Activation of Protein Kinase C-ζ by Insulin and Phosphatidylinositol-3,4,5-(PO$_4$)$_3$ Is Defective in Muscle in Type 2 Diabetes and Impaired Glucose Tolerance

Amelioration by Rosiglitazone and Exercise

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Insulin resistance in type 2 diabetes is partly due to impaired glucose transport in skeletal muscle. Atypical protein kinase C (aPKC) and protein kinase B (PKB), operating downstream of phosphatidylinositol (PI) 3-kinase and its lipid product, PI-3,4,5-(PO$_4$)$_3$ (PIP$_3$), apparently mediate insulin effects on glucose transport. We examined these signaling factors during hyperinsuline-mic-euglycemic clamp studies in nondiabetic subjects, subjects with impaired glucose tolerance (IGT), and type 2 diabetic subjects. In nondiabetic control subjects, insulin provoked twofold increases in muscle aPKC activity. In both IGT and diabetes, aPKC activation was markedly (70–80%) diminished, most likely reflecting impaired activation of insulin receptor substrate (IRS)-1–dependent PI 3-kinase and decreased ability of PIP$_3$ to directly activate aPKCs; additionally, muscle PKC-ζ levels were diminished by 40%. PKB activation was diminished in patients with IGT but not significantly in diabetic patients. The insulin sensitizer rosiglitazone improved insulin-stimulated IRS-1–dependent PI 3-kinase and aPKC activation, as well as glucose disposal rates. Bicycle exercise, which activates aPKCs and stimulates glucose transport independently of PI 3-kinase, activated aPKCs comparably to insulin in nondiabetic subjects and better than insulin in diabetic patients. Defective aPKC activation contributes to skeletal muscle insulin resistance in IGT and type 2 diabetes, rosiglitazone improves insulin-stimulated aPKC activation, and exercise directly activates aPKCs in diabetic muscle. *Diabetes* 52:1926–1934, 2003

Skeletal muscle is the major organ for insulin-stimulated glucose disposal (1), and glucose transport is rate limiting for such disposal (2). Impaired activation of glucose transport in muscle contributes importantly to insulin resistance in type 2 diabetes (2), in both later and earlier phases, including impaired glucose tolerance (IGT), wherein blood glucose levels are only minimally increased.

The mechanisms underlying defects in insulin-stimulated glucose transport in IGT and type 2 diabetes are uncertain. Except for morbid obesity, insulin-sensitive GLUT4 glucose transporter levels in skeletal muscle are not altered (3), and further studies (see below) have suggested that there may be defects in insulin signaling and translocation of glucose transporters to the plasma membrane.

Recent studies suggest that insulin stimulates glucose transport through insulin receptor–mediated tyrosine phosphorylation of insulin receptor substrate (IRS)-1 or other intermediates that activate phosphatidylinositol (PI) 3-kinase, which, through increases in PI-3,4,5-(PO$_4$)$_3$ (PIP$_3$), activate downstream effectors protein kinase B (PKB/Akt) (4–7) and atypical protein kinase Cs (aPKCs) ζ and λ/ι (8–12). Although defects in IRS-1–dependent PI 3-kinase activation by insulin in muscle of type 2 diabetic human subjects have been reported (13–15), information on downstream activators of glucose transport is controversial or lacking. Thus, defective PKB activation was seen during incubation of muscle strips of nonobese type 2 diabetic humans (14), whereas PKB activation was undiminished in muscle biopsies taken during clamp studies in obese type 2 diabetic humans (15). Further, it is currently unknown whether aPKC activation is defective in muscles of type 2 diabetic subjects.

Concerning IGT, decreased incremental but normal absolute levels of IRS-1–dependent PI 3-kinase and no significant reduction in PKB phosphorylation were seen in human muscle in a hyperinsulinemic clamp study (16), suggesting that other factors may contribute more directly to defects in insulin-stimulated glucose disposal. On the other hand, defects in glucose transport and IRS-2–dependent PI 3-kinase and aPKC activation were observed in

TABLE 1
Patient characteristics

	Nondiabetic control subjects	Subjects with IGT	Type 2 Diabetic patients
Age (years)	44 ± 2 (18)	43 ± 3 (6)	50 ± 3 (10)
BMI	27 ± 1 (18)	34 ± 2 (6)	32 ± 2 (10)
		$P < 0.004$	$P < 0.03$
Fasting plasma glucose (mmol/l)	5.1 ± 0.2 (18)	6.2 ± 0.1 (6)	8.7 ± 1.4 (10)
			$P < 0.001$
Serum insulin (pmol)	111 ± 16 (17)	346 ± 124 (6)	235 ± 43 (10)
		$P < 0.004$	$P < 0.05$
Serum C-peptide (ng/ml)	1.46 ± 0.17 (17)	3.63 ± 0.97 (6)	2.63 ± 0.34 (10)
		$P < 0.001$	$P < 0.03$
HbA$_{1c}$ (%)	4.79 ± 0.13 (18)	5.42 ± 0.25 (6)	7.00 ± 0.71 (10)
			$P < 0.0003$
Triglycerides (mg/dl)	141 ± 16 (18)	209 ± 39 (6)	209 ± 36 (10)
Systolic blood pressure (mmHg)	127 ± 3 (18)	136 ± 76 (6)	139 ± 6 (10)
Diastolic blood pressure (mmHg)	76 ± 2 (18)	82 ± 3 (6)	85 ± 2 (10)
			$P < 0.02$
Serum free fatty acids (mmol)	0.44 ± 0.03 (17)	0.52 ± 0.08 (4)	0.51 ± 0.04 (10)
Glucose disposal rate $(mg \cdot kg^{-1} \cdot min^{-1})$	11.60 ± 1.11 (18)	5.92 ± 1.01 (6)	4.90 ± 0.74 (10)
		$P < 0.004$	$P < 0.0001$

Data are means $\pm$ SE (number of determinations). P values versus normal nondiabetic control subjects, by ANOVA.

cultured myocytes obtained from obese/impaired glucose tolerant humans (17).

In the current study, we obtained skeletal muscle biopsies during hyperinsulinemic-euglycemic clamp studies in humans and found that *1*) insulin-induced activation of aPKCs was defective in muscles of subjects with IGT and type 2 diabetic patients, *2*) this defect in muscle aPKC activation was accompanied by and appeared to be at least partly due to impaired activation of IRS-1–dependent PI 3-kinase and diminished responsiveness of aPKCs to PIP$_3$, and *3*) defects in insulin-stimulated PI 3-kinase and aPKC activation were improved by rosiglitazone treatment. We also found that exercise, which acutely increases aPKC activity and GLUT4 translocation/glucose transport by a PI 3-kinase–independent pathway in rodent muscle (18), was as effective as insulin in acutely activating aPKCs in muscles of nondiabetic patients and more effective than insulin in muscles of diabetic patients.

RESEARCH DESIGN AND METHODS

Patients. Type 2 diabetic patients who were reasonably well controlled (HbA$_{1c}$ levels between 5 and 8% in 8 of 10 diabetic patients and >8% in 2 patients; mean values shown in Table 1) with diet alone (4 patients), diet combined with sulfonylureas (3 patients), or sulfonylureas plus metformin (2 patients) and free of cardiovascular, renal, neuropathic, and other medical problems were studied. Antidiabetic medications were discontinued 2 weeks before the studies. Nondiabetic patients were matched for age and sex. Patients with IGT had fasting plasma glucose levels of 6.1–6.9 mmol/l and/or plasma glucose levels between 7.8 and 11.1 mmol/l at 2 h of a standard oral glucose tolerance test (75-g glucose load). Type 2 diabetic patients had fasting plasma glucose levels >6.9 mmol/l. Eighteen nondiabetic control subjects (15 men and 3 women), 10 diabetic patients (9 men and 1 women), and 6 patients with IGT (3 men and 3 women) were studied. Results of assays of signaling factors in women and men were similar and therefore pooled. Seven diabetic patients (six of whom had been on sulfonylurea and/or metformin treatment until 2 weeks before the clamp study) were studied both before and 1 month after treatment with rosiglitazone (8 mg/day). Eight nondiabetic and six diabetic patients were subjected to acute 20-min bicycle ergometry exercise (see below).

Informed consent was obtained before entry into the study. All procedures were reviewed, approved, and monitored by the Institutional Review Board of the University of South Florida College of Medicine and the Research Committee of the James A. Haley Veterans Hospital. Studies were performed in accordance with the Declaration of Helsinki and Good Clinical Practice.

Clamp/biopsy studies. Hyperinsulinemic-euglycemic clamp/vastus lateralis muscle biopsy studies were conducted in the morning after an overnight fast, as described (15). While receiving physiological saline intravenously, and after anesthetizing the biopsy site with subcutaneous injection of 0.5% lidocaine (without epinephrine), a percutaneous biopsy of the vastus lateralis muscle was performed with a 14-gauge biopsy needle in the basal state. Insulin was then given intravenously in a priming dose over 10 min, followed by a constant rate proportional to body weight (42 pmol $\cdot$ kg body wt^{-1} $\cdot$ min^{-1}) to rapidly raise (within 15 min) and steadily maintain serum insulin concentrations at ~5–6 nmol/l. Insulin concentrations (means $\pm$ SE) for nondiabetic control subjects ($n = 17$), patients with IGT ($n = 6$), and diabetic patients ($n = 10$) immediately before and during insulin administration in the clamp procedure were 0.13 ± 0.01 and 6.17 ± 0.47, 0.22 ± 0.04 and 5.61 ± 0.43, and 0.12 ± 0.03 and 6.38 ± 1.24 nmol/l, respectively. Plasma glucose levels were clamped at 5–6.1 mmol/l (measured every 5 min) by varying the rate of the glucose infusion. Forty minutes after starting insulin, a second biopsy was obtained. This amount of time was selected because aPKC activation has been found to be maximal after 15–30 min and steadily maintained during insulin treatment for 2 h in nondiabetic monkeys (19), 0.67–3 h in humans (as evidenced by comparison of results in the current study and those of an accompanying study in which aPKC activity was measured at 3 h [20]), and 1 h in rats (21). The clamp was continued for another 90–120 min to estimate steady-state insulin-stimulated glucose disposal rates during the final 30 min. Muscle samples were rapidly frozen in liquid N$_2$, stored at $-70°C$, and assayed within 2–4 weeks. Kinase activities appeared to be stable in these conditions, as evidenced by the fact that results of repeat aPKC assays were not appreciably different in repeat assays conducted 2–12 months after the initial assays (ratio of initial values to values in repeat assays = 1.03 ± 0.08 [means $\pm$ SE of 14 comparisons]).

Exercise/biopsy studies. Patients were exercised in the fasting state for 20 min at ~60% of VO$_{2max}$ (range 50–70%) on a stationary Fitron Cycle-Ergometer (Cybex) operating at 90 rpm, and an approximate work rate of 900 kg $\cdot$ m^{-1} $\cdot$ min^{-1}, or a work load of 150 W, was attained. Muscle biopsies were taken immediately before and within 5 min of completing the standard bicycle exercise test. This bicycle exercise was routinely performed with both legs, except in one case in which the same level of exercise was performed with one leg, and both exercised and rested legs were biopsied within 5 min of completing the procedure.

Muscle kinase analyses. Samples were homogenized with a Polytron in buffer containing 20 mmol/l Tris/HCl (pH 7.5), 50 mmol/l sucrose, 2 mmol/l EDTA, 2 mmol/l EGTA, 2 mmol/l Na$_3$VO$_4$, 2 mmol/l NaF, 2 mmol/l Na$_4$P$_2$O$_7$, 1 mmol/l phenylmethylsulfonyl fluoride, 20 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 1 μmol/l LR-microcytstin, as described (18,19,22). Muscle homogenates were supplemented with 150 mmol/l NaCl, 1%Triton X-100, and 0.5%

Nonidet, and 500 µg lysate was protein immunoprecipitated at 4°C with antisera for determination of total aPKC, IRS–1–dependent PI 3-kinase, or PKB-α enzyme activity. Precipitates were collected on Sepharose-AG beads (Santa Cruz Biotechnologies) and assayed as described below. In each assay, two to four replicate precipitates prepared from muscles of two to four nondiabetic control subjects and two to four diabetic patients or patients with IGT were assayed simultaneously to provide intra-assay comparisons.

PKC-ζ/λ/ι activation. PKC-ζ/λ/ι activity was measured as described previously (8–11,16,18,19,21–24). In brief, PKC-ζ/λ/ι was immunoprecipitated with a rabbit polyclonal antiserum (Santa Cruz Biotechnologies) that recognizes a common epitope in the COOH-termini of PKC-ζλ and -λ/ι, collected on Sepharose-AG beads, and incubated for 8 min at 30°C in 100 µl buffer containing 50 mmol/l Tris/HCl (pH,7.5), 100 µmol/l Na_3VO_4, 100 µmol/l $Na_4P_2O_7$, 1 mmol/l NaF, 100 µmol/l phenylmethylsulfonyl fluoride, 4 µg phosphatidylserine (Sigma), 50 µmol/l [γ-^{32}P]ATP (NEN/Life Science Products), 5 mmol/l $MgCl_2$, and, as substrate, 40 µmol/l serine analog of the PKC-ε pseudosubstrate (BioSource). After incubation, ^{32}P-labeled substrate was trapped on P-81 filter paper and counted in a liquid scintillation counter.

In some assays (sample availability permitting) 10 µmol/l PIP$_3$ (Matreya), a maximally effective concentration as currently determined in immunoprecipitates prepared from human muscle (10,19,21,25), was added to directly activate aPKCs in immunoprecipitates prepared from unstimulated muscles, as described (10,19,21,25).

As reported (8,10,19), aPKC recovery during immunoprecipitation is partial (50–60%) using antiserum concentrations as per the supplier's instructions. Thus, with this limiting antibody concentration, equal amounts of aPKCs were immunoprecipitated from all groups, regardless of differences in aPKC levels of lysates; therefore, aPKC values reflect enzyme specific activity.

PKB activation. PKB enzyme activity was measured using a kit obtained from Upstate Biotechnologies, as described previously (22–25). In brief, PKB was immunoprecipitated with sheep polyclonal antisera raised to PKBα (Upstate Biotechnologies), collected on Sepharose-AG beads, and incubated for 10 min at 30°C in 40 µl of buffer containing 75 mmol/l MOPS (pH 7.2), 93 mmol/l β-glycerophosphate, 19 mmol/l EGTA, 3.75 mmol/l Na_3VO_4, 3.75 mmol/l dithiothreitol, 10 µmol/l cyclic-AMP–dependent protein kinase inhibitor peptide, 100 µmol/l PKB-specific substrate (RPRAATF), 16 mmol/l $MgCl_2$, 112 µmol/l ATP, and 10 µCi [γ-^{32}P]ATP. Following incubation, ^{32}P-labeled substrate was trapped on P-81 filter paper and counted in a liquid scintillation counter.

PKB activation was also assessed by Western analysis and immunoblotting for serine-473 phosphorylation (rabbit polyclonal antiserum from Cell Signaling Technology).

IRS-1–dependent PI 3-kinase activation. IRS-1–dependent PI 3-kinase was precipitated with rabbit polyclonal antiserum (Upstate Biotechnologies) and assayed in the presence of PI, $MgCl_2$, and [γ-^{32}P]ATP, as previously described (19,20,22,26). ^{32}P-labeled PI-3-PO$_4$ was isolated by thin-layer chromatography and quantified with a BioRad PhosphoImager/Molecular Analyst Program.

Western analyses. As described (19,21,22,26), muscle lysates were immunoblotted for PKC-ζ/λ/ι (COOH-terminal rabbit polyclonal antiserum from Santa Cruz Biotechnologies that recognizes aPKCs ζ, λ, and ι), PKC-ζ (isoform-specific NH_2-terminal rabbit polyclonal antiseru,m kindly supplied by Dr. Todd Sacktor, State University of New York, Downstate Medical Center, Brooklyn, NY), PKC-λ/ι (isoform-specific mouse monoclonal antibodies from Transduction Labs), GLUT4 (mouse monoclonal antibodies from Biogenesis), p85 subunit of PI 3-kinase (rabbit polyclonal antiserum from Upstate Biotechnologies), PDK-1 (sheep polyclonal antiserum from Upstate Biotechnologies), IRS-1 (rabbit polyclonal antiserum from Upstate Biotechnologies), and PKB-α (rabbit polyclonal antiserum from Upstate Biotechnologies). Samples from the nondiabetic group, the group with IGT, and the diabetic group or from the untreated and rosiglitazone-treated diabetic groups were simultaneously blotted and resulting enhanced chemiluminescence was quantified with a BioRad PhosphoImager/Chemiluminescence Molecular Bioanalyst Program.

Serum analyses. Levels of immunoreactive insulin, C-peptide, and free fatty acids were determined as described (19,21).

PKC-ζ mRNA measurements. Muscle RNA was isolated and PKC-ζ mRNA was quantified by real-time RT-PCR using forward primer 5′-AGGAGACA GATGGAATTGCTTACAT-3′ and reverse primer 5′-CCTCCGAGTCGTCTT TAATGCT-3′ to produce the selected PKC-ζ cDNA fragment, followed by annealing with a probe containing fluorescent dyes coupled to a complementary sequence of human PKC-ζ mRNA, 5′-FAM (reporter fluorescein dye)-CCTCATCCCGGAAGC-3′-MGB (quencher fluorescein dye).

Statistical methods. Data are reported as actual counts per minute per immunoprecipitate observed in aPKC and PKB-α assays and as relative values (i.e., compared with the basal unstimulated nondiabetic control level) in assays of IRS-1–dependent PI 3-kinase. Data are expressed as means ± SE.

Statistical differences between two means, and relative differences between two samples, were determined by Student's unpaired and paired t tests, respectively. Statistical differences between three or more groups were determined by one-way ANOVA and the least-significant multiple comparison method.

RESULTS

Patient characteristics. As seen in Table 1, relative to nondiabetic control subjects, BMI was increased by 30% in the group with IGT ($P < 0.004$) and by 19% in the diabetic group ($P < 0.03$). Fasting plasma glucose levels were significantly increased by 70% in the diabetic group ($P < 0.001$) and mildly but not significantly in the group with IGT. Serum insulin and C-peptide levels, respectively, were increased approximately threefold in the group with IGT ($P < 0.004$ and $P < 0.001$) and twofold in the diabetic group ($P < 0.05$ and $P < 0.03$). HbA$_{1c}$ levels were increased in the diabetic group ($P < 0.0003$) and slightly but not significantly in the group with IGT. Diastolic blood pressure was mildly increased in the diabetic group ($P < 0.02$) and slightly but not significantly in the group with IGT. Glucose disposal rates were decreased in both the group with IGT ($P < 0.004$) and the diabetic group ($P < 0.0001$). Serum levels of triglycerides and free fatty acids, as well as levels of systolic blood pressure, trended upward in both the group with IGT and the diabetic group, but these changes were not statistically significant.

Insulin-induced alterations in skeletal muscle PKC-ζ/λ/ι enzyme activity. Administration of insulin during the clamp procedure increased muscle aPKC activity approximately twofold ($P < 0.0001$) in nondiabetic patients (Fig. 1A). Although basal (i.e., before insulin administration) levels of aPKC activity were not significantly different among groups (Fig. 1A), insulin failed to increase aPKC activity significantly in patients with IGT and diabetic patients. Moreover, relative to that seen in nondiabetic patients, insulin-stimulated aPKC activity was diminished by ~70–80% in both diabetic patients ($P < 0.0001$) and those with IGT ($P < 0.0007$) (Fig. 1A).

It may be noted that the mean BMI of diabetic patients was significantly greater than that of nondiabetic patients (Table 1). Moreover, obesity, in the absence of diabetes, has been found to be associated with diminished activation of aPKCs in monkeys (19) and humans (20). It may therefore be questioned whether obesity alone could have accounted for the diminution in aPKC activation observed in diabetic subjects. Germane to this question, there was significant overlap of individual BMI values in our nondiabetic and diabetic groups, and this overlap allowed us to evaluate the role of obesity by comparing six diabetic and six nondiabetic patients who had identical BMIs (29 ± 2 kg/m^2) and comparable ages (diabetic subjects 54 ± 5 and nondiabetic subjects 53 ± 4 years). The basal and insulin-stimulated muscle aPKC activity in these weight- and age-matched nondiabetic subjects and diabetic patients were as follows: $4,431 \pm 749$ (basal) and $8,728 \pm 1,478$ (insulin stimulated) cpm/immunoprecipitate in nondiabetic patients and $3,130 \pm 410$ (basal) and $4,114 \pm 412$ (insulin stimulated) cpm/immunoprecipitate in diabetic patients. These values in these weight- and age-matched subgroups were comparable to, and not significantly different from, those seen in the full groups depicted in Fig. 1A. It may therefore be surmised that simple obesity did
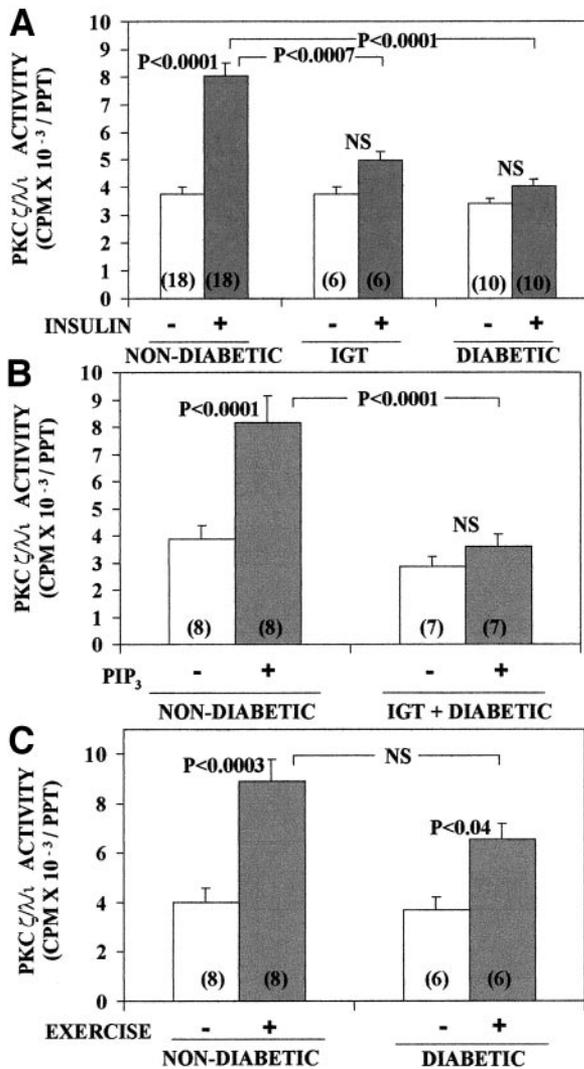
FIG. 1. Effects of insulin administration in vivo (*A*), PIP$_3$ treatment in vitro (*B*), and acute exercise (*C*) on PKC-ζ/λ/ι activity in vastus lateralis muscles of control nondiabetic patients and in patients who have IGT or type 2 diabetes. *A*: Insulin was administered during the clamp procedure. *B*: PIP$_3$ (10 μmol/l) was added to assays of equal amounts of aPKCs immunoprecipitated from basal/unstimulated muscle lysates of nondiabetic control subjects and patients with IGT/ diabetic patients (results of one patient with IGT and five diabetic patients were comparable and therefore combined). *C*: Nondiabetic and diabetic patients were subjected to 20 min bicycle exercise. Clear bars reflect basal/unstimulated values; shaded bars reflect stimulated values. Values are means ± SE of the number of patients shown in parentheses. *P* values were determined by ANOVA.

not account for the presently observed defect in aPKC activation seen in muscles of diabetic patients.

PIP$_3$-induced alterations in skeletal muscle PKC-ζ/λ/ι enzyme activity. It was of interest to see if PIP$_3$, the lipid product of PI 3-kinase, could directly activate aPKCs in immunoprecipitates prepared from human muscle, as has been observed in immunoprecipitates prepared from rodent (21) and monkey (19) muscle; such activation by PIP$_3$ could theoretically bypass defects in upstream activators of PI 3-kinase in muscles of patients with IGT and diabetic patients. Indeed, PIP$_3$ directly activated aPKCs in vitro in immunoprecipitates prepared from unstimulated muscles of nondiabetic patients ($P < 0.0001$) to an extent comparable to that observed with insulin treatment in vivo (Fig. 1*A* and *B*). In contrast, PIP$_3$ failed to activate aPKCs

significantly in aPKCs immunoprecipitated from unstimulated muscle samples of two IGT subjects and five diabetic patients ($P = 0.18$), and the level of PIP$_3$-stimulated aPKC activity in impaired glucose tolerant and diabetic muscle was significantly different ($P < 0.0001$) from that seen in nondiabetic muscle (Fig. 1*B*).

It is noteworthy that apart from stimulatory effects of PIP$_3$ on aPKC activity in unstimulated muscle, PIP$_3$ has previously been found to have little or no stimulatory effect on aPKC activity in immunoprecipitates prepared from insulin-stimulated adipocytes (10) and muscles (21) of rodents (presumably reflecting maximal activation). Similarly, we presently found that PIP$_3$ did not activate aPKCs in immunoprecipitates prepared from insulin-stimulated human muscles (ratio of aPKC activity, as assayed with PIP$_3$, to aPKC activity, as assayed without PIP$_3$ = 0.88 ± 0.04 [means ± SE of seven comparisons]). In this respect, note that PIP$_3$ may be slightly inhibitory when added to assays of aPKCs immunoprecipitated from insulin-stimulated muscle (10,21 and current study).

Exercise-induced alterations in skeletal muscle PKC-ζ/λ/ι enzyme activity. Like insulin, moderate treadmill exercise has been found to acutely activate aPKCs in mouse muscle (18), and, analogously to insulin, aPKCs may be important in activating glucose transport during exercise. It was therefore of interest to find that moderate bicycle ergometric exercise activated aPKCs in muscles of nondiabetic patients ($P < 0.0003$) (Fig. 1*C*), and this activation of aPKCs by exercise was comparable to that seen with insulin treatment of nondiabetic patients (Fig. 1*A* and *C*). Exercise also activated aPKCs in muscles of diabetic patients ($P < 0.04$) (Fig. 1*C*), and this activation of aPKCs by exercise was more effective than that observed with insulin ($P < 0.003$, ANOVA comparison of data in Fig. 1*A* and *C*). On the other hand, this aPKC-activating effect of exercise in diabetic muscle was less, on the average, than that seen in nondiabetic muscle, but this difference was not statistically significant ($P = 0.07$) (Fig. 1*C*).

To be certain that the presently observed effects of bicycle ergometry on aPKCs were due to the exercise as such, rather that to alterations in levels of systemic factors during exercise, one nondiabetic patient performed this same bicycle exercise on separate occasions, both with one leg and two legs (i.e., the standard method). The activation of aPKCs in the one-legged exercise (exercised versus rested muscle, 7,769 and 3,936 cpm/immunoprecipitate, respectively) was essentially the same as that observed in standard two-legged exercise (postexercise versus preexercise, 7,735 and 3,363 cpm/immunoprecipitate, respectively). Thus, aPKCs appeared to be activated by local (presumably exercise-induced muscle contraction) rather than systemic factors.

Insulin-induced alterations in skeletal muscle IRS-1–dependent PI 3-kinase enzyme activity. Insulin increased IRS-1–dependent PI 3-kinase activity by 2.4-fold ($P < 0.0006$) in muscle of nondiabetic patients (Fig. 2) and by 3.1-fold ($P < 0.007$) in muscles of patients with IGT (Fig. 2); this difference in IRS-1–dependent PI 3-kinase activation in muscles of nondiabetic patients and subjects with IGT was not statistically significant. In contrast to nondiabetic patients and those with IGT, insulin failed to activate IRS-1–dependent PI 3-kinase significantly in dia-
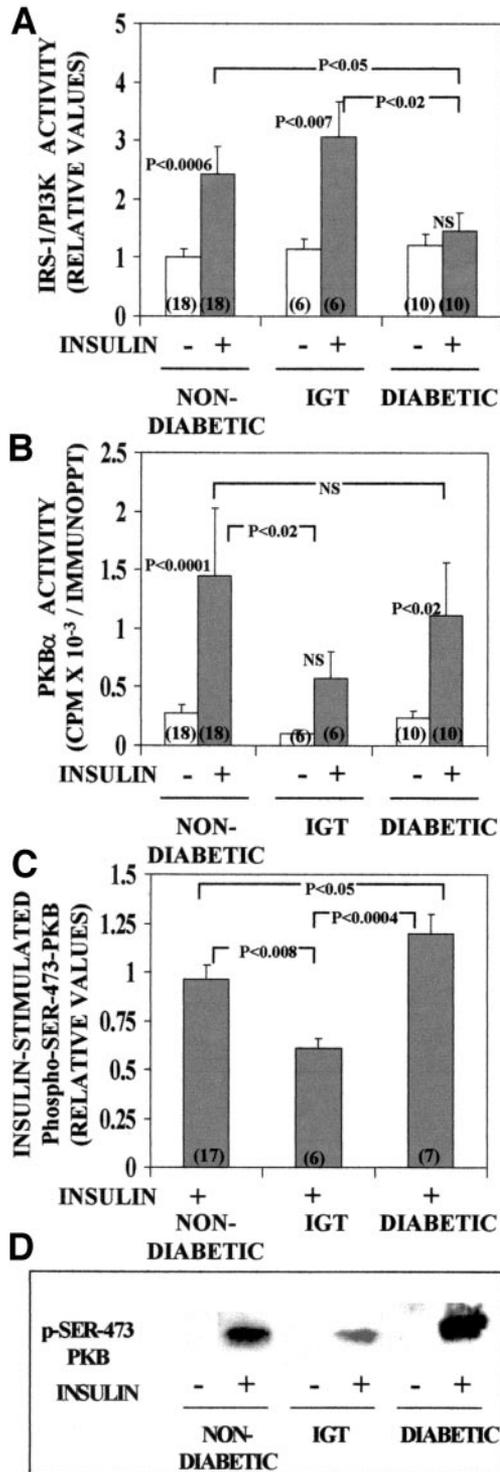
FIG. 2. Effects of insulin administration on IRS-1–dependent PI 3-kinase activity (*A*), PKB-α enzyme activity (*B*), and phosphorylation of serine-473 in PKB (*C* and *D*) in vastus lateralis muscles of control nondiabetic patients and in patients who have IGT or type 2 diabetes. Insulin was administered during the clamp procedure, and muscle samples were the same as those used in Fig. 1. Clear bars reflect basal/unstimulated values; shaded bars reflect stimulated values. Values are means ± SE of the number of patients shown in parentheses. *P* values were determined by ANOVA. Note that there was little or no measurable PKB phosphorylation in the absence of insulin treatment, as shown in *D* (which shows representative immunoblots).

betic patients, and this difference in activation was significantly different from that observed in both nondiabetic patients ($P < 0.05$) and patients with IGT ($P < 0.02$) (Fig. 2).

Insulin-induced alterations in skeletal muscle PKB-α enzyme activity/phosphorylation. Insulin increased PKB-α enzyme activity 5.3-fold ($P < 0.0001$) in muscles of nondiabetic patients and 4.7-fold ($P < 0.02$) in muscles of diabetic patients (Fig. 2*B*); this difference in PKB-α activation was not significantly different. In contrast, insulin failed to activate PKB-α significantly in muscles of patients with IGT, and this differed significantly ($P < 0.02$) from that seen in nondiabetic (but not diabetic) patients (Fig. 2*B*).

In keeping with changes in PKB-α activity, relative to phosphorylation seen in nondiabetic muscle, insulin-induced phosphorylation of serine-473 in PKB was significantly diminished by $36 \pm 12\%$ in impaired glucose tolerant muscle ($P < 0.008$) (Fig. 2*C* and *D*). Insulin-induced phosphorylation of serine-473 in PKB in muscles of patients with IGT was also significantly ($P < 0.0004$) less than that seen in muscles of diabetic patients (Figs. 2*C* and *D*). Surprisingly, insulin-induced phosphorylation of serine-473 in PKB in muscles of diabetic patients was $20 \pm 6\%$ greater ($n = 7$, $P < 0.05$) than that seen in muscles of nondiabetic patients (Fig. 2*C* and *D*). It is noteworthy that basal phosphorylation of serine-473 in PKB was very low and could not be reliably measured in muscle samples; consequently, comparisons could only be made between insulin-stimulated samples.

Alterations in immunoreactive levels of insulin-sensitive signaling/transport factors in skeletal muscles. PKC-ζ, migrating on SDS-PAGE at 80 kDa, was decreased by $35 \pm 5\%$ ($n = 9$, $P < 0.001$, ANOVA) and $26 \pm 10\%$ ($n = 6$, $P < 0.01$, ANOVA) in muscles of diabetic patients and those with IGT, respectively (Fig. 3). PKC-λ/ι, migrating at 75 kDa, was considerably less abundant than PKC-ζ (upper predominantly ζ and lower predominantly λ/ι bands in anti–COOH-terminal PKC-ζ/λ/ι blots) was not significantly altered (Fig. 3). Like PKC-ζ, and presumably reflecting that PKC-ζ is more abundant than PKC-λ in human muscle, total aPKC levels (i.e., PKC-ζ/λ/ι, as determined with the anti–COOH-terminal antiserum) were diminished by ~40% in diabetic patients and patients with IGT (Fig. 3). (PKC-ζ and -λ function interchangeably for insulin-stimulated glucose transport [11].) Levels of immunoreactive IRS-1, the p85 subunit of PI 3-kinase, PDK-1, PKB-α, and GLUT4 glucose transporters were comparable in muscles of nondiabetic control subjects, patients with IGT, and diabetic patients (Fig. 3).

Alterations in PKC-ζ mRNA in vastus lateralis skeletal muscles. In nondiabetic patients, the level of PKC-ζ mRNA was 481 ± 89 ($n = 12$) arbitrary units/ng rRNA. Surprisingly, PKC-ζ mRNA levels were increased nearly twofold in muscles of diabetic patients and patients with IGT (831 ± 102; $n = 8$, $P < 0.01$ vs. nondiabetic, ANOVA) and 715 ± 115 arbitrary units/ng rRNA ($n = 4$, not statistically different from nondiabetic), respectively.

Effects of rosiglitazone on glucose disposal rates and serum free fatty acids. Insulin-stimulated glucose disposal rates were increased ($P < 0.025$; unpaired t test) after rosiglitazone treatment (8 mg/day for 1 month) (Fig. 4). Although not shown, serum free fatty acid concentrations decreased, albeit not significantly, from 0.51 ± 0.04
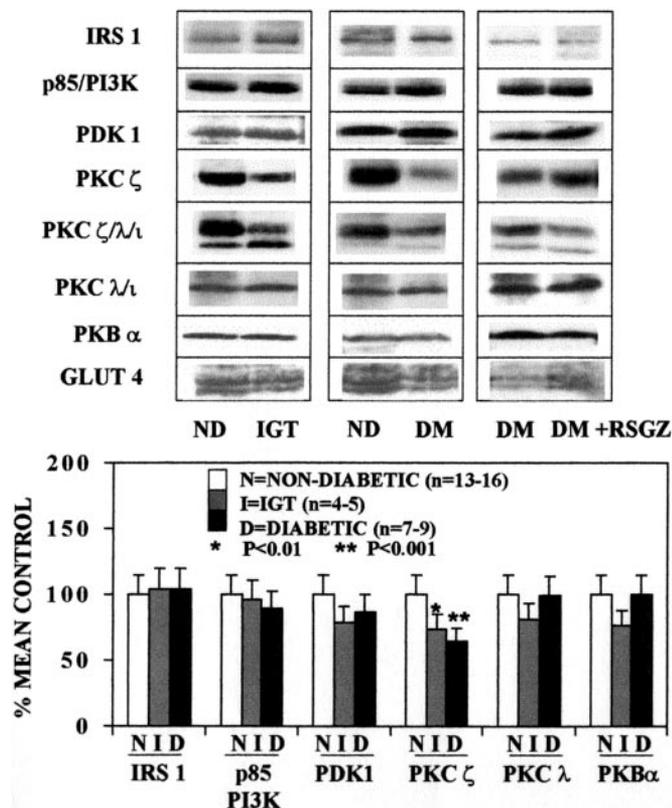
FIG. 3. Western analyses of IRS-1, p85 PI 3-kinase, PDK-1, PKC-ζ, combined PKC-ζ/λ/ι, PKC-λ/ι, PKB-α, and GLUT4 glucose transporters in muscles of nondiabetic control subjects (ND or N), patients with IGT, type 2 diabetic patients (DM or D), and type 2 diabetic patients treated with rosiglitazone (RSGZ). Note that comparisons were made between samples of nondiabetic control subjects versus patients with IGT or diabetic patients, or diabetic patients before and after rosiglitazone (RSGZ) treatment, as analyzed on the same immunoblot (note that there are different degrees of chemiluminescence development on different blots, as it is necessary to make comparisons within the same blot). *Top*: Representative immunoblot comparisons. *Bottom*: Bar graphs depicting relative means ± SE values of multiple (the number of patients examined is shown in parentheses) immunoblot analyses of insulin-sensitive signaling factors, with nondiabetic values set at 100%. Asterisks indicate *P* values, as determined by ANOVA.

($n = 7$) to 0.34 ± 0.07 mmol/l ($n = 7$), following rosiglitazone treatment.

Effects of rosiglitazone on activation and levels of skeletal muscle signaling factors and glucose transporters. Following rosiglitazone treatment, insulin-induced increases in IRS-1–dependent PI 3-kinase ($P < 0.001$, paired *t* test) and aPKC activities ($P < 0.04$, ANOVA) were significantly increased, albeit not to normal, in diabetic patients (Fig. 4). PKB-α activation and phosphorylation at serine-473 were increased in some, but not all, rosiglitazone-treated patients (changes not significant). Levels of IRS-1, p85 subunit of PI 3-kinase, PDK-1, PKB-α, PKC-ζ, PKC-λ/ι, and GLUT4 glucose transporters were unchanged by rosiglitazone treatment (Fig. 3).

Rosiglitazone-induced alterations in glucose disposal rates and signaling factors in individual patients are shown in Fig. 5.

DISCUSSION

The present findings suggested that diminished activation of aPKCs in skeletal muscle contributes importantly to decreases in insulin-stimulated glucose disposal rates in

diabetic patients. This conclusion follows if it is accepted that *1*) glucose transport is rate limiting for glucose uptake in skeletal muscle, *2*) whole-body glucose disposal rates at high levels of insulin stimulation are importantly determined by glucose uptake into muscle, and *3*) aPKCs are required for insulin-stimulated glucose transport. In support of the latter premise, aPKCs are required for insulin-stimulated glucose transport in L6 muscle cells (9,23), rat and 3T3/L1 adipocytes (8,10–12), cultured human adipocytes (24), and murine brown adipocytes (27).

Remarkably, insulin-stimulated aPKC activation was comparably impaired in patients with IGT and type 2 diabetic patients. Thus, defective aPKC activation occurs early in the development of IGT and type 2 diabetes. In this regard, it may be noted that defective activation of aPKCs has been observed in cultured myotubes that were derived from obese, glucose-intolerant humans (17) (i.e., even after removal of these cells from the influences of glucose, fatty acids and other circulating factors that may down-regulate insulin action). It is also important to note that defective activation of aPKCs has been observed in obese glucose-tolerant humans (20) and in obese glucose-tolerant women who have polycystic ovary syndrome (PCOS) (M.B., S.M.P., M.D., D.G., J.P., S. Lucidi, A.M., Y.K., G.B., M.L.S., R.V.F., unpublished observations).

Defects in aPKC activation in diabetic patients were associated with, and most likely at least partly due to, defects in upstream signaling by the insulin receptor acting through IRS-1 and PI 3-kinase, as presently noted and previously described (13–15). However, insulin-induced activation of IRS-1–dependent PI 3-kinase was not diminished in patients with IGT and the presently observed defect in aPKC activation in these patients cannot be explained by a simple defect in IRS-1–dependent PI 3-kinase activation.

In searching for other potential reasons for diminished aPKC activation, we found that the direct activation of aPKCs by PIP$_3$, the lipid product of PI 3-kinase, was impaired in subjects with IGT and diabetic patients. Interestingly, poor responsiveness of aPKCs to PIP$_3$ has also been seen in muscles of obese insulin-resistant glucose-intolerant type 2 diabetic monkeys (19), obese insulin-resistant but glucose-tolerant women who have PCOS (M.B., S.M.P., M.D., D.G., J. P., S. Lucidi, A.M., Y.K., G.B., M.L.S., R.V.F., unpublished observations), and insulin-resistant normoglycemic high fat–fed rats (21). The reasons for poor responsiveness to PIP$_3$ in these diverse states are uncertain, but in high fat-fed rats this defect is not due to diminished ability of aPKCs to be activated by 3-phosphoinositide–dependent protein kinase-1 (21), which phosphorylates the activation loop site of aPKCs. Moreover, this defect in aPKC activation in fat-fed rats occurs in the absence of a defect in either IRS-1– or -2–dependent PI 3-kinase activation (21). Of further note, we have not observed poor responsiveness of aPKCs to PIP$_3$ in skeletal muscles of IRS-1 knockout mice (M.P.S., M.L.S., C.R. Kahn, R.V.F., unpublished observations), and a loss of this important upstream signaling factor does necessarily result in diminished responsiveness of aPKCs to PIP$_3$. In any event, it seems likely that a defect in aPKC activation by PIP$_3$ contributes importantly to impaired activation of aPKCs in a variety of insulin-resistant states.
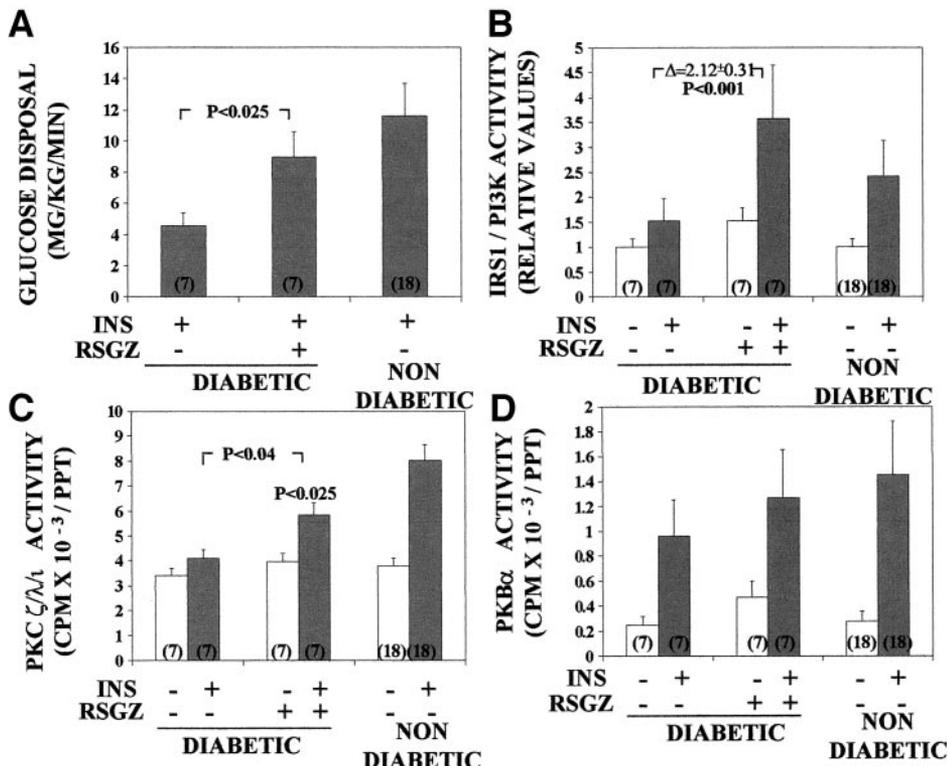
FIG. 4. Effects of rosiglitazone (RSGZ) on insulin-stimulated total-body glucose disposal rates (*A*), and insulin-stimulated activities of IRS-1–dependent PI 3-kinase (*B*), PKC-ζ/λ/ι (*C*), and PKB (*D*) in vastus lateralis muscle during clamp studies in diabetic patients. Values are means ± SE of the number of patient studies, which is shown in parentheses. *P* values were determined by ANOVA in *A* and *D* and by paired *t* test in *B*. For comparison, data from untreated nondiabetic control subjects are included in the figure.

Further studies are needed to determine whether obesity, increases in circulating lipids or intramyocellar lipids, or other factors are responsible for the defect in responsiveness of aPKCs to PIP_3 in diabetic muscle.

In addition to defective activation of aPKCs by upstream factors and PIP_3, the level of PKC-ζ, but not PKC-λ/ι, was diminished in muscles of diabetic patients and those with IGT. Accordingly, decreases in PKC-ζ levels may conceivably add further insult to decreases in insulin/PIP_3-stimulated aPKC enzyme activity, depending on the availability of aPKCs in muscle. The reason for the diminution in PKC-ζ levels in muscles of subjects with IGT and diabetic subjects is unclear; however, PKC-ζ mRNA levels were increased, suggesting a defect in translation/synthesis or stability of PKC-ζ.

As in diabetic rats (22), rosiglitazone ameliorated the defect in insulin-induced aPKC activation in muscles of diabetic patients, and this provided a reasonable explanation for increases in insulin-stimulated glucose disposal following rosiglitazone treatment. The improvement in aPKC activation was most likely at least partly due to increases in IRS-1–dependent PI 3-kinase activity. (Troglitazone also increases PI 3-kinase activation in human muscle [28].) However, rosiglitazone uses a variety of mechanisms to activate aPKCs in adipocytes (26), and further studies are needed to evaluate alternative mechanisms in muscle.

The ability of exercise to activate aPKCs in diabetic muscle is noteworthy. Although exercise-induced aPKC activation in diabetic muscle showed a tendency to be less than that seen in nondiabetic muscle, it nevertheless was substantial and significantly greater than that provoked by maximal insulin stimulation in diabetic muscle. If aPKC activation is in fact linked to GLUT4 translocation during exercise, the present findings may be relevant to the observation that exercise-stimulated GLUT4 translocation exceeds that seen with insulin in type 2 diabetic muscle (29). On the other hand, the presently observed tendency of exercise-induced aPKC activation to be less in diabetic, as opposed to nondiabetic, muscle is also in agreement with the reported tendency for a lesser effect of exercise on GLUT4 translocation in diabetic muscle (29).

As in certain studies of type 2 diabetic humans (15) and rats (22), alterations in insulin-stimulated PKB activation were relatively small and statistically insignificant in diabetic muscle. This is surprising in view of concomitant decreases in insulin-stimulated IRS-1–dependent PI 3-kinase in muscles of these same diabetic patients. It is uncertain whether this difference between PKB and aPKC activation in these diabetic muscles reflects that a lower level of IRS-1–dependent PI 3-kinase activation is required for PKB activation or whether factors other than IRS-1–dependent PI 3-kinase maintain PKB activation in diabetic muscle. Our equally surprising finding of diminished activation of PKB in subjects with IGT but not diabetic patients is in agreement with the latter possibility. This finding suggests that PKB-activating mechanisms may change during progression of IGT to overt type 2 diabetes, and these changes in PKB activation appear to be unrelated to changes in IRS-1–dependent PI 3-kinase.

Although the current study does not examine the effects of obesity on aPKC activation, our findings in weight-matched diabetic and nondiabetic patients indicated that obesity alone did not account for the defect in aPKC activation observed in diabetic patients. On the other hand, obesity was present in all patients with IGT and all of our diabetic patients were either overweight (four patients with BMI of 25–30 kg/m^2) or obese (six patients with BMI >30); accordingly, obesity may have served as a contributing factor for observed defects in aPKC activa-
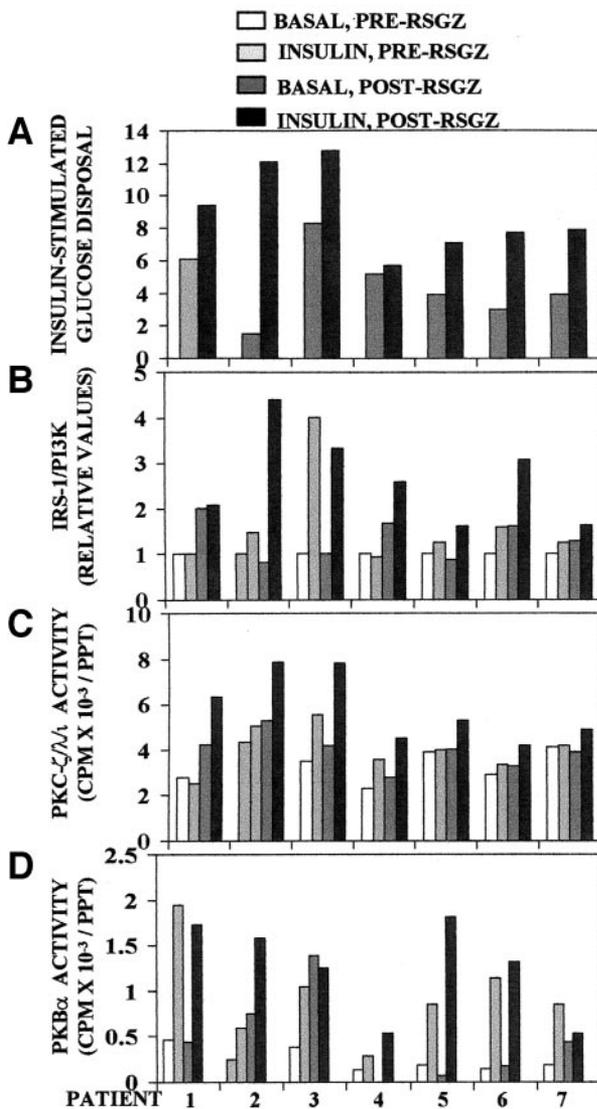
FIG. 5. Effects of rosiglitazone (RSGZ) on insulin-stimulated total-body glucose disposal rates (A), and insulin-stimulated activities of IRS-1–dependent PI 3-kinase (B), PKC-ζ/λ/ι (C), and PKB (D) in vastus lateralis muscle during clamp studies in diabetic patients. Shown are findings in individual patients (see Fig. 4 for mean values).

tion in our patients with IGT and our diabetic patients. In regards to the latter, we have found markedly defective aPKC activation, with only mild insignificant decreases in IRS-1–dependent PI 3-kinase and PKB activation, in insulin-resistant, glucose-tolerant obese women who have PCOS (M.B., S.M.P., M.D., D.G., J.L.P., S. Lucidi, A.M., Y.K., G.B., M.C.S., R.V.F., unpublished observations). In addition, we have found that caloric restriction prevents the appearance of obesity and diabetes and associated defects in IRS-1–dependent PI 3-kinase and aPKC activation in monkeys (19). Thus, in agreement with the accompanying report (20), we believe that obesity is frequently, although not invariably, associated with defects in aPKC activation and glucose disposal. Moreover, these obesity-related defects are preventable or reversible by dietary means, at least in some patients.

To summarize, we found defects in the activation and levels of aPKCs in muscles of type 2 diabetic patients. Both defects occurred early in the pathogenesis of type 2

diabetes, as they were of comparable severity in subjects with IGT and overtly diabetic patients. The defect in aPKC activation in type 2 diabetes was probably at least partly due to decreased activation of IRS-1–dependent PI 3-kinase and decreased responsiveness of aPKCs to PIP_3. The defect in aPKC levels is unexplained but not due to a decrease in PKC-ζ mRNA. Although underlying mechanisms remain uncertain, the presently observed defect in aPKC activation provides a reasonable proximate explanation for defects in glucose transport in muscles of patients with IGT and type 2 diabetic patients, as well as subsequent systemic insulin resistance.

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