36). IRS-1 phosphorylation in obese subjects before and after weight loss was determined using a polyclonal IRS-1 [pY⁶¹²] phosphospecific antibody (Biosource International, Camarillo, CA).

Determination of PI3K activity. Muscle lysates (500 μ g protein) were subjected to immunoprecipitation with a polyclonal IRS-1 antibody, and PI3K activity was determined as described (8).

Determination of PKC α/ζ and PDK-1 activity. Muscle lysates (200 μ g protein) were subjected to immunoprecipitation with a polyclonal PKC ζ antibody (Santa Cruz Biotechnology, Santa Cruz, CA) that recognizes both PKC α and ζ or a polyclonal PDK-1 antibody (Upstate Biotechnology, Lake Placid, NY) coupled to protein A-Sepharose beads (Sigma) or G-Sepharose beads (Amersham Pharmacia Biotechnology, Piscataway, NJ). Immune pellets were washed, and PKC α/ζ activity was determined (22). PDK-1 activity was determined according to the manufacturer's instructions (Upstate Biotechnology). With this protocol the efficiency of immunoprecipitation of PKC α/ζ and PDK-1 is ~90% (not shown).

PKC α/ζ , PKC α , PKC ζ , PKC ϵ , PKC θ , and PDK-1 protein levels. Tissue lysate protein (50 μ g per lane) was resolved by SDS-PAGE and transferred to nitrocellulose membranes. The nitrocellulose membranes were blocked with 5% nonfat dry milk and incubated with antibodies against PKC α/ζ (recognizes both PKC α and PKC ζ ; Santa Cruz Biotechnology), PDK-1 (Upstate Biotechnology), PKC α (Santa Cruz Biotechnology), PKC ζ (Transduction laboratories, San Jose, CA; recognizes human PKC ζ and mouse PKC α , which are 97% homologous), PKC ϵ (Santa Cruz Biotechnology), or PKC θ (Transduction Laboratories). The membranes were washed and bands visualized with enhanced chemiluminescence (Amersham) and quantified by densitometry (Molecular Dynamics) (37).

Statistical analysis. Statistical analyses including correlation coefficients were performed using the Stat View program (Abacus Concepts, Berkeley, CA). Statistical significance was tested with ANOVA and paired Student's *t* test when appropriate.

RESULTS

The mean age was similar in all groups (Table 1). BMI was increased in obese nondiabetic and obese type 2 diabetic subjects compared with lean subjects. BMI was not different between obese nondiabetic and diabetic subjects. HbA_{1c} and fasting serum glucose were normal in obese nondiabetic subjects but elevated in type 2 diabetic subjects. Fasting plasma insulin levels tended to be increased in obese nondiabetic subjects and were 2.9-fold elevated in diabetic subjects compared with lean subjects (Table 1). Clamp insulin levels were the same in lean and obese nondiabetic subjects. Although they were reduced in diabetic subjects, the concentration was still in the maximally stimulating range. Fasting plasma FFA levels were normal in obese nondiabetic subjects but were elevated in diabetic subjects. GDR was reduced 26% in obese nondiabetic subjects compared with lean subjects, 62% in obese diabetic subjects compared with lean subjects, and 48% in

A Insulin receptor and IRS-1 phosphorylation and total protein

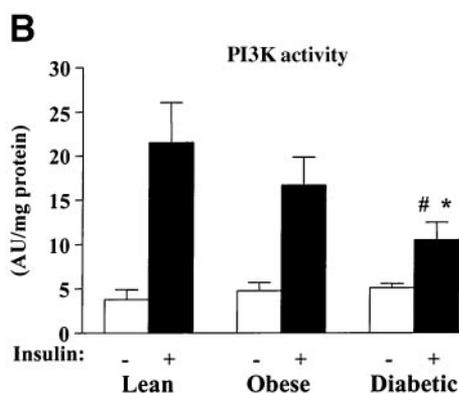
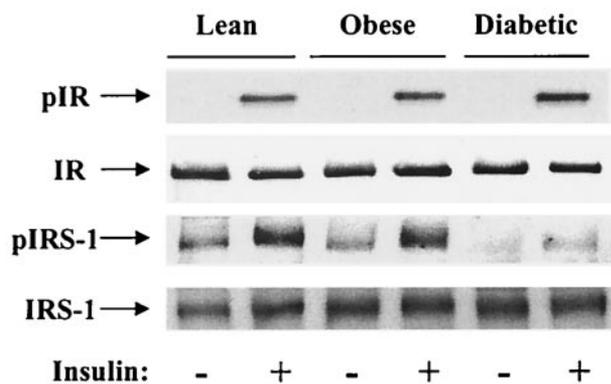


FIG. 1. Insulin receptor and IRS-1 tyrosine phosphorylation (A) and IRS-1-associated PI3K activity (B) in skeletal muscle of lean, obese nondiabetic and obese diabetic subjects. All subjects underwent a 3-h hyperinsulinemic-euglycemic clamp, and biopsies of vastus lateralis muscle were performed before and at the end of the clamp. A: Muscle lysates (250 μ g protein) were subjected to immunoprecipitation with an IRS-1 or insulin receptor antibody. The precipitated proteins were separated by SDS/PAGE on 4–12% gels and immunoblotted with an antiphosphotyrosine antibody. B: PI3K activity was measured in muscle lysates (500 μ g) that were subjected to immunoprecipitation with an IRS-1 antibody. Results are means \pm SE for 7–17 subjects per group. PI3K data from 16 of the 35 subjects were included in our previous study (8). * $P < 0.01$ vs. lean; # $P < 0.05$ vs. obese.

obese diabetic compared with obese nondiabetic subjects (Table 1). Thus, obese nondiabetic subjects were mildly insulin resistant, whereas diabetic subjects were more severely insulin resistant.

Insulin receptor and IRS-1 tyrosine phosphorylation. Insulin stimulated insulin receptor tyrosine phosphorylation 2.8- to 3.7-fold in muscle of all groups, with no impairment in obese nondiabetic or diabetic subjects (Fig. 1A). Insulin also stimulated IRS-1 tyrosine phosphorylation \sim 2.5-fold in lean and obese nondiabetic subjects. IRS-1 phosphorylation was decreased 41–48% in diabetic subjects compared with lean or obese nondiabetic subjects. The total amounts of insulin receptor and IRS-1 proteins were unaltered among the groups (Fig. 1A). These data confirm other previous findings (9–11).

PI3K activity. Insulin increased IRS-1-associated PI3K activity 5.7-fold in lean, 3.5-fold in obese nondiabetic, and only 2.1-fold in obese diabetic subjects (Fig. 1B). Insulin-stimulated IRS-1-associated PI3K activity tended to be lower in muscle from obese nondiabetic subjects, but this

A PKC λ/ζ activity

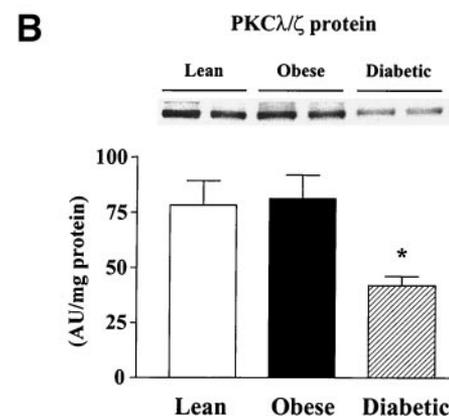
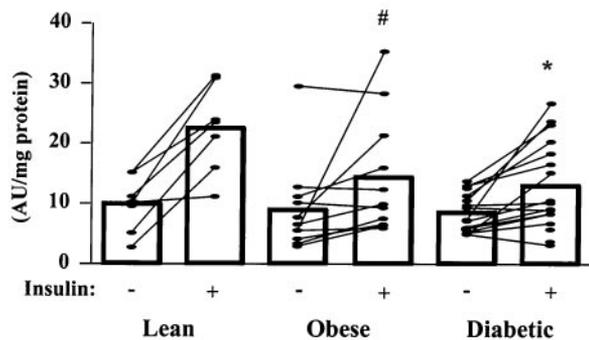


FIG. 2. PKC λ/ζ activity (A) and PKC λ/ζ protein amounts (B) in skeletal muscle of lean, obese nondiabetic, and obese diabetic subjects. Subjects underwent clamp and biopsies as in Fig. 1. A: PKC λ/ζ activity was measured in muscle lysates (200 μ g) that were subjected to immunoprecipitation with an antibody that recognizes both PKC λ and PKC ζ . The immunoprecipitated pellets were assayed for kinase activity using a PKC ϵ pseudosubstrate as substrate. Results are means \pm SE for 7–19 subjects per group. * $P < 0.01$ vs. lean; # $P < 0.05$ vs. lean. We see the normal variation among human subjects with several obese nondiabetic and obese diabetic subjects who respond relatively well to insulin. However, three-quarters of these subjects have markedly reduced responses to insulin. We could not identify any metabolic characteristics that distinguished the few responders. B: Proteins in muscle lysates (50 μ g) were separated by SDS/PAGE on 8% gels and transferred to nitrocellulose membranes. PKC λ/ζ was visualized by immunoblotting. Representative autoradiogram of PKC λ/ζ levels in muscle of six subjects; bars show densitometric quantitation of PKC λ/ζ protein levels in 7–19 subjects per group. The autoradiogram is representative of three different gels. * $P < 0.01$ vs. lean and obese nondiabetic.

did not reach statistical significance. PI3K was reduced 51% in obese diabetic subjects compared with lean subjects and 37% compared with obese nondiabetic subjects. The insulin-stimulated increment in PI3K activity above basal was decreased 70% in diabetic compared with lean subjects and 60% in diabetic compared with obese nondiabetic subjects. This is most likely due to decreased IRS-1 tyrosine phosphorylation (Fig. 1A). These data are also consistent with the previous findings (9–11). However, we felt it was important to quantitate the degree of impairment in these proximal signaling steps in the same samples in which we measured PKC λ/ζ activity and PDK-1 activity.

PKC λ/ζ activity and protein. Basal activity of PKC λ/ζ was not different among the three groups (Fig. 2A). Insulin-stimulated PKC λ/ζ activity was 2.3-fold in muscle of lean subjects but only 1.6-fold in obese and 1.5-fold in diabetic

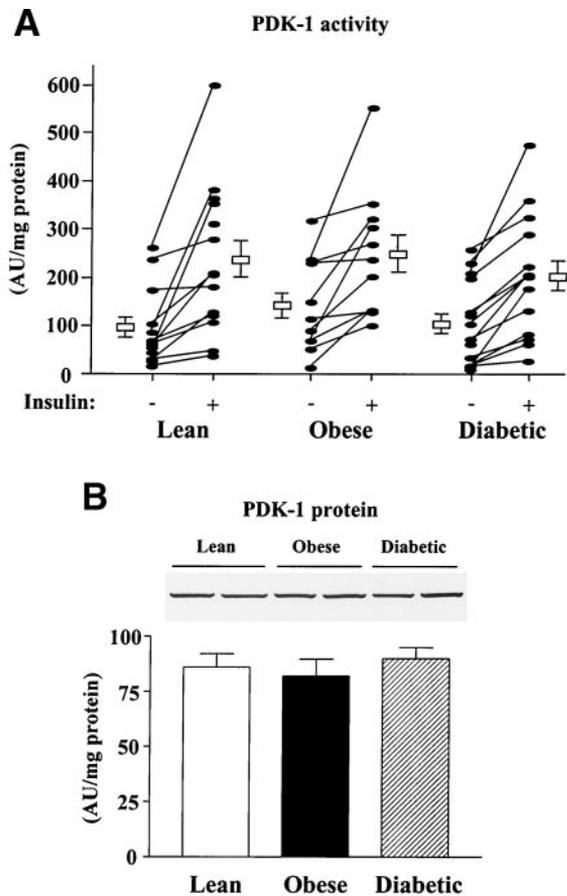


FIG. 3. PDK-1 activity (A) and PDK-1 protein amounts (B) in skeletal muscle of lean, obese nondiabetic, and obese diabetic subjects. Subjects underwent clamp and biopsies as in Fig. 2. A: PDK-1 activity was measured in muscle lysates (200 μ g) that were subjected to immunoprecipitation with a PDK-1 antibody. The immunoprecipitated pellets were assayed for kinase activity using Akt as substrate. For two lean nondiabetic subjects, biopsy specimens were available only in the insulin-stimulated state because of low yields in the basal state. Therefore, these two values are included without corresponding basal values. These values do not change the means for insulin-stimulated PDK-1 activity, which are similar in all three groups. Results are means \pm SE for 11–14 subjects per group. B: Proteins in muscle lysates (50 μ g) were separated by SDS/PAGE on 8% gels and transferred to nitrocellulose membranes. PDK-1 was visualized by immunoblotting. Representative autoradiogram of PDK-1 levels in muscle of 6 subjects; bars show densitometric quantitation of PDK-1 protein levels in 11–14 subjects per group. The autoradiogram is representative of three different gels.

subjects. The insulin-stimulated increment in PKC α/ζ activity above basal was decreased 57% in obese and 65% in diabetic subjects compared with lean subjects (Fig. 2A). This suggests that reduced PKC α/ζ activity may contribute to insulin resistance in muscle in humans. PKC α/ζ protein amount was normal in obese subjects but was reduced 46% in diabetic subjects compared with lean subjects (Fig. 2B). When PKC α/ζ activity was normalized for the protein amount, the increment of insulin-stimulated PKC α/ζ activity over basal was still reduced 33% in diabetic subjects [lean 144 ± 28 and diabetic 96 ± 17 increase over basal (arbitrary units), $P < 0.05$].

A positive relationship was seen between insulin-stimulated PKC α/ζ activity and insulin-stimulated GDR ($r = 0.70$, $P < 0.08$) in lean subjects. This correlation was not present in obese nondiabetic or obese diabetic subjects (data not shown). We found a similar correlation between

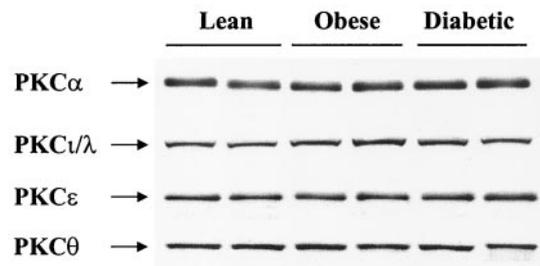


FIG. 4. PKC α , PKC ι/λ , PKC ϵ , and PKC θ protein amounts in skeletal muscle of lean, obese nondiabetic, and obese diabetic subjects. Proteins in muscle lysates (50 μ g) were separated by SDS/PAGE on 8 or 10% gels and transferred to nitrocellulose membranes. PKC α , PKC ι/λ , PKC ϵ , and PKC θ were visualized by immunoblotting with specific antibodies. This blot is representative of three blots on 11–14 subjects per group.

the amounts of PKC α/ζ protein and insulin-stimulated GDR in lean subjects ($r = 0.53$, $P < 0.002$).

PDK-1 activity and protein. Insulin increased PDK-1 activity in muscle of nearly every subject. Neither basal nor insulin-stimulated PDK-1 activity was different among the three groups (Fig. 3A). In addition, the insulin-stimulated increment above basal was unchanged with obesity or diabetes. There was also no difference in the amount of PDK-1 protein in muscle among the groups (Fig. 3B).

PKC α , PKC ι , PKC ϵ , and PKC θ protein levels. To determine the specificity of the effect to decrease PKC α/ζ in muscle of obese diabetic subjects, we measured protein levels for specific PKC isoforms in the same samples. PKC α , PKC ι/λ , PKC ϵ , and PKC θ protein amounts were not altered by obesity or diabetes (Fig. 4). PKC ι is the human homologue of the mouse PKC λ ; these proteins are 97% homologous (38,39). The total amount of PKC ι/λ was not decreased in diabetic subjects (Fig. 4), whereas immunoreactivity with the antibody that recognizes PKC λ and ζ showed a 43% decrease (Fig. 2B). Transfection studies in COS-7 cells showed equal affinity of this latter antibody for PKC λ and PKC ζ (not shown). Therefore, these data imply that PKC ζ is specifically reduced in muscle of diabetic subjects.

TABLE 2

Clinical and metabolic characteristics of obese subjects before and after a VLCD treatment

| | VLCD | |
|--|-----------------|-----------------|
| | Pretreatment | Posttreatment |
| <i>n</i> | 6 | 6 |
| Age (years) | 45 \pm 3 | 45 \pm 3 |
| Body weight (kg) | 95.0 \pm 6.3 | 86.7 \pm 6.0* |
| BMI (kg/m ²) | 34.5 \pm 1.7 | 31.8 \pm 2.2* |
| Skinfold thickness (mm) | 206 \pm 15 | 179 \pm 13† |
| HbA _{1c} (%) | 5.5 \pm 0.2 | 5.4 \pm 0.1 |
| Glucose (mmol/l) | 5.2 \pm 0.1 | 5.0 \pm 0.12 |
| Insulin (pmol/l) | 96.0 \pm 13.4 | 84 \pm 7.8 |
| Clamp insulin (pmol/l) | 6,931 \pm 726 | 5,998 \pm 693 |
| Triglyceride (mg/dl) | 183 \pm 51 | 115 \pm 20 |
| Free fatty acid (mmol/l) | 0.53 \pm 0.08 | 0.52 \pm 0.06 |
| GDR (mg \cdot kg ⁻¹ \cdot min ⁻¹) | 8.2 \pm 0.7 | 10.2 \pm 1.3† |

Data are means \pm SD. Glucose, insulin, free fatty acid, and triglycerides were measured after an overnight fast. Skinfold thickness = abdominal + subscapular + suprailliac + chest. * $P < 0.01$ vs. pretreatment; † $P < 0.05$ vs. pretreatment.

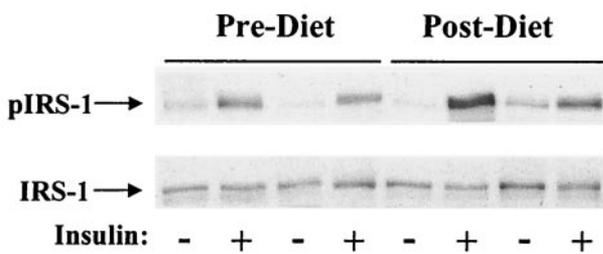
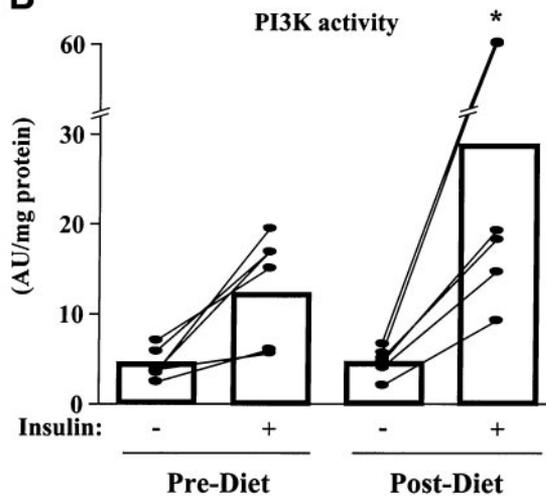
A IRS-1 Phosphorylation and Total Protein**B**

FIG. 5. IRS-1 tyrosine phosphorylation (A) and IRS-1-associated PI3K activity (B) in skeletal muscle of obese subjects before and after a VLCD treatment. Subjects underwent clamp and biopsies as in Fig. 1. A: Proteins in muscle lysates (50 μ g) were separated by SDS/PAGE on 7.5% gels and transferred to nitrocellulose membranes. IRS-1 was visualized by immunoblotting with phosphospecific IRS-1 or total IRS-1 antibody. Representative results for two subjects pre- and postdiet are shown. B: PI3K activity was measured as in Fig. 1A. Results are means \pm SE for six subjects per group * P < 0.05 vs. pretreatment for insulin-stimulated condition.

VLCD treatment. Only obese nondiabetic subjects were treated with VLCD. Subjects lost 4.3–14.4 kg. After 24 weeks of VLCD treatment, body weight, BMI, and skinfold thickness were decreased (P < 0.01), but HbA_{1c}, glucose, insulin, clamp insulin, and free fatty acid concentrations were unaltered (Table 2). Triglyceride concentrations tended to be reduced and GDR increased by 24% after VLCD treatment (P < 0.05) (Table 2).

VLCD treatment significantly increased IRS-1 tyrosine phosphorylation compared with pretreatment levels (Fig. 5A). In parallel, insulin-stimulated IRS-1-associated PI3K activity was increased \sim 2.4-fold (P < 0.05) posttreatment with VLCD compared with pretreatment (Fig. 5B). The total amount of IRS-1 protein was unaltered by the diet treatment (Fig. 5A).

Basal activity of PKC ζ was not altered by VLCD treatment, but insulin-stimulated activity was increased nearly twofold over pretreatment levels (P < 0.01) and was similar to the effect in lean control subjects (Figs. 2A and 6A). The amount of PKC ζ protein in muscle was unchanged between both groups (Fig. 6B), indicating that the ability of insulin to activate PKC ζ was improved by VLCD. This could be due to increased signaling upstream of PKC ζ (Fig. 5B).

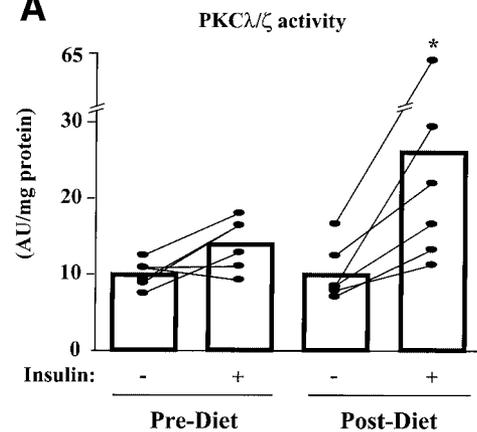
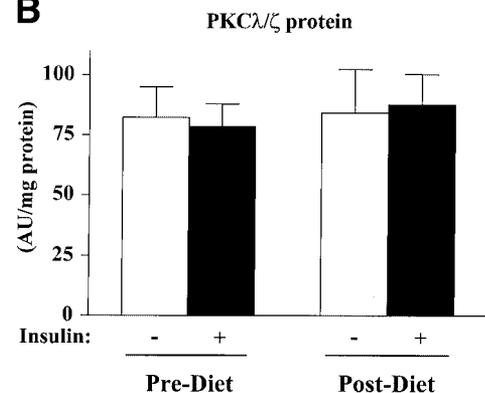
A**B**

FIG. 6. PKC ζ activity (A) and PKC ζ protein amounts (B) in skeletal muscle of obese subjects before and after a VLCD treatment. Subjects underwent clamp and biopsies as in Fig. 1. A: PKC ζ activity was measured as in Fig. 2A. Results are means \pm SE for six subjects per group. * P < 0.01 vs. pretreatment for insulin-stimulated condition. B: PKC ζ protein amount was measured as in Fig. 3B. Bars show densitometric quantitation of PKC ζ protein levels in six subjects per group.

DISCUSSION

Because PI3K is important for insulin action to maintain glucose homeostasis (2,3) and because activation of PI3K is defective in obese (8,9) and nonobese type 2 diabetic humans (10,11), elucidation of the potential downstream mediators of PI3K that contribute to the insulin resistance of obesity and type 2 diabetes is of great interest. While recent studies in knockout mice indicate that Akt2 is important for the metabolic effects of insulin at least in the liver, it is not clear whether the mild defect in skeletal muscle is directly due to the absence of Akt2 in muscle or is secondary to the insulin-resistant state (13). Furthermore, we found no impairment in insulin-stimulated Akt1, Akt2, or Akt3 activation in skeletal muscle of obese nondiabetic or obese diabetic humans despite decreased PI3K activation in diabetic humans (8). However, a study in muscle of lean type 2 diabetic subjects incubated with very high insulin concentrations showed decreased Akt activation (14). In the current study we investigated the potential role of the atypical PKC isoforms, PKC ζ , and of PDK-1, which are also downstream targets of PI3K.

Our data are the first to demonstrate that in vivo insulin administration in humans activates PKC ζ and PDK-1 in skeletal muscle. Importantly, insulin-stimulated PKC ζ

activity is reduced in muscle of obese nondiabetic and obese diabetic subjects. This contrasts with our previous findings that Akt activation is normal in obese and diabetic subjects with similar metabolic characteristics (8). Thus, although IRS-1-associated PI3K activity is reduced in diabetic subjects, not all downstream pathways are similarly affected. Consistent with our current results in humans, several studies in insulin-resistant rodent models including GK diabetic rats (22), high-fat-fed rats (40), free fatty acid-infused rats (41), and Zucker obese rats (Y.-B.K., B.B.K., unpublished data) and in obese diabetic monkeys (25) also show decreased insulin-stimulated PKC α / ζ activity in skeletal muscle. Taken together, these data suggest that reduced insulin-stimulated atypical PKC activity may play an important role in insulin resistance in vivo. Importantly, the impaired PKC α / ζ activation in obese humans is reversed with weight reduction. This, combined with the fact that rosiglitazone treatment of diabetic subjects reverses the defect in PKC α / ζ activity (42), suggests that reversal of the PKC α / ζ defect might enhance insulin sensitivity in insulin-resistant states.

In obese subjects, the amount of PKC α / ζ protein is normal, indicating that the entire defect in PKC α / ζ activation is due to impaired insulin action on the enzyme. In our diabetic subjects, PKC α / ζ protein in muscle is reduced. This may explain part of the defect in PKC α / ζ activation, but the reduction in the insulin-stimulated increment in PKC α / ζ activation above basal levels is greater than the reduction in PKC α / ζ protein. Therefore, there appears to also be a defect in insulin action on PKC α / ζ activity. The fact that basal PKC α / ζ activity is not reduced in diabetic subjects in spite of lower PKC α / ζ protein levels could be due to the elevated fasting insulin levels (Table 1). We see a similar reduction in PKC α / ζ protein in muscle of obese Zucker rats with severe insulin resistance (Y.-B.K., B.B.K., unpublished data).

The reduction in PKC α / ζ protein in diabetic humans appears to be relatively specific because protein levels of PKC α , PKC β , PKC ϵ , and PKC θ , as well as Akt isoforms (8), are not affected. We did not measure the activity of these other PKC isoforms because the aim of our study was to identify pathways downstream of PI3K that have been implicated in mediating insulin-stimulated glucose transport. Our data do not exclude the possibility that the activity or membrane localization of other PKC isoforms could also be altered and could possibly impair insulin action at other steps in the pathway.

The impairment in PKC α / ζ activation in diabetic subjects appears to be due to, at least in part, decreased upstream signaling including PI3K. Interestingly, PKC α / ζ activity is impaired in obese subjects who have no defect in PI3K activity. This discrepancy may reflect the fact that a PI3K-independent pathway can be involved in PKC α / ζ stimulation by insulin. The potential for metabolic regulation of PKC α / ζ activation by alternate pathways is demonstrated by the fact that glucose at high concentration rapidly activates PKC α / ζ in vitro in skeletal muscles through proline-rich tyrosine kinase-2, extracellular signal-related kinase-1/2 and phospholipase D pathways that are distinct from the PI3K pathway (43).

Because PI3K activates PKC α / ζ by producing PI(3,4,5)P $_3$, which binds directly to and activates PDK-1 (26–29),

another possible mechanism for discordance between normal activation of PI3K and impaired PKC α / ζ activity could be impaired PDK-1 activation. In the present study, we show that insulin stimulates PDK-1 activity in human skeletal muscle in vivo. Support for an insulin effect comes from in vitro studies showing that insulin increases phosphorylation and catalytic activity of PDK-1 in isolated rat adipocytes (32) and skeletal muscle cells (44). However, some studies indicated that PDK-1 is a constitutively active enzyme whose catalytic activity is not stimulated by growth factors such as insulin or IGF-1 in 293 cells or CHO cells or 3T3-L1 adipocytes (45–49). The difference may be due to differences in the type of cells or assay methodology in these studies. In addition, more recent studies in HeLa cells and 293 cells show insulin-stimulated translocation of PDK-1 to the plasma membrane, where it binds lipid products of PI3K and activates downstream signaling (50,51). In our study, insulin-dependent activation of PDK-1 is normal in muscle of obese nondiabetic and obese diabetic individuals. This combined with the impaired PKC α / ζ activation in obese and diabetic subjects suggests that PKC α / ζ activity can be regulated independent of PDK-1. Possibly, a defect in activation of another PDK isoform might explain the decrease in PKC α / ζ activation or altered subcellular localization of PDK-1 could modify PKC α / ζ activation. Although recent studies suggest that PDK-1 activity may be important for insulin stimulation of glucose transport and GLUT4 translocation in adipocytes and muscle cells (32–34), our data suggest that PDK-1 activation is not involved in the pathogenesis of insulin resistance in obesity and type 2 diabetes in humans.

In summary, in vivo administration of insulin in humans stimulates PKC α / ζ and PDK-1 activity. In insulin-resistant obese subjects with or without type 2 diabetes and with the characteristic impairments in glucose disposal in skeletal muscle, insulin-stimulated PKC α / ζ activity is reduced. Whereas PKC α / ζ protein content is normal in obese nondiabetic subjects, it is reduced in diabetic subjects. However, this cannot fully account for the defect in PKC α / ζ activation in diabetic subjects, and an impairment in insulin action on PKC α / ζ appears to also be present. This impairment can be reversed in obese subjects treated with a VLCD. Thus, reduced PKC α / ζ activity due to impaired activation and/or decreased expression may be an important factor in insulin resistance in muscle of obese nondiabetic and obese type 2 diabetic humans. Targeting PKC α / ζ itself could potentially have side effects, since atypical PKCs mediate other biologic processes such as cell growth and proliferation (52–54). However, the emergence of PKC α / ζ as a potentially important step in the pathogenesis of insulin resistance could lead to new treatment approaches for obesity and type 2 diabetes.

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