Increased Protein Kinase C Theta in Skeletal Muscle of Diabetic Patients

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In this study we have investigated whether protein kinase C (PKC) protein and activity are increased in skeletal muscle of human diabetic patients. The protein content of different PKC isoforms (β, θ, ϵ, δ, μ, and ζ) in the particulate fraction was measured, using Western analysis, in human rectus abdominus skeletal muscle from obese (hyperinsulinemic, normoglycemic) and obese diabetic (hyperinsulinemic, hyperglycemic) subjects. PKC θ protein content was significantly higher in the particulate fraction of muscle from diabetic patients compared with the nondiabetic controls. PKC θ was immunoprecipitated and its activity was measured in muscle from diabetic and nondiabetic controls. There was a significant increase in PKC θ activity in muscle from diabetic patients compared with muscle from nondiabetic controls. Therefore, both PKC θ protein content and activity were significantly increased in the particulate fraction in muscle from diabetic patients, suggesting the involvement of this isoform in diabetes. Most of the PKC θ protein was found in the cytosol. There was no change in cytosolic PKC θ protein content in muscle from diabetic patients compared with muscle from nondiabetic controls. Thus, the increase in particulate-associated PKC θ was likely due to translocation and activation rather than an increase in protein mass.

NON–INSULIN-DEPENDENT diabetes mellitus (NIDDM) is accompanied by insulin resistance, hyperinsulinemia, and hyperglycemia. Chronic hyperglycemia has harmful effects on insulin secretion and insulin action, a concept known as glucose toxicity. The concept of glucose toxicity refers to hyperglycemia being a self-perpetuating factor responsible for the diabetic state rather than simply being a manifestation of diabetes.[1] Various studies showed that increased flux of glucose through the hexosamine pathway may impair insulin-dependent glucose transport in adipocytes,[2] rat muscles,[3] rats infused with glucose,[4] and rats infused with glucosamine.[5,6] The mechanism(s) responsible for the hyperglycemia-induced defect in insulin secretion is not well known but may be related to a defect in phosphoinositide metabolism. In this scheme, phospholipase C is activated after glucose interacts with a specific receptor on the cell surface. Activated phospholipase C catalyses the hydrolysis of membrane phosphoinositides generating diacylglycerol (DAG) and inositol phosphate. Inositol phosphate may increase intracellular calcium, which together with DAG may activate protein kinase C (PKC) in the membrane fraction.[7]

The PKC gene family is divided into 3 subfamilies: "conventional" or "classical" (PKC α, βI, βII, and γ), "novel" (PKC δ, ϵ, η, θ, and μ), and the "atypical" (PKC ζ, λ, and ι). These 3 subfamilies differ in their N-terminal regulatory domain. The conventional PKCs have a calcium binding site and a DAG/phorbol ester binding site. The novel PKCs lack the calcium binding site and therefore are not activated by calcium. The atypical PKCs lack the calcium binding site and the phorbol ester binding site, but are activated by phospholipids such as phosphatidylserine.

PKC is released in the cytosol as an inactive protein. Phosphorylation of this protein on serine/threonine residues and the availability of second messengers such as DAG increases its affinity towards the membrane fraction, which leads to its translocation. Binding of ligands such as lipids in the membrane fraction leads to its activation. Activated PKC has the ability to phosphorylate target proteins on serine/threonine residues.[8] Susceptibility to proteolytic activation, substrate specificity, and cellular and subcellular distribution differ from one isoform to another.[9] Therefore, subcellular distribution of both the enzyme and substrate may be key regulators of PKC function in vivo.

In this study, we investigated the involvement of PKC θ, ϵ, μ, ζ, δ, and β in type 2 diabetes in human skeletal muscle. PKC θ protein and activity were increased in the particulate fraction of muscle from diabetic patients, suggesting the involvement of this isoform in diabetes.

MATERIALS AND METHODS

Materials

The PKC assay kit was purchased from Amersham Life Science (Arlington Heights, IL). PKC antibodies (polyclonal) were purchased from Santa Cruz (Santa Cruz, CA). Unless otherwise specified, all other reagents were purchased from either Sigma Chemical (St Louis, MO) or Fisher Scientific (Springfield, NJ).

Human Subjects

Obese normoglycemic patients and obese diabetic patients undergoing elective surgeries (primarily hysterectomies and gastric bypass) served as subjects. The experimental protocol for obtaining the muscle biopsies was explained to all patients, and informed consent was obtained. This procedure was approved by the University and Medical Center Institutional Review Board. When surgery was performed on these patients, a muscle biopsy held by a clamp was obtained from the rectus abdominus muscle, and placed in a container with oxygenated Krebs-Henseleit buffer (KHB) for transport to the laboratory. Muscle fiber strips were "teased" from the muscle sample, clamped in plastic clips, and placed into separate wells containing KHB buffer (plus 1% bovine serum albumin, 1.0 mmol/L pyruvate) at 29°C under normoxic (95% O_2, 5% CO_2) conditions.

Measurement of glucose transport into human muscle in our labo-

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Table 1. Patients Characteristics

Characteristic	Obese Normoglycemic (n = 12)	Obese Diabetic (n = 11)
Age	42 ± 2	47 ± 2
BMI	51 ± 6	47 ± 3
Glucose (mg/dL)	91 ± 9	168 ± 30*
Insulin (μU/mL)	9 ± 3	18 ± 7

NOTE. Values are expressed as means ± SE, with number of observations in parentheses. There were 12 females in the normoglycemic group and 10 females and 1 male in the diabetic group. Muscle from the male diabetic patient was used for the measurement of PKC θ activity (see legend to Fig 4).

*Significantly different from corresponding obese normoglycemic values ($P < .05$).

ratory has been described elsewhere.[10] In this study, unincubated muscle fiber strips were frozen in liquid nitrogen followed by storage at -80°C until further analysis (≈3 to 12 months).

Western Analysis

Western analysis was performed as described elsewhere.[11] Frozen rectus abdominus skeletal muscle was homogenized and the protein content was determined using a Bio-rad protein assay (Bio-rad Laboratories, Hercules, CA). Samples were separated using 8% sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) and electrotransfered to a polyvinylidene fluoride (PVDF) microporous membrane. Isoform-specific PKC antibodies at a dilution factor of 1:1,000 (unless otherwise stated) in 5% nonfat milk were added to the membranes. Secondary antibody conjugated to horseradish peroxidase was added at a dilution of 1:5,000 and membranes were subjected to ECL (Western blot chemiluminescence reagent; Dupont, Wilmington, DE). Autoradiographs were quantitated on a Hewlett Packard (Palo Alto, CA) Scan Jet IIcx/T using Imagequant software (Molecular Dynamics, Sunnyvale, CA).

PKC Enzyme Assay

PKC activity was determined using the PKC enzyme assay kit (Amersham Life Science). Rectus abdominus skeletal muscle was homogenized and the protein content was determined using Bio-rad protein assay. For PKC θ activity, the membrane fraction from 25 mg muscle homogenate plus 5 μg of PKC θ antibodies were used to immunoprecipitate PKC θ. Measurement of PKC θ activity was conducted as described in the Amersham protocol kit. Myelin basic protein substrate was phosphorylated by $^{32}P[\gamma]$-adenosine triphosphate (ATP) and phosphorylated peptides were separated using binding paper discs and the disks were counted in 5 mL of scintillant using Beckman LS 6500 Multi-purpose Scintillation Counter (Beckman Instruments, Fullerton, CA).

Statistics

The protein content of various PKC isoforms, insulin-stimulated glucose transport, insulin levels, glucose levels, age, body mass index (BMI), and PKC θ activity data were analyzed with Mann-Whitney tests.

RESULTS

Rectus abdominus skeletal muscle from obese (normoglycemic) and obese diabetic (hyperglycemic) patients was used in various experiments. Patient characteristics are summarized in Table 1. There was no significant difference in age, BMI, and insulin levels in obese diabetic subjects compared with obese normoglycemic controls. Blood glucose levels were significantly higher in obese diabetic patients compared with obese normoglycemic controls. Patients were classified as diabetic by the criteria of the American Diabetes Association.[12] Glycosylated hemoglobin was 9.7% ± 1.7% (n = 9) in the diabetic group, compared with 6.1% ± 0.3% (n = 7) in the controls. Two of the diabetic patients were being treated with insulin, 1 was on glucotrol, and 1 was on a combination therapy of both insulin and glucotrol. Seven of the patients were not taking medication for treatment of their diabetes. Since most of the patients were not being treated with medication, we do not believe that medical treatment of the diabetic group was a factor in interpretation of the results.

There was no significant difference in insulin-stimulated glucose transport rates in muscle from diabetic patients compared with nondiabetic controls (Fig 1). Both groups were insulin-resistant as shown by the lack of stimulation of glucose transport by insulin.

Western blot analysis was used to measure the protein content of different PKC isoforms in muscle from obese diabetic and obese normoglycemic patients (6 patients per group). PVDF blots of cytosolic and particulate fractions were probed with antibodies for the different PKC isoforms. Only PKC θ protein content was significantly higher ($P < .05$) in the particulate fraction of skeletal muscle from obese diabetic patients compared with muscle from obese normoglycemic controls (Fig 2 and Table 2). There was no significant difference in

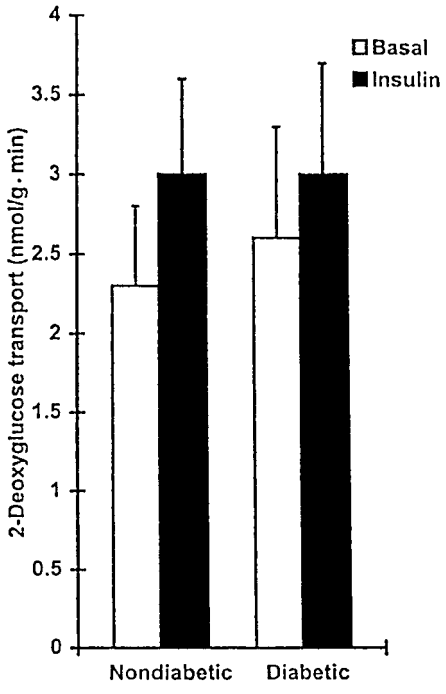

Fig 1. Effect of insulin on glucose transport. Rectus abdominus skeletal muscle samples were obtained from obese normoglycemic (n = 12) and obese diabetic subjects (n = 11). Muscle fiber strips were treated with 10^{-7} mol/L insulin during the last 10 minutes or without (basal) insulin. 2-Deoxyglucose transport was measured as described in the Methods.

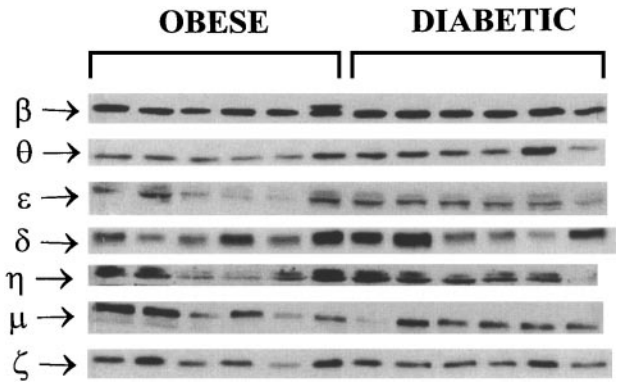

Fig 2. Western blot of the various PKC isoforms investigated in the particulate fraction. Rectus abdominus skeletal muscle samples were obtained from obese normoglycemic and obese diabetic subjects. The particulate proteins (100 μg) were separated by SDS-PAGE electrophoresis, transferred to an Immobilon-P transfer membrane, and probed with various PKC isoform antibodies. N = 6 for all observations.

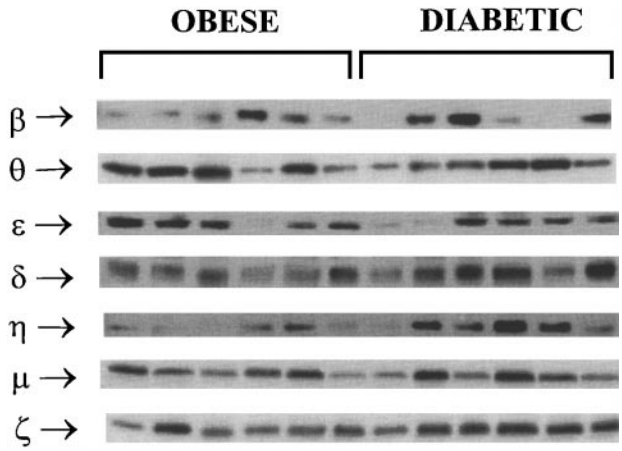

Fig 3. Western blot of the various PKC isoforms investigated in the cytosolic fraction. Rectus abdominus skeletal muscle samples were obtained from obese normoglycemic and obese diabetic subjects. The cytosolic proteins (100 μg) were separated by SDS-PAGE electrophoresis, transferred to an Immobilon-P transfer membrane, and probed with various PKC isoform antibodies. N = 6 for all observations.

cytosolic PKC content of various PKC isoforms measured (Fig 3 and Table 3).

To confirm that PKC θ was increased in muscle of diabetic patients, we measured the enzyme activity after immunoprecipitation with a PKC θ-specific antibody. Unfortunately, there was not enough muscle from the original samples used for Western blot analyses for measurement of activity and another set of muscles was used. PKC θ enzyme activity was significantly higher ($P < .05$) in the particulate fraction of skeletal muscle from obese diabetic patients compared with muscle from obese normoglycemic controls (Fig 4).

Discussion

We report the novel finding of this study that PKC θ protein content and activity were significantly increased in human rectus abdominus skeletal muscle from obese diabetic patients as compared with muscle from obese normoglycemic controls. There were no significant alterations in PKC ϵ, μ, ζ, δ, and β in muscle from obese diabetic patients as compared with muscle from obese normoglycemic controls. The diabetic patients and the normoglycemic controls were obese. Although PKC θ protein content/activity was significantly increased in muscle from obese diabetic patients compared with muscle from obese normoglycemic controls, there was no difference in 2-deoxyglucose transport. This suggests that hyperglycemia may be activating PKC θ in muscle from obese diabetic subjects.

Feeding rats a fructose-enriched diet induced insulin resistance in rat skeletal muscle, which was associated with increased DAG and PKC θ mass in the membrane fraction of white muscle.[13] Rats chronically infused with glucose developed hyperinsulinemia, an increase in the level of malonyl-coenzyme A (CoA), an increase in long-chain acyl-CoA (LCAC) concentration, activation of PKC θ in the red gastrocnemius, and skeletal muscle insulin resistance.[14] This leads to the hypothesis that glucose infusion may increase the level of malonyl-CoA, which may inhibit the transfer of LCAC into the mitochondria leading to its accumulation in the cytosol and activation of PKC.

Studies showed that membrane PKC α, β, ϵ, and ζ were increased in the Zucker diabetic fatty rat as compared with lean and obese controls.[15] In the type II diabetic Goto-Kakizaki (GK) rat soleus muscle, PKC α, β, ϵ, θ, and ζ translocated from the cytosol to the membrane fraction. PKC α, β, ϵ, and δ enzyme activity and level were increased in the membrane

Table 2. Particulate PKC Content in Skeletal Muscle From Obese Normoglycemic Compared With Obese Diabetic Subjects

PKC Isoform	Obese	Diabetic
θ	705 ± 100	1,287 ± 168*
ϵ	2,463 ± 657	2,931 ± 312
μ	4,647 ± 1,088	3,563 ± 662
ζ	2,691 ± 641	3,504 ± 304
δ	4,571 ± 909	5,716 ± 1,165
β	3,467 ± 726	3,340 ± 581

NOTE. Values are expressed as means (arbitrary units) ± SE. N = 6 for all observations.

*Significantly different from corresponding obese normoglycemic value ($P < .05$).

Table 3. Cytosolic PKC Content in Skeletal Muscle From Obese Normoglycemic Compared With Obese Diabetic Subjects

PKC Isoform	Obese	Diabetic
θ	5,021 ± 857	4,017 ± 449
ϵ	3,198 ± 644	2,538 ± 561
μ	3,179 ± 434	4,044 ± 590
ζ	4,085 ± 424	4,808 ± 268
δ	4,648 ± 186	4,598 ± 674
β	3,708 ± 745	4,102 ± 1,387

NOTE. Values are expressed as means (arbitrary units) ± SE. N = 6 for all observations.

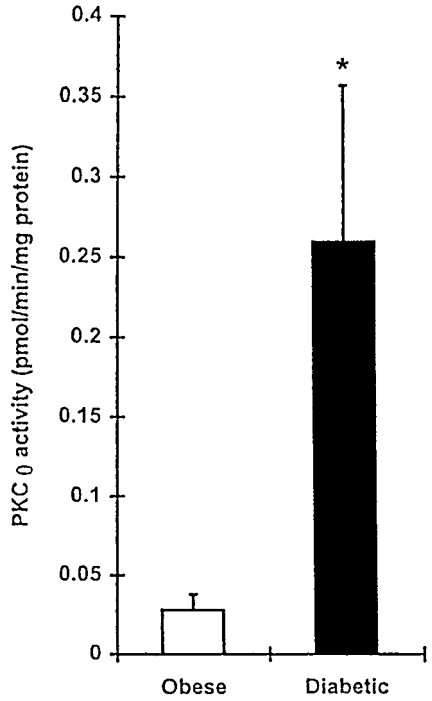

Fig 4. Effect of diabetes on PKC θ activity in the particulate fraction. Isoform specific PKC θ activity was measured in 25 mg of rectus abdominus skeletal muscle samples from obese normoglycemic and obese diabetic subjects. Activity was normalized to the protein content of each sample. Data are expressed as means (pmol/min/mg protein) with N=6 for all observations. There were 6 females in the normoglycemic group and 5 females and 1 male in the diabetic group. The PKC θ activity for the male subject was 0.13 pmol/min/mg protein. *Significantly different than the corresponding obese normoglycemic value ($P < .05$).

fraction and decreased in the cytosolic fraction as compared with nondiabetic control. In addition, PKC ζ levels were not changed and PKC θ levels were decreased in both the membrane and cytosolic fractions of GK rats.[16]

In the human liver, PKC α, ϵ, and ζ were increased in the membrane fraction from obese diabetic subjects.[15] In rectus abdominus skeletal muscle, we found no differences in the protein level of PKC ϵ and ζ. In addition, we found no significant differences in the particulate or cytosolic protein levels of PKC β, ϵ, and δ in rectus abdominus muscle of diabetic patients. This suggests that alterations in PKC in the liver of diabetic patients is different from those occurring in muscle. This is not surprising since PKC differs in its localization and subcellular distribution from one model to another. In agreement with the rat GK model, PKC ζ levels were not changed in either fraction, whereas particulate PKC θ level and activity were increased in muscle from obese diabetic patients compared with obese nondiabetic controls.

The increase in the activity of PKC θ was greater than the increase in protein mass. In addition, there was no significant difference in the protein level of PKC θ in the cytosolic fraction. Since a large part of the PKC protein is present in the cytosolic fraction ($\approx$9,000 μg total protein), it seems unlikely that altered expression of PKC θ caused the increase in the particulate fraction ($\approx$200 μg total protein). This suggests translocation and activation of PKC θ rather than increased PKC θ expression. This increase in PKC θ content/activity and its translocation to the particulate fraction and subsequent activation could be due to the hyperglycemic state of the diabetic patients. This suggestion was substantiated by the observation that incubation of rat-1 fibroblasts overexpressing the insulin receptor with high glucose concentration–induced translocation and activation of PKC α, δ, ϵ, and ζ to the membrane fraction.[17]

In conclusion, we report that PKC θ protein content and activity were significantly increased in human rectus abdominus muscle from obese diabetic patients compared with muscle from obese normoglycemic controls, suggesting the possible involvement of this PKC isoform in diabetes and/or diabetic complications.

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