

Skeletal Muscle Insulin Resistance in Obesity-Associated Type 2 Diabetes in Monkeys Is Linked to a Defect in Insulin Activation of Protein Kinase C- $\zeta/\lambda/\iota$

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Rhesus monkeys frequently develop obesity and insulin resistance followed by type 2 diabetes when allowed free access to chow. This insulin resistance is partly due to defective glucose transport into skeletal muscle. In this study, we examined signaling factors required for insulin-stimulated glucose transport in muscle biopsies taken during euglycemic-hyperinsulinemic clamps in nondiabetic, obese prediabetic, and diabetic monkeys. Insulin increased activities of insulin receptor substrate (IRS)-1-dependent phosphatidylinositol (PI) 3-kinase and its downstream effectors, atypical protein kinase Cs (aPKCs) ($\zeta/\lambda/\iota$) and protein kinase B (PKB) in muscles of nondiabetic monkeys. Insulin-induced increases in glucose disposal and aPKC activity diminished progressively in prediabetic and diabetic monkeys. Decreases in aPKC activation appeared to be at least partly due to diminished activation of IRS-1-dependent PI 3-kinase, but direct activation of aPKCs by the PI 3-kinase lipid product PI-3,4,5-(PO_4)₃ was also diminished. In conjunction with aPKCs, PKB activation was diminished in prediabetic muscle but, differently from aPKCs, seemed to partially improve in diabetic muscle. Interestingly, calorie restriction and avoidance of obesity largely prevented development of defects in glucose disposal and aPKC activation. Our findings suggest that defective activation of aPKCs contributes importantly to obesity-dependent development of skeletal muscle insulin resistance in prediabetic and type 2 diabetic monkeys. *Diabetes* 51:2936–2943, 2002

The pathogenesis of insulin resistance in type 2 diabetes and related obesity is poorly understood, particularly in primates. In human type 2 diabetic subjects, defective insulin-stimulated glucose transport has been observed in skeletal muscle (1), the major organ for glucose disposal (2). However, the

mechanisms underlying this defect remain unclear. Except for morbidly obese humans, alterations in muscle insulin-sensitive GLUT4 glucose transporters are not apparent in human type 2 diabetes (3), and the translocation of these transporters to the plasma membrane, or the underlying signaling mechanisms that activate this translocation, seems more likely to be immediately responsible for defects in insulin-stimulated glucose transport in these diabetic muscles.

Concerning insulin signaling to the glucose transport system, it is generally accepted that 1) phosphatidylinositol (PI) 3-kinase plays a key role by generating PI-3,4,5-(PO_4)₃ (PIP_3) in response to activation by insulin receptor-mediated tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and other signaling factors, and that 2) PIP_3 activates downstream effectors, viz., protein kinase B (PKB/Akt) isoforms α/β (4–7) and atypical protein kinase C (aPKC) isoforms $\zeta/\lambda/\iota$ (8–12). Defects in insulin activation of IRS-1-dependent PI 3-kinase have been observed in skeletal muscle of lean and obese type 2 diabetic humans (13–15), and decreases in PKB activation were observed in muscle of lean (14) but not obese (15) diabetic humans (14). Information on aPKC activation is limited to a preliminary report of diminished activation of aPKCs in muscles of obese diabetic humans (16), and there is no published information on alterations of aPKCs in other primates. On the other hand, the activation of aPKCs in skeletal muscle by insulin was found to be defective in nonobese type 2 diabetic Goto-Kakazaki rats (17) and in rats fed a high-fat diet (18).

In this study, we examined the activation of IRS-1-dependent PI 3-kinase, PKB, and aPKCs ($\zeta/\lambda/\iota$) in skeletal muscle during hyperinsulinemic-euglycemic clamp studies in a group of monkeys (nondiabetic, prediabetic, diabetic, and calorie-restricted) in which “prediabetes,” i.e., a state of obesity and associated insulin resistance attended by compensatory β -cell hyper-responsiveness and maintenance of relatively normal plasma glucose levels, followed by overt type 2 diabetes, occurs at high prevalence rates (>60%) in the absence of caloric restriction (19–21). In these studies, which were both cross-sectional and, in some monkeys, longitudinal, we found that insulin-induced activation of aPKCs diminished partially in prediabetic monkeys and then more severely as monkeys became overtly diabetic. In contrast, insulin activation of

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aPKC, atypical protein kinase C; IRS, insulin receptor substrate; K_{glu} , glucose disappearance rate; PDK-1, 3-phosphoinositide-dependent protein kinase-1; PI, phosphatidylinositol; PIP_3 , PI-3,4,5-(PO_4)₃; PKB, protein kinase B.

TABLE 1
Monkey characteristics

Parameter	Nondiabetic	Calorie restricted	Prediabetic	Diabetic
<i>n</i>	6	6	9	6
Age (years)	6.3 ± 0.7	20.3 ± 0.9*	14.2 ± 2.1†	20.1 ± 1.8*
Body weight (kg)	9.8 ± 1	11.3 ± 0.2	19.0 ± 1.3*	14.3 ± 0.9‡
Body fat (%)	16.1 ± 3.2	21.2 ± 1.6	28.8 ± 2.6*	29.6 ± 1.3§
Fasting plasma glucose (mg/dl)	63 ± 3	69 ± 3	71 ± 4	167 ± 21*
Fasting plasma insulin (μU/ml)	47 ± 5	51 ± 12	144 ± 18	110 ± 60
Acute insulin release (μU · ml ⁻¹ · min ⁻¹)	223 ± 67	146 ± 39	384 ± 120	38 ± 14‡
Glucose disposal (mg · kg FFM ⁻¹ · min ⁻¹)	10.00 ± 0.84	7.63 ± 2.25	5.60 ± 0.28†	2.61 ± 1.05*
<i>K</i> _{glu} (%/min)	3.79 ± 0.38	3.32 ± 0.13	2.88 ± 0.19‡	1.27 ± 0.08*

Values are means ± SE. **P* < 0.0001, †*P* < 0.005, ‡*P* < 0.01, §*P* < 0.001, ||*P* < 0.05 vs. nondiabetic group.

aPKCs was largely preserved in calorie-restricted monkeys.

RESEARCH DESIGN AND METHODS

Procedures in monkeys. Male rhesus monkeys (*Macacca mulatta*) were individually housed and maintained in accordance with National Academy of Sciences guidelines for care and use of laboratory animals. The monkeys were allowed unlimited access to Purina monkey chow, except those in the calorie-restricted group, in which chow was given in limited amounts to keep body weight at ~10–11 kg (22). Chronic calorie restriction of rhesus monkeys (i.e., prevention of obesity) has been shown to prevent the development of type 2 diabetes (23) and to improve insulin sensitivity as measured during a euglycemic clamp (24). Diabetic monkeys characteristically had fasting plasma glucose levels >110 mg/dl, decreased acute insulin release, and decreased glucose disappearance rates (*K*_{glu}) during an intravenous glucose tolerance test and decreased insulin-stimulated glucose disposal rates during euglycemic-hyperinsulinemic clamp studies (19,25). Prediabetic monkeys were obese and had increased acute insulin release rates, marked hyperinsulinemia, and decreased *K*_{glu} and insulin-stimulated glucose disposal rates (21).

Euglycemic-hyperinsulinemic clamp studies with skeletal muscle biopsies were conducted after an overnight fast as described (26). Biopsies of the vastus lateralis muscles were obtained just before and at 90–120 min of insulin administration. Muscle samples were rapidly frozen in liquid N₂, lyophilized, and stored at -196°C. Activities of PKC-ζ/λ/ι, PKB, and PI 3-kinase appeared to be stable during such storage.

Enzyme assays. Muscle samples were homogenized with a polytron in appropriate buffers as described (17,27). In all aPKC and PKB assays, two to four replicates of basal and insulin-stimulated samples from two to four diabetic, prediabetic, and/or calorie-restricted monkeys were directly compared with replicates from two to four nondiabetic monkeys.

aPKC activity was measured as described (17,27). In brief, aPKCs ζ, λ, and ι were immunoprecipitated from cell lysates with a rabbit polyclonal antiserum (Santa Cruz Biotechnologies) that recognizes the nearly identical COOH-termini of PKC-ζ, -λ, and -ι. (Note that it is currently not possible to separately measure activity of the individual aPKCs, but they apparently function interchangeably during insulin-stimulated glucose transport [11].) Precipitates were 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 Na₂VO₄, 100 μmol/l Na₄P₂O₇, 1 mmol/l NaF, 100 μmol/l PMSF (phenylmethylsulfonyl fluoride), 4 μg phosphatidylserine (Sigma), 50 μmol/l [γ-³²P]ATP (NEN Life Science Products), 5 mmol/l MgCl₂, 1 mmol/l EGTA, and, as substrate, 40 μmol/l serine analog of the PKC-ε pseudosubstrate (BioSource), a preferred substrate for aPKCs. In some assays, PIP₃ (Matreya) was added to activate aPKCs, as described (10). After incubation, ³²P-labeled substrate was trapped on P-81 filter paper and counted. Note that we found that the amounts of aPKCs recovered in these immunoprecipitates were equal, regardless of whether lysates were prepared from nondiabetic, calorie-restricted, prediabetic, or diabetic muscles, which were found (see below) to contain different concentrations of specific aPKC isoforms. This equal precipitation of aPKCs with anti-COOH-terminal antiserum may be due to the fact that, as originally reported (8), the immunoprecipitation is not quantitative and the recovery of aPKC is ~50–60% when precipitated according to the instructions provided by the supplier. (In fact, much higher amounts of antiserum are needed to more fully precipitate aPKCs.) Thus, anti-aPKC antibodies may have been limiting. In any event, because equal amounts of aPKC were precipitated, the presently reported levels of aPKC activity reflect the specific activity of the enzyme.

PKB enzyme activity was measured using a kit obtained from Upstate

Biotechnologies (Lake Placid, NY), as described previously (17,27). In brief, PKBα was immunoprecipitated with sheep polyclonal anti-PKBα antiserum (Upstate Biotechnologies), collected on Sepharose-AG beads, and assayed as per kit directions. PKB activation was also assessed by immunoblotting for phosphorylation of serine-473 and, in some instances, threonine-308, as described (17,27).

Immunoprecipitable IRS-1-dependent PI 3-kinase (rabbit polyclonal antiserum was purchased from Upstate Biotechnologies) was determined as previously described (17). In this case, samples from each monkey were assayed simultaneously, and results (as determined in a BioRad PhosphorImager/Molecular Analyst Program) were expressed relative to the nondiabetic control (i.e., unstimulated) samples developed on the same thin-layer chromatography plate.

Western analyses. Lysate proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted as described (17,27). Antibodies used for blotting included rabbit polyclonal anti-COOH-terminal PKC-ζ/λ/ι antiserum (Santa Cruz Biotechnologies) (PKCs ζ, λ, and ι have nearly identical COOH-termini that are recognized by this antiserum), mouse monoclonal isoform-specific anti-PKC-λ/ι antibodies that recognize both PKC-λ and -ι, which are 98% homologous (Transduction Labs), rabbit polyclonal isoform-specific anti-NH₂-terminal PKC-ζ antiserum (kindly supplied by Dr. Todd Sacktor), sheep polyclonal anti-PKBα antiserum (Upstate Biotechnologies), sheep polyclonal anti-3-phosphoinositide-dependent protein kinase-1 (PDK-1) antiserum (Upstate Biotechnologies), rabbit polyclonal anti-p85 subunit/PI 3-kinase antiserum (Upstate Biotechnologies), rabbit polyclonal anti-IRS-1 antiserum (Upstate Biotechnologies), rabbit polyclonal anti-phospho-ser-473-PKB antiserum (Cell Signaling, Beverly, MA), and rabbit polyclonal anti-phospho-thr-308-PKB antiserum (Biosource, Camarillo, CA). Immunoblots were quantified by measurement of chemiluminescence in a BioRad Molecular Analyst Chemiluminescence/Phosphorescence Imaging System.

Statistical methods. Data are expressed as means ± SE. Basal versus insulin-stimulated means were compared by Student's *t* test for paired samples. Group differences were compared by a one-way ANOVA and subsequently by the least-significant difference multiple comparison method. Pearson's correlation coefficient was used to test for significant linear relationships between variables.

RESULTS

Monkey characteristics. As seen in Table 1, in the absence of caloric restriction and relative to nondiabetic monkeys, there were marked increases in body weight and percent body fat in prediabetic monkeys and more modest increases in diabetic monkeys. Relative to nondiabetic monkeys, fasting plasma glucose levels were slightly but not significantly (*P* = 0.58) increased in obese prediabetic monkeys and more clearly increased in diabetic monkeys. As a manifestations of insulin resistance 1) serum insulin levels were significantly (*P* < 0.05) increased in obese prediabetic monkeys but only modestly and not significantly (*P* = 0.17) increased in diabetic monkeys, presumably reflecting a decrease in insulin secretion with progression to the diabetic state, and 2) glucose disappearance rates during intravenous glucose tolerance test-

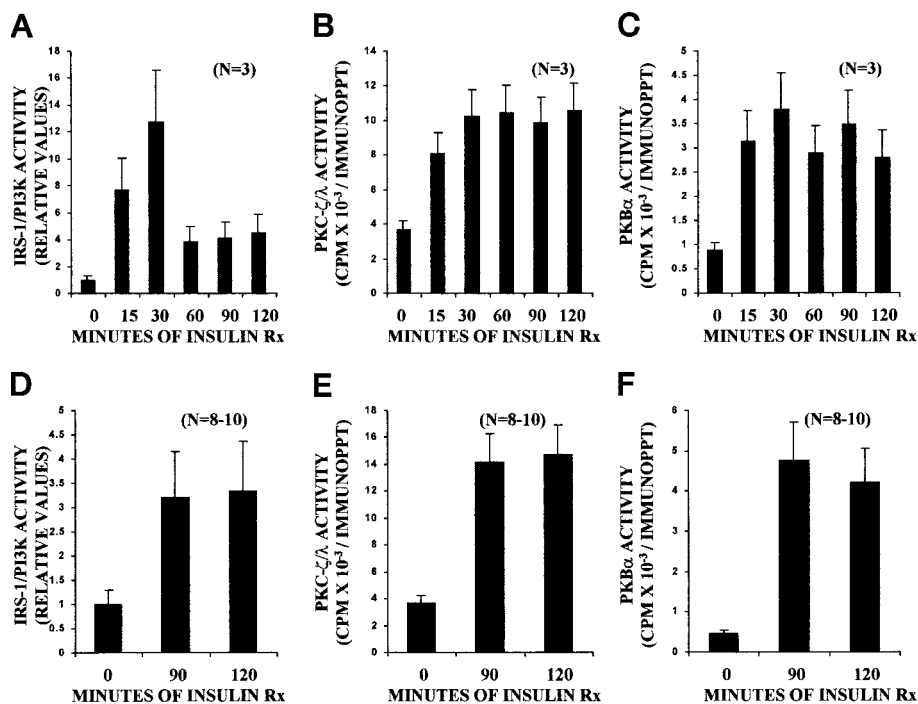


FIG. 1. Time-dependent changes in activities of IRS-1-dependent PI 3-kinase, aPKCs, and PKB in vastus lateralis muscles of nondiabetic monkeys during the hyperinsulinemic-euglycemic clamp. Biopsies were taken at different muscle sites at the indicated times. Values are means \pm SE of the number of monkeys (*N*) represented in each group.

ing (K_{glu}) and insulin-stimulated glucose disposal rates during euglycemic-hyperinsulinemic clamp studies were markedly diminished in diabetic monkeys and moderately decreased in prediabetic monkeys. Of particular interest, in calorie-restricted monkeys, despite an age comparable to that of diabetic monkeys, plasma glucose and insulin levels, body weight, and percent body fat were only slightly, but not significantly, increased as compared with younger nondiabetic monkeys.

Time-related activation of IRS-1-dependent PI 3-kinase, PKB, and aPKCs by insulin. As seen in assays of samples taken from muscles of three nondiabetic monkeys at various times of the 2-h clamp (Fig. 1A–C), insulin-induced increases in aPKC activity and PKB activity were maximal at 30 min and then were maintained with little or no significant change during the remainder of the clamp. IRS-1-dependent PI 3-kinase activity, on the other hand, showed an initial peak at 15–30 min of insulin treatment and then returned to a lower level that was then maintained with little or no significant change during the remainder of the clamp. As seen in Fig. 1D–F, there were no significant differences in activities of IRS-1-dependent PI 3-kinase, aPKCs, and PKB in samples obtained at 90 or 120 min of the clamp. Note that glucose disposal rates were calculated during the last 30–40 min of the clamp, when insulin's effect on all three signaling factors was readily apparent and steadily maintained, i.e., at 90–120 min of the clamp.

Alterations in aPKC activation and levels in vastus lateralis skeletal muscles. In muscles of nondiabetic monkeys, aPKC activity increased approximately twofold in response to insulin stimulation during the clamp (Fig. 2A). In obese prediabetic monkeys, there were moderate but significant ($P < 0.01$) decreases in insulin-stimulated aPKC activity and modest but insignificant ($P = 0.14$) decreases in basal aPKC activity (however, also note high basal insulin levels in these monkeys, suggesting resistance of aPKC activation to basal endogenous insulin)

(Fig. 2A). In diabetic monkeys, there were further significant decreases in insulin-stimulated ($P < 0.001$) and basal ($P < 0.05$) aPKC activity, such that the insulin-stimulated

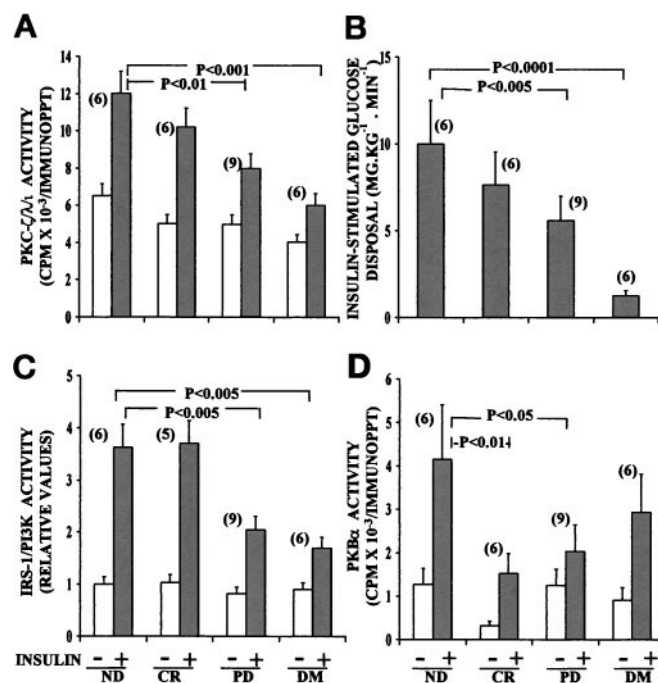


FIG. 2. Effects of insulin (INS) on activities of PKC- $\zeta/\lambda/\iota$ (A), PKB (D), and IRS-1-dependent PI 3-kinase (C) in vastus lateralis muscles and glucose disposal rates (B) of dietary unrestricted nondiabetic (ND), prediabetic (PD), and diabetic (DM) monkeys and calorie-restricted (CR) nondiabetic monkeys. Muscle biopsies were taken before (basal, clear bars) and 90–120 min after (insulin-stimulated, shaded bars) the start of insulin administration during the hyperinsulinemic-euglycemic clamp procedure. Glucose disposal rates are based on fat-free body weight. Insulin-stimulated IRS-1-dependent PI 3-kinase values are relative to simultaneously assayed nondiabetic basal (unstimulated) values. Values are mean \pm SE of the number (*N*) of monkeys shown in parentheses. *P* values (as per ANOVA) reflect differences between indicated groups. Also see text for other relevant *P* values.

increment was reduced by 60% and the absolute level of stimulated aPKC activity was reduced to the basal unstimulated level seen in nondiabetic monkeys (Fig. 2A). In calorie-restricted nondiabetic monkeys, basal and insulin-stimulated aPKC activities were diminished only slightly, but not significantly ($P < 0.24$ and $P < 0.18$, respectively).

In addition to cross-sectional studies, in some cases, it was possible to follow alterations in aPKC activation longitudinally. Thus, insulin-stimulated aPKC activity diminished from $12,268 \pm 930$ to $7,566 \pm 890$ cpm/immunoprecipitate, and basal aPKC activity diminished from $6,182 \pm 407$ to $5,063 \pm 846$ cpm/immunoprecipitate (means \pm SE), as four monkeys progressed from the lean nondiabetic to the obese prediabetic state over 4–7 years. Similarly, basal and insulin-stimulated aPKC activity diminished from 4,201 and 6,279 to 1,423 and 3,495 cpm/immunoprecipitate, as one monkey progressed from a prediabetic to a frankly diabetic state over 6 years.

Decreases in insulin-stimulated aPKC activity in diabetic muscles could not have resulted from decreases in levels of aPKCs and subsequently diminished availability of aPKCs to serve as activation targets for PI 3-kinase and PDK-1. As seen in Fig. 3 and Table 2, the content of the major aPKC in monkey muscle, viz., PKC- ζ , as measured by specific anti-NH₂-terminal antibodies, was increased significantly (by 60%, $P < 0.01$) in diabetic muscles but not significantly in prediabetic muscles. The content of PKC- λ/ι , the less abundant aPKC, as assessed by a specific antibody, seemed to be increased slightly but not significantly in diabetic and prediabetic muscles. Note that 1) PKC- ζ , as indicated by blotting with the isoform-specific anti-NH₂-terminal anti-PKC- ζ antiserum, migrated on SDS-PAGE largely at 80 kDa, corresponding to the more abundant higher Mr aPKC seen on blots developed with the anti-COOH-terminal antiserum, which does not distinguish between PKC- ζ , - λ , and - ι ; and 2) PKC- λ and - ι , which are 98% homologous and recognized similarly by the presently used λ/ι -specific monoclonal antibodies, migrated largely at a lower Mr of 75 kDa, corresponding to the less abundant lower band seen with anti-COOH-terminal antiserum. However, also note that, as measured with anti-COOH-terminal antiserum, the upper 80-kDa band was increased and the lower 75-kDa band was slightly decreased in the blots (Fig. 3), and the total aPKC level, as measured by this antiserum, was increased slightly but not significantly, viz., $22 \pm 13\%$ (Table 2).

Alterations in IRS-1-dependent PI 3-kinase activation in vastus lateralis muscles. Insulin-induced activation of IRS-1-dependent PI 3-kinase was diminished in both the prediabetic and diabetic groups ($P < 0.005$ for both) (Fig. 2C). In addition, there were marked decreases ($P < 0.025$) in IRS-1 levels in diabetic monkeys and lesser insignificant decreases in prediabetic monkeys (Fig. 3 and Table 2). In contrast, there were no consistent differences in levels of immunoreactive p85 subunit of PI 3-kinase in diabetic, obese prediabetic, and nondiabetic skeletal muscles (Fig. 3 and Table 2).

Alterations in PKB- α activation in vastus lateralis muscles. PKB- α activity increased nearly fourfold in response to insulin administration during the clamp in nondiabetic monkeys (Fig. 2D). (Note that we did not measure PKB- β activity, as it is very low in skeletal

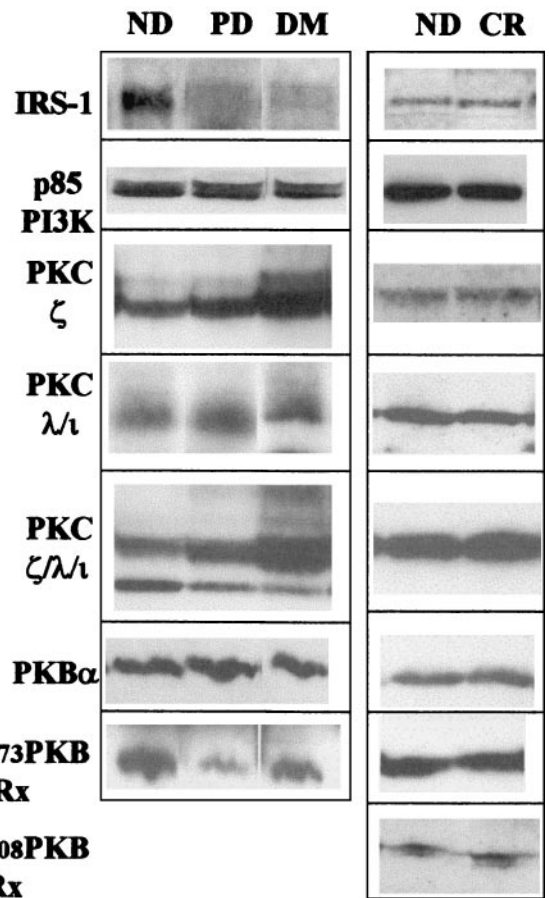


FIG. 3. Western analyses of IRS-1, p85 subunit of PI 3-kinase, PKB- α , phospho-serine-473-PKB α/β , phospho-threonine-308-PKB α/β , PKC- ζ (blotted with specific anti-NH₂-terminal antiserum), PKC- λ/ι (blotted with antibodies that specifically recognize common internal sequences of PKC- λ and - ι), and combined PKB- $\zeta/\lambda/\iota$ (blotted with anti-COOH-terminal antiserum) in vastus lateralis muscles of nondiabetic (ND), prediabetic (PD), diabetic (DM), and calorie-restricted (CR) monkeys. Note that the portrayed phospho-PKB samples had little or no pPKB signal and, for the sake of simplicity, are not shown. Shown here are representative blots. Note that nondiabetic samples were compared with both prediabetic and diabetic samples (left panels) and calorie-restricted samples (right panels). Because these comparisons were entirely independent, immunoreactivity can only be compared between samples within the same comparison group. See Table 2 for quantitative results from multiple analyses.

muscle, and, moreover, knockout of the PKB- β gene in mice does not impair insulin-stimulated glucose transport at high maximally effective insulin concentrations [28] that would be comparable to the high maximally effective levels of plasma insulin achieved during the present clamps.) Interestingly, the activation of PKB- α by insulin was significantly diminished in obese prediabetic ($P < 0.05$) but not diabetic ($P = 0.21$) monkeys. Similarly, insulin-stimulated PKB- α/β phosphorylation, as judged by immunoblotting for phospho-serine-473 content, was diminished by 67% in muscles of prediabetic monkeys and 36% in diabetic monkeys (Fig. 3 and Table 2); however, because of wide variations within groups, changes in PKB phosphorylation were not statistically significant.

In calorie-restricted monkeys, insulin-stimulated phosphorylation of serine-473 of PKB- α/β (Fig. 3 and Table 2) was well maintained relative to nondiabetic monkeys, but, surprisingly, insulin-stimulated PKB enzyme activity was

TABLE 2
Levels of immunoreactive signaling factors in vastus lateralis muscles of monkeys

Factor	Calorie			
	Nondiabetic	restricted	Prediabetic	Diabetic
<i>n</i>	6	6	9	6
IRS-1	100 ± 13	85 ± 4	79 ± 14	53 ± 8*
p85 subunit of PI 3-kinase	100 ± 28	87 ± 6	102 ± 28	124 ± 21
PKB- α	100 ± 15	92 ± 5	99 ± 10	96 ± 10
p-Ser-473-PKB (insulin stimulated)	100 ± 35	134 ± 51	33 ± 8	64 ± 19
PKC- ζ	100 ± 10	111 ± 9	124 ± 14	162 ± 15†
PKC- λ/ι	100 ± 6	77 ± 4‡	116 ± 42	115 ± 27
PKC- $\zeta/\lambda/\iota$	100 ± 12	95 ± 18	100 ± 28	122 ± 13

Values are means ± SE. Each blot contained samples from each group. Values reflect immunoreactivity relative to the nondiabetic mean value on each blot, set at 100. * $P < 0.025$, † $P < 0.01$, ‡ $P < 0.005$ vs. nondiabetic group.

significantly ($P < 0.01$) diminished (fold-wise, however, it was still very responsive to insulin) (Fig. 2D). This decrease in PKB enzyme activity in calorie-restricted monkeys could not be explained by an altered effect of insulin on phosphorylation of the threonine-308 activation loop site of PKB, as this PDK-1-dependent phosphorylation, like that of serine-473, was comparable in muscles of nondiabetic and calorie-restricted monkeys (Fig. 3).

There were no significant differences between immunoreactive PKB- α levels in muscles of any of the groups (Fig. 3 and Table 2).

Alterations in insulin-stimulated glucose disposal rates. Insulin-stimulated glucose disposal rates were diminished slightly but not significantly ($P = 0.64$) in calorie-restricted monkeys, moderately ($P < 0.005$) in obese prediabetic monkeys, and more severely ($P < 0.0001$) in diabetic monkeys (Fig. 2B). These changes in glucose disposal rates were similar to those of aPKC activation. Both glucose disposal rates in the hyperinsulinemic clamp procedures and glucose disappearance rates in the intravenous glucose tolerance test correlated well with insulin-induced increments in aPKC activity (Fig. 4).

Activation of aPKCs by PIP₃ in vitro. Our findings indicated that decreases in aPKC activity were apparent, despite increases in aPKC levels in skeletal muscles of prediabetic and diabetic monkeys. This divergence raised the possibility that in addition to decreases in IRS-1 levels and IRS-1-dependent PI 3-kinase activity in prediabetic and diabetic muscles, there may be a diabetes-associated defect in the activity of aPKCs or in their ability to respond to the major product of PI 3-kinase, PIP₃. Indeed, the activation of immunoprecipitated aPKCs by PIP₃ was diminished in basal (unstimulated) immunoprecipitates taken from prediabetic and diabetic monkeys relative to immunoprecipitates from nondiabetic monkeys (Fig. 5A). Note that the immunoprecipitates prepared from these groups contained equal amounts of aPKCs and coprecipitated PDK-1 (inset, Fig. 5A); however, because of inordinately high immunoreactive nonspecific background levels in aPKC immunoprecipitates developed from muscle samples, we were unable to detect immunoreactive phosphothreonine-410 to determine whether altered levels of PDK-

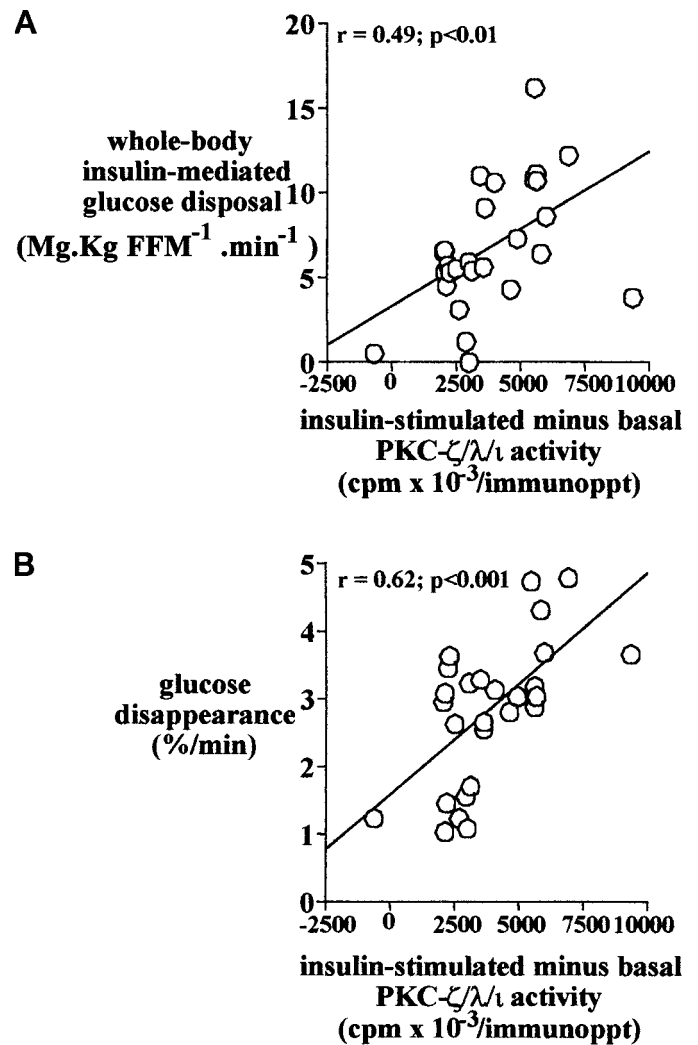


FIG. 4. Correlation of insulin-stimulated glucose disposal (A) and disappearance (B) rates to increments of aPKC activity. Glucose disposal and disappearance rates were determined during the hyperinsulinemic-euglycemic clamp procedures and intravenous glucose tolerance tests, respectively. Increments in aPKC activity were determined during the clamp procedures. immunoppt, immunoprecipitate.

1-dependent phosphorylation of this activation loop site may have contributed significantly to changes in PIP₃-induced increases in aPKC activity. In contrast to prediabetic and diabetic monkeys, in aPKC immunoprecipitates prepared from calorie-restricted monkeys, PIP₃ provoked increases in aPKC activity that were comparable to those seen in nondiabetic monkeys (Fig. 5A and B). Also note that we recovered comparable amounts of aPKCs and PDK-1 in immunoprecipitates prepared from calorie-restricted and nondiabetic monkeys (inset, Fig. 5B).

DISCUSSION

As presently observed in both cross-sectional and longitudinal studies, when placed on an unrestricted standard monkey chow diet, monkeys initially developed obesity and severe insulin resistance, as evidenced by marked hyperinsulinemia and diminished insulin-stimulated glucose disposal rates. In this prediabetic state, insulin resistance appeared to be reasonably well compensated for by increases in plasma insulin levels, as there were only slight, if any, increases in fasting plasma glucose levels. On

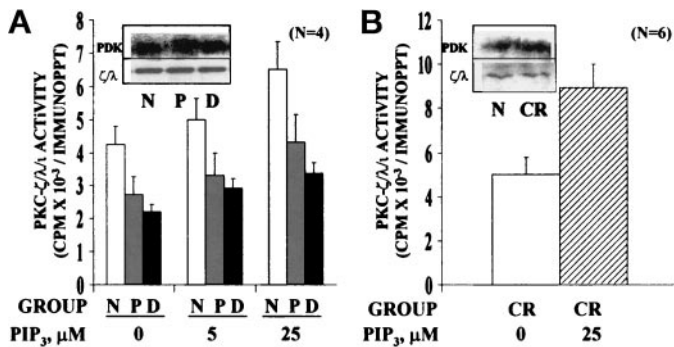


FIG. 5. Activation of aPKCs by PIP₃ in immunoprecipitates prepared from vastus lateralis muscles of nondiabetic (N, clear bars), prediabetic (P, shaded bars), and diabetic (D, black bars) monkeys (A) and calorie-restricted (CR, diagonally striped bars) monkeys (B). aPKCs were immunoprecipitated with anti-COOH-terminal antiserum from equal amounts of lysates prepared from indicated muscles taken in the basal unstimulated state. Precipitates containing equal amounts of immunoreactive aPKCs (ζ/λ) (see insets and RESEARCH DESIGN AND METHODS) were assayed in the presence of indicated concentrations of PIP₃. Note that the assay and immunoblots of calorie-restricted muscle shown in B were conducted independently of those shown in A; thus, immunoblots shown in A cannot be compared with those shown in B. Nevertheless, as seen in the insets shown in B, the recovery of immunoreactive aPKCs was comparable in calorie-restricted and nondiabetic monkeys (B) and diabetic, prediabetic, and nondiabetic monkeys (A). Also note that PDK-1, which is known to immunoprecipitate with aPKCs, was recovered in equal amounts in these aPKC immunoprecipitates. Values are means \pm SE of duplicate assays of muscles from four or six monkeys in each group, as indicated.

the other hand, there were significant decreases in insulin-induced activation of aPKCs and PKB in skeletal muscles of prediabetic monkeys, most likely at least partially due to diminished levels and activation of IRS-1-dependent PI 3-kinase. Interestingly, the development of this insulin-resistant prediabetic, as well as the diabetic, state could be largely prevented by caloric restriction and avoidance of obesity (19,24).

With further progression of the prediabetic to an overtly diabetic state, there was a drop in serum insulin levels, apparently reflecting diminished insulin secretion. However, there was an added worsening of insulin resistance, as evidenced by further decreases in insulin-stimulated glucose disposal and muscle aPKC activation. As PKB activation did not diminish further and, in fact, may have improved during progression from the prediabetic to the diabetic state, the observed decreases in skeletal muscle aPKC activation, rather than alterations in PKB activation, seemed more likely to have contributed to the deterioration in insulin-stimulated glucose disposal in the diabetic state. However, it is possible that a specific pool(s) of PKB, or its coupling to metabolic processes such as glucose transport and glycogen synthesis, may have been altered in the diabetic state.

Taken together, our findings suggested that defective activation of aPKCs in skeletal muscle by insulin played an important role in the pathogenesis of peripheral insulin resistance in both obese prediabetic and diabetic monkeys. Most likely, the importance of aPKCs in this pathogenic process reflected the apparent requirement for aPKCs during insulin-stimulated glucose transport, as deduced from studies of expression of kinase-inactive PKC- ζ or λ in both rodent (8–12,27) and human cell types (29). This does not necessarily imply that alterations in aPKCs did not influence parameters of glucose metabolism other

than transport, and in this respect, although it is unlikely that glycogen synthesis per se is activated by aPKCs, it remains a possibility that glycolysis and/or glucose oxidation may be regulated by aPKCs.

We have previously shown that 1) activation of skeletal muscle glycogen synthase during the euglycemic-hyperinsulinemic clamp is diminished in insulin-resistant prediabetic and diabetic monkeys (26,30) and 2) chronic caloric restriction significantly increases basal skeletal muscle glycogen synthase fractional activity and apparent affinity of glycogen synthase for glucose-6-PO₄, relative to all (i.e., normal, prediabetic, and diabetic) ad libitum-fed monkeys (30,31). Thus, previously reported changes in glycogen synthase do not appear to correlate well with presently observed changes in PKB activation in monkey muscle. However, as discussed above, changes in overall PKB activation may not be reflective of PKB pools that are important for specific metabolic processes.

As a further indication of the inconclusive nature of the present PKB findings, the lower activity but normal phosphorylation of basal and insulin-stimulated PKB in calorie-restricted monkeys deserves further comment. First, the normal phosphorylation state of PKB in calorie-restricted monkeys is probably an accurate reflection of its activation by upstream activators, including IRS-1, PI 3-kinase, and PDK-1, whose activation appears to be essentially normal, as determined by measurement of IRS-1-dependent PI 3-kinase and phosphorylation of the threonine-308 activation loop site in PKB. Second, the decrease in PKB enzyme activity in calorie-restricted monkeys is more difficult to assess because the in vitro assay does not necessarily reflect the ability of PKB to phosphorylate endogenous substrates. Third, insulin-stimulated increases in PKB activity were, fold-wise, comparable to those observed in nondiabetic monkeys. Finally, such a dichotomy between PKB enzyme activity and phosphorylation is not without precedent, e.g., during high-fat feeding in rats, PKB phosphorylation was maintained despite a 50% decrease in PKB enzyme activity (18). Obviously, further studies are needed to evaluate the cause and significance of alterations in PKB enzyme activity in calorie-restricted monkeys.

A particularly notable aspect of the present studies was our ability to examine insulin signaling mechanisms in skeletal muscles of primates who have an extraordinarily high prevalence rate for development of obesity-dependent insulin resistance and overt type 2 diabetes. With this predictable outcome in monkeys fed an unrestricted chow diet, we were able to study relevant insulin signaling mechanisms before, during, and after the development of the obesity/type 2 diabetes syndrome. Accordingly, the defect in insulin signaling via aPKCs to the glucose transport system in this obesity/diabetes syndrome in monkeys appeared to be acquired, at least in the sense that this defect became apparent only with development of obesity in prediabetic monkeys. Moreover, since this defect in signaling via aPKCs was largely prevented by caloric restriction and avoidance of obesity, it is plausible to suggest that it may have been secondary to obesity per se. However, it remains possible that the signaling defect contributed to the development of obesity, either as a

primary initiating factor or a secondary compounding factor.

As alluded to, the defects in activation of aPKCs by insulin in muscles of prediabetic and diabetic monkeys appeared to be at least partly due to defective activation of IRS-1-dependent PI 3-kinase. However, there also appeared to be a defect in the direct activation of aPKCs by PIP₃, presumably the major relevant lipid product of PI 3-kinase. As previously reported (32), PIP₃ activates aPKCs through at least three mechanisms that become operative upon binding of PIP₃ to the regulatory domain of aPKCs and presumed molecular unfolding, viz., increased phosphorylation of the activation loop site by PDK-1, increased auto(trans)phosphorylation, and release of the catalytic domain from autoinhibition by the pseudosubstrate sequence present in the regulatory domain. Further studies are needed to determine the reason for the presently observed defect in responsiveness of aPKCs to PIP₃ in muscles of diabetic monkeys.

It should be noted that some of the present findings may not be relevant to certain groups of humans in whom type 2 diabetes prevalence rates are much lower and that the development of obesity does not necessarily lead to significant insulin resistance and type 2 diabetes. Indeed, in a comparable hyperinsulinemic-euglycemic clamp study of humans who were nondiabetic or had impaired glucose tolerance or frank diabetes, we observed that, unlike monkeys, there were diminished rather than increased levels of PKC- ζ in muscles of glucose intolerant and diabetic humans (M. Beeson, M.P.S., Y.K., Jennifer Powe, G.B., M.L.S., R.V.F., unpublished observations). Nevertheless, similar to the situation in monkeys, aPKC activation is severely compromised in vastus lateralis muscles of both obese/glucose intolerant and type 2 diabetic human subjects.

In summary, the activation of aPKCs in skeletal muscle during euglycemic-hyperinsulinemic clamp procedures was progressively impaired as monkeys developed obesity-dependent insulin resistance and overt type 2 diabetes. This defect in aPKC activation (1) appeared to be partly a result of defects in IRS-1-dependent PI-3-kinase activation and diminished responsiveness of aPKCs to the major lipid product of PI 3-kinase, PIP₃, and (2) correlated well with defects in glucose disposal rate and K_{glu} in diabetic monkeys. Of particular interest, defects in muscle aPKC activation and the development of insulin resistance and diabetes were largely prevented by calorie restriction and avoidance of obesity.

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