Protein Kinase C Activity Is Acutely Regulated by Plasma Glucose Concentration in Human Monocytes In Vivo

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Activation of protein kinase C (PKC) by hyperglycemia is implicated in the pathogenesis of long-term diabetic complications. Monocyte activation and transformation into macrophages is a key step in the atherosclerotic process. Therefore, in this study, we sought to determine *1*) the effect of hyperglycemia on monocyte PKC activity and on the distribution of Ca^{2+}-dependent and diacylglycerol-sensitive PKC isoforms; and *2*) whether the effects on these parameters are determined by hyperglycemia per se, independent of the diabetic state. The studies were performed in 19 type 2 diabetic patients and 14 control subjects. Plasma glucose concentration was higher and insulin sensitivity lower (both $P < 0.01$) in diabetic patients than in control subjects. Monocytes from diabetic patients showed similar cytosol PKC activity to those from control subjects but higher membrane PKC activity (78 ± 6 vs. 50 ± 5 pmol · min^{-1} · mg^{-1} protein; $P < 0.01$). A direct correlation was observed between fasting plasma glucose and membrane PKC activity ($r^2 = 0.4008$, $P = 0.0001$). In contrast, a reciprocal correlation was observed between membrane PKC activity and insulin sensitivity index ($r^2 = 0.28$, $P < 0.05$). Using immunoblotting analysis, we found that membrane β_2, but not α, isoform of PKC was more abundant in monocytes from diabetic patients. In diabetic patients, when euglycemia was acutely induced, membrane PKC activity decreased by ~42% and β_2 isoform by ~15%. In two normal subjects in whom hyperglycemia was induced, membrane PKC increased from 63 and 57 to 92 and 128.6 pmol · min^{-1} · mg^{-1} protein, respectively. This increase was associated with an increase in the membrane isoform β_2; α isoform was unchanged. We conclude that *1*) monocytes express the glucose-sensitive β_2 isoform of PKC; *2*) the prevailing plasma glucose acutely regulates the activity of the membrane PKC and the content of membrane PKC β_2 isoform; and *3*) this effect appears to be a direct effect of glucose per se, since the phenomenon was observed in normal control subjects when hyperglycemia was induced. Monocyte PKC activation may account for the accelerated atherosclerosis of patients with type 2 diabetes. *Diabetes* 48:1316–1322, 1999

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BSA, bovine serum albumin; DAG, diacylglycerol; PBS, phosphate-buffered saline; PKC, protein kinase C; Pser, L-phosphatidyl-L-serine.

Vascular complications of diabetes occur in both micro- and macrovessels, with hyperglycemia being an important risk factor (1). Several studies have demonstrated that hyperglycemia could cause its adverse effects by activating the diacylglycerol (DAG)-sensitive protein kinase C (PKC) (2). PKC activity is increased in the retina (3), aorta and heart (4), and renal glomeruli of diabetic rats (5) as well as in cultured vascular cells or tissues exposed to elevated levels of glucose (4,6).

Little information is available on the effects of hyperglycemia and diabetes on PKC activity in circulating monocytes. The assessment of PKC in monocytes appears to be particularly important for two reasons. First, their PKC response may mirror that of vascular cells; second, an increased monocyte PKC activity enhances their initial adhesion to the vascular wall and fibrinogen binding (7) and their differentiation into macrophages (8). Recently, Morigi et al. (9) showed that PKC inhibitor staurosporine markedly decreases the number of leukocytes adhering to high glucose–treated cells. Therefore, elevated plasma glucose concentration would favor the earliest events of atherosclerotic lesion by stimulating the PKC activity in monocytes. Indeed, this array of events is accelerated in humans with insulin resistance and diabetes (10). In particular, the monocyte binding to human aortic endothelial cells is enhanced in diabetes (11). Besides the compelling evidence of a closed relationship between hyperglycemia, PKC activation, and monocytes–endothelium interaction, circulating monocytes might represent a potentially suitable cell model to detect modifications in kinase activities in response to changes of the metabolic environment.

PKC, a serine/threonine kinase, consists of a family of at least 12 isoforms divided into three subfamilies—conventional, novel, and atypical—according to their calcium and phospholipid dependence (12). When intracellular levels of the activators are elevated, PKC isozymes can translocate from cytosol to membrane and assume an active conformation.

Therefore, in this in vivo study, we sought to determine *1*) the effect of hyperglycemia on PKC activity and on the distribution of Ca^{2+}- and phospholipid-sensitive PKC isoforms in monocytes from type 2 diabetic patients and *2*) whether the effects on these parameters are determined by hyperglycemia per se, independent of diabetes.

RESEARCH DESIGN AND METHODS

We recruited for this study 19 patients with a known diagnosis of type 2 diabetes who were free from clinical and instrumental evidence of atherosclerotic cardiovascular disease (Table 1). Diabetic control was achieved with diet alone or

with diet plus sulfonylureas or biguanide preparations or both. Pharmacologic treatment for hyperglycemia was stopped at least 7 days before the study; antihypertensive drugs were continued. All participants were asked to fast for at least 12 h before the examination. Fourteen healthy control volunteers, comparable for age, BMI, and lifestyle, were recruited from the local community (Table 1). All participants underwent a full medical history and physical examination. All subjects followed an isocaloric diet, recorded by a dietitian, with three meals daily (50% carbohydrate, 35% fat, and 15% protein) for at least 30 days before the study.

The patients filled in a complete lifestyle questionnaire to gain information on medical histories, parental history of cardiovascular disease, smoking habits, and physical activity. Peripheral vascular disease was ruled out both by minimum criteria such as the absence of peripheral pulses of the lower extremity (dorsal pedal, posterior tibial, popliteal, and femoral arteries), which were examined through manual palpation, and by ankle-brachial pressure indices. Atherosclerotic involvement was also excluded by Doppler ultrasound. A resting 12-lead electrocardiogram was performed and angina was excluded in each patient according to the World Health Organization Rose questionnaire (13). Patients with proliferative retinopathy or significant renal impairment were also excluded. The local ethics committee approved the study protocol. Smoking and alcohol intakes were prohibited at least 24 h before the study.

Experimental procedures

Study 1. On the day of the study, at 7:00 A.M. after an overnight fast, 19 type 2 diabetic patients and 14 normal subjects were admitted to the Divisione di Malattie del Metabolismo of the University of Padova. A 20-gauge butterfly needle was inserted into a dorsal hand vein at 7:30 A.M. The hand was heated to 60°C to arterialize venous blood. The patency of the needle was maintained with a controlled saline infusion throughout the study. Subjects had a baseline blood sampling (50 ml) for the determination of circulating glucose, insulin, C-peptide, and lipids and of PKC activity and immunoblotting in circulating monocytes. Then they received an intravenous glucose tolerance test. Insulin sensitivity index, the S_I parameter derived from the minimal model analysis of intravenous tolerance test, was determined in 11 diabetic patients and 10 normal subjects, as previously described (14,15).

Study 2. Nine diabetic patients were studied while hyperglycemic (19.72 ± 2.45 mmol/l), after an overnight fast. They had a baseline sampling for the determination of PKC activity and immunoblotting in circulating monocytes. Once the baseline sampling was completed, a continuous intravenous infusion of short-acting insulin was begun at a rate of 1.5 $mU \cdot kg^{-1} \cdot min^{-1}$ to reestablish euglycemia. Plasma glucose was determined every 10 min. After 4 ± 0.3 h from the beginning of insulin infusion, once the patients achieved euglycemia, blood sampling for the determination of PKC activity and immunoblotting in circulating monocytes was repeated.

Study 3. Two normal subjects were studied after an overnight fast. At 7:30 A.M., after the baseline sampling for the determination of PKC activity and immunoblotting in circulating monocytes, a combined exogenous intravenous continuous infusion of glucose (10% wt/vol) and somatostatin (250 µg/h) was begun to raise the plasma glucose from a normal mean value of 4.7 mmol/l to a mean value of 13.05 mmol/l. Plasma glucose was determined every 10 min. After 4.2 h from the beginning of glucose and somatostatin infusions, blood sampling for the determination of PKC activity and immunoblotting in circulating monocytes was repeated.

Laboratory analysis. Plasma glucose was measured with a glucose oxidase method on a Beckman Glucose analyzer (Beckman Instruments, Palo Alto, CA). Plasma insulin and C-peptide were measured by polyclonal radioimmunoassay (RIA) (16). HbA_{1c} (reference range 4.5–6%) was measured by ion exchange high performance liquid chromatography (17). Cholesterol and triglycerides in the plasma were measured by enzymatic methods (18,19). HDL cholesterol was determined according to the method of Kostner (20).

Determination of PKC activity in circulating monocytes

Monocyte preparation. Mononuclear cells were prepared from heparinized blood by centrifugation (400*g* for 30 min at room temperature) over Histopaque-1077 (Sigma), as previously described (21). Mononuclear cells were washed once with phosphate-buffered saline (PBS) and twice with RPMI 1640, then resuspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mmol/l L-glutamine, 100 U/ml penicillin, and 10 µg/ml streptomycin (complete medium). Cells were plated at 1×10^7 cells/ml in 60-mm culture dishes and incubated for 30 min at 37°C in a humidified atmosphere with 5% CO_2. Nonadherent cells were removed by washing with PBS, and adherent cells were incubated in the same fresh RPMI 1640 for 3–4 h. More than 90% of adherent cells were monocytes by morphologic examination and by staining with Diff-Quik. More than 95% of monocytes were viable as determined by trypan blue exclusion.

Cell fractionation. Monocytes were rapidly lysed by the addition of ice-cold extraction buffer containing 12.5 mmol/l Tris-HCl (pH 7.4), 250 mmol/l sucrose, 2 mmol/l EDTA, 2 mmol/l EGTA, 25 mmol/l β-glycerophosphate, 2 mmol/l sodium vanadate, 10 µmol/l phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml leupeptin, and 5 µg/ml aprotinin. Cells were disrupted with a Dounce homogenizer (20 strokes) on ice for 1 min. The homogenate was centrifuged at 800*g* for 10 min at 4°C to remove cell debris and nuclei. The homogenate was then centrifuged at 100,000*g* for 1 h at 4°C in a Beckman centrifuge (TI 50). The resultant supernatant (cytosol fraction) was removed and stored at –80°C. The pellet (membrane fraction) was resuspended in extraction buffer with 1% Triton X-100 and incubated with shaking at 4°C for 1 h. The membrane fraction was centrifuged at 100,000*g* for 30 min at 4°C, and the supernatant (containing membrane PKC) was stored at –80°C. Protein concentration was determined with Biorad DC assay (Biorad, Hercules, CA) using bovine serum albumin (BSA) as standard.

PKC assay. Ca^{2+}- and phospholipid-dependent PKC activity was measured as previously described (22). Briefly, phosphorylation reactions were performed in the mixture, final volume 175 µl, containing 12 mmol/l calcium acetate, 30 mmol/l dithiothreitol, 50 mmol/l Tris-HCl, 900 µmol/l substrate peptide (R-K-R-T-L-R-R-L), a detergent-dispersed solution of 400 µmol/l L-phosphatidyl-L-serine (Pser) and DAG, and [γ-^{32}P]ATP. In the assays, PKC activity was also measured in the absence of Ca^{2+} (i.e., in the presence of EGTA) and phospholipids. The synthetic octapeptide is based on the sequence surrounding a PKC phosphorylation site within the epidermal growth factor receptor. It is specific for PKC, since it is marginally phosphorylated by cAMP-dependent protein kinase (8%), phosphorylase kinase (13%), and proteolytic PKC fragment (30%) and not by hexokinase, myosin light chain kinase, Ca^{2+}-calmodulin, serine kinase, or casein kinase I and II (22,23).

The reaction was initiated by adding 25 µl of the cytosol or membrane fractions (30 µg protein). After 15-min incubation at 25°C, the reaction was stopped with trichloroacetic acid. An aliquot of the reaction medium was spotted on a phosphocellulose paper. The paper was washed with 5% vol/vol acetic acid and assayed for ^{32}P by scintillation counting. PKC activity was expressed as picomoles of ^{32}P incorporated into peptide per milligram of protein per minute, calculated in the presence of Ca^{2+}/phospholipid minus the activity obtained in the absence of Ca^{2+}, Pser, and DAG.

To rule out a possible interfering effect of glucose in the RPMI medium (which usually contains 11.1 mmol/l glucose), monocytes, after the removal of nonadherent cells as previously described, were incubated in glucose-deficient RPMI 1640 with 5, 10, and 20 mmol/l glucose added, respectively, with or without

TABLE 1
Characteristics of the study population

	Control subjects	Diabetic patients	*P* value
Age (years)	49 ± 5	52 ± 3	NS
Plasma glucose (mmol/l)	4.94 ± 0.22	13.00 ± 1.05	<0.01
BMI (kg/m^2)	27.4 ± 0.7	29.9 ± 1.1	<0.05
Waist-to-hip ratio	0.89 ± 0.01	0.96 ± 0.02	<0.05
Total cholesterol (mmol/l)	4.45 ± 0.17	4.65 ± 0.22	NS
HDL cholesterol (mmol/l)	1.22 ± 0.07	0.95 ± 0.10	<0.05
Triglycerides (mmol/l)	1.52 ± 0.13	3.79 ± 0.16	<0.01
HbA_{1c} (%)	4.8 ± 0.1	8.7 ± 0.6	<0.01
Plasma insulin (pmol/l)	36 ± 6	78 ± 5	<0.05
Plasma C-peptide (nmol/l)	0.73 ± 0.14	0.65 ± 0.08	NS
S_I ($10^4 \cdot min^{-1} \cdot \mu U^{-1} \cdot ml$)	4.76 ± 0.55	0.72 ± 0.18	<0.01

Data are means ± SE. Statistical comparisons are for control subjects versus diabetic patients.

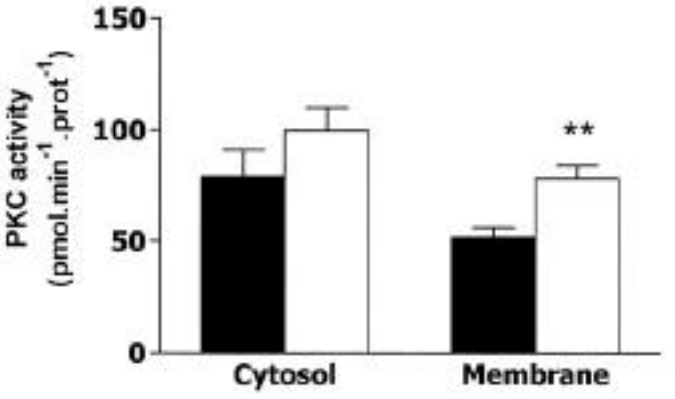

FIG. 1. PKC activity in human monocytes of normal (■) and type 2 diabetic (□) subjects. PKC activity was determined in the cytosol and membrane fractions as described in METHODS. Data are means ± SE from 14 normal and 19 diabetic patients. **P < 0.01.

600 pmol/l insulin, for 3 h. The membrane PKC activity was 25, 32, and 34 pmol · min^{-1} · mg $protein^{-1}$ in the presence of 5, 10, and 20 mmol/l glucose, respectively, without insulin. With insulin added to the medium, membrane PKC activity was 33, 20, and 24 pmol · min^{-1} · mg^{-1} protein.

Immunoblot analysis of PKC isoenzymes. Cytosol and membrane fractions from human monocytes were immunoblotted with rabbit polyclonal antibodies for PKC α and β_2 (Santa Cruz Biotechnology, Santa Cruz, CA). Cytosol and membrane protein extracts (30 µg) were solubilized in Laemmli buffer and then separated by electrophoresis through a 10% polyacrylamide gel. Proteins separated on the gels were electroblotted onto nitrocellulose membrane (Hybond ECL; Amersham Pharmacica Biotech, Buckinghamshire, England) in Tris-glycine transfer buffer with 20% methanol for 2 h at 100 V in the cold, using a BioRad Transblot cell. The membranes were blocked overnight at 4°C in PBS containing 0.05% (vol/vol) Tween (T-PBS) and 5% BSA. Membranes were exposed to primary antibody (1:2,000 dilution) for anti–PKC α and anti–PKC β2 overnight at 4°C. Membranes were washed (4× for 20 min) with the same buffer and then incubated with 1:4,000 goat anti-rabbit antibody conjugate to horseradish peroxidase. Detection was made using the enhanced chemiluminescence (ECL) system from Amersham. Blots were scanned and quantified with a BioRad Chemiluminescence Molecular Imaging System, and results were expressed relative to the control, on the same blot, set at 100%.

Statistics. The differences were analyzed by two-tailed paired and unpaired t test. Linear regression was assessed using a linear fit with GraphPad version 2.00 statistical software.

RESULTS

Metabolic parameters and insulin sensitivity index. As shown in Table 1, patients with diabetes had significantly higher BMI, waist-to-hip ratio, and HbA_{1c}. Plasma glucose concentration was significantly higher in diabetic patients than in normal control subjects (13.00 ± 1.05 vs. 4.94 ± 0.22 mmol/l; P < 0.0001). Likewise, insulin concentration was significantly higher in diabetic patients than control subjects (78 ± 5 vs. 36 ± 6 pmol/l; P < 0.05).

S_I was significantly lower in diabetic patients than in normal control subjects (0.72 ± 0.18 vs. 4.76 ± 0.55 10^4 · min^{-1} · $µU^{-1}$ · ml; P < 0.0001) (Table 1).

In study 2, diabetic patients showed a mean baseline plasma glucose level of 19.72 ± 2.45 mmol/l, which was reduced to 5.2 ± 0.3 by exogenous insulin infusion. In study 3, in two normal subjects, plasma glucose was raised from a mean value of 4.7 to 13.05 mmol/l.

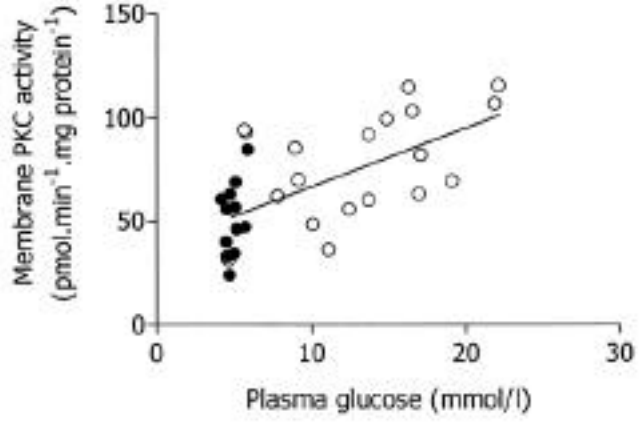

FIG. 2. Linear regression between membrane monocyte PKC activity and fasting plasma glucose. Data points represent the pooled experimental observations in 19 patients with diabetes (○) and 14 control subjects (●) (r^2 = 0.4008, P = 0.0001).

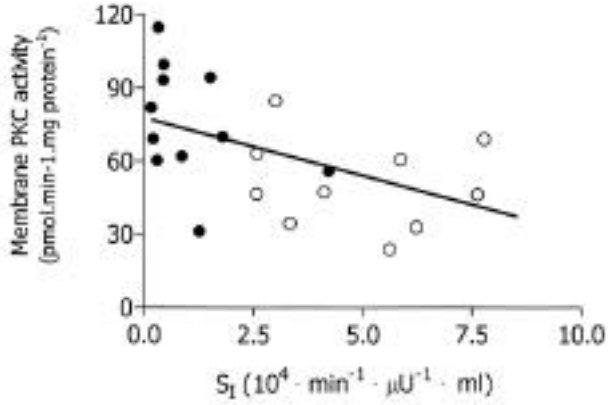

FIG. 3. Linear regression between membrane monocyte PKC activity and S_I, an index of insulin sensitivity. S_I is a parameter provided by the minimal model of glucose disappearance, which measures the ability of insulin to enhance plasma glucose disappearance and to inhibit hepatic glucose production. Data points represent the pooled experimental observations in 11 patients with diabetes (●) and 10 control subjects (○) (r^2 = 0.28, P < 0.05).

PKC assay and immunoblot analysis of PKC isoenzymes

Basal condition. As shown in Fig. 1, membrane-associated PKC activity in the diabetes group was significantly higher than in the control group (78.1 ± 5 vs. 51.7 ± 4 pmol · min^{-1} · mg^{-1} protein; P < 0.01), whereas cytosol PKC activity was not significantly different (99.7 ± 10 vs. 84.5 ± 11 pmol · min^{-1} · mg^{-1} protein). In addition, the membrane versus cytosol ratios of PKC activity in the diabetes group were higher than in the control group (0.93 ± 0.1 vs. 0.64 ± 0.07; P < 0.05). A highly significant positive correlation (Fig. 2) was observed between fasting plasma glucose and membrane PKC activity in the monocytes (r^2 = 0.4008, P = 0.0001). Again, as shown in Fig. 3, membrane-associated PKC activity in both groups was significantly correlated with S_I, an estimate of insulin sensitivity (r^2 = 0.28, P < 0.05).

Immunoblotting analysis was carried out on cytosol and membrane fractions from human monocytes to determine which Ca^{2+}/phospholipid-dependent PKC isoforms contribute to the activity measured. Previous study in human monocytes has identified the presence of Ca^{2+}/phospholipid-dependent PKC α and β isoforms, whereas PKC γ isoform was absent (21).

Therefore, we decided to investigate PKC α isoform and the glucose-sensitive PKC β_2 isoform (4). Figure 4 shows immunoblots for these PKC isoforms in cytosol and membrane fractions from human monocytes. The positive rat brain control is shown in each blot, and the molecular size of individual PKC

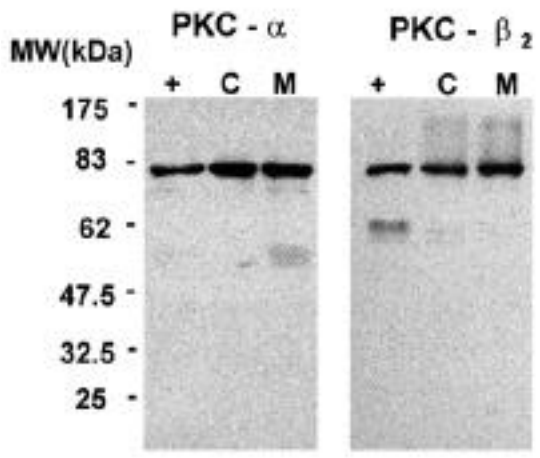

FIG. 4. Western blot of PKC α and β_2 in human monocytes. Cells were homogenized, and the cytosol and membrane fractions were prepared as described in METHODS. Protein samples (30 µg) were resolved on 10% polyacrylamide gels and transferred onto nitrocellulose membrane. The blots were probed with PKC isozyme-specific antibodies. The sizes of protein markers are indicated at the left. +, rat brain control; C, cytosol fraction; M, membrane fraction.

isoform is indicated. Antibodies to PKC α and β_2 detected two bands of 82 and 80 kDa, respectively, in cytosol and membrane fractions of monocytes from normal control subjects.

The amounts of PKC α and β_2 isoforms were compared in 14 normal control subjects and 19 diabetic patients by densitometric analysis. Figure 5 summarizes immunoblot densitometry analysis of PKC α and β_2 in the cytosol and membrane fractions. In the cytosol fraction from diabetic patients, PKC α and β_2 contents were similar to those of control subjects. In the membrane, the protein content of PKC α was not significantly different, while PKC β_2 was significantly increased by 45 ± 4% ($P < 0.05$) compared with control subjects.

Effect of euglycemia. To evaluate the possibility that the increase in the PKC activity and in the expression of membrane PKC β_2 was due to hyperglycemia, we evaluated the PKC activity and the quantitative amounts of this isoform of PKC in nine diabetic patients in whom an insulin infusion was given to restore euglycemia, as described in METHODS. As shown in Fig. 6, after insulin infusion, PKC activity was significantly reduced (42 ± 8%), while only a modest and not significant decrease (15 ± 2%) was observed in the protein content of membrane PKC β_2.

Effect of hyperglycemia. In vitro, elevated extracellular glucose has been reported to activate PKC in VSM cells (6) and endothelial cells (9). We therefore investigated PKC activity in two normal subjects when hyperglycemia was induced, as described in METHODS. As shown in Fig. 7, cytosol PKC activity was unchanged, whereas membrane PKC markedly increased from 63 and 57 to 92 and 128.6 pmol · min^{-1} · mg^{-1} protein, respectively.

To determine which isoform might mediate the increase in the membrane-associated PKC activity induced by hyperglycemia, we measured the amount of PKC α and β_2 isoforms in monocytes from these two subjects. As shown in Fig. 8, PKC α was unchanged by glucose treatment in the cytosol and in membrane fractions, whereas hyperglycemia increased levels of the PKC β_2 isoform in the membrane fraction by 75 ± 5%.

DISCUSSION

The present study shows that *1*) membrane PKC activity is increased in monocytes from diabetic patients; *2*) monocytes express the glucose-sensitive PKC β_2 isoform, which is increased in diabetic patients; *3*) in these patients, the normalization of circulating plasma glucose by insulin infusion results in a slight reduction of PKC β_2 isoform along with a significant reduction of PKC activity; and *4*) in normal subjects, an acute rise of plasma glucose increases PKC activity and membrane β_2 isoform. These data therefore indicate that PKC

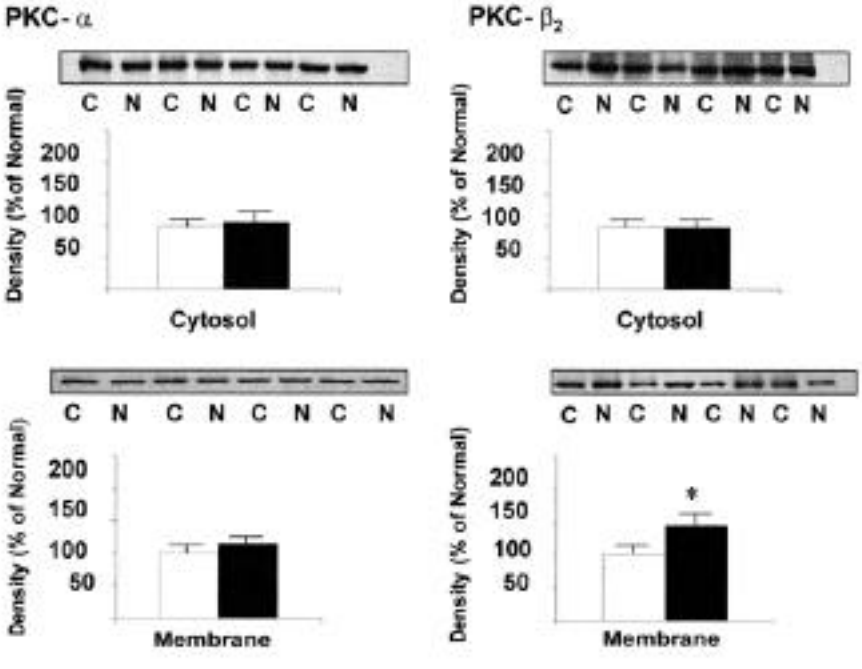

FIG. 5. Immunoblot of Ca^{2+}/Pser-dependent PKCα and β_2 isoforms in human monocytes from control (C) and diabetic (N) patients. The panels show representative immunoblots of PKCα and β_2 in the cytosol and membrane fractions of monocytes. The graphs illustrate the densitometry measurements (mean ± SE) expressed as percent of normal patients (taken as 100%) for each PKC isoform. Diabetes PKC isoforms are compared with normal. *$P < 0.05$.

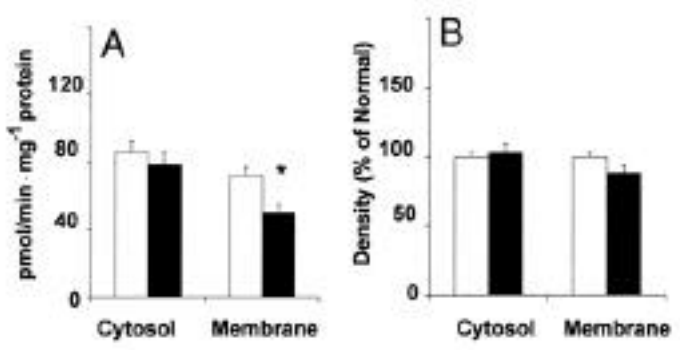

FIG. 6. PKC activity (*A*) and densitometric measurements of immunoblot analysis of PKC β_2 isoform (*B*) at hyperglycemia (□) and euglycemia (■) in human monocytes from diabetic patients. Density data are expressed as a percent of values at hyperglycemia (taken as 100%). MW, molecular weight. *$P < 0.05$.

activation in type 2 diabetes is largely accounted for by hyperglycemia. This observation is also strengthened by the positive correlation observed between the membrane PKC activity in the monocytes and fasting plasma glucose concentration.

Previous reports showed that PKC activity is increased in liver cells from diabetic patients (24) and in the skeletal muscle of high fat–fed rats, a well-characterized model of insulin resistance (25). In contrast, Nagy et al. (26) found decreased PKC activity in mononuclear cells from very poorly controlled diabetic patients. Their patients had a mean HbA_{1c} level of 12%, and the mean HbA_{1c} of our patients was 8.7%: therefore, it is conceivable that such a worsening of metabolic control may induce a progressive downregulation of PKC activity, at least in monocytes.

Membrane PKC activity was negatively correlated with insulin sensitivity. This finding suggests that PKC may undergo long-term regulation, in keeping with the data of Heydrick et al. (27), who linked an enhanced DAG-PKC signaling with insulin resistance. In fact, insulin resistance through chronic hyperglycemia might stimulate DAG-PKC system within the cell (28). Likewise, insulin resistance through hyperinsulinemia might be associated with a persistent translocation and activation of PKC as recently observed in soleus muscle in diabetic GK rats (29).

Membrane-to-cytosol PKC ratio in monocytes from diabetes was increased, suggesting an enhanced translocation of one or more isoforms of PKC from the cytosol to the membrane. By immunoblotting technique, Ca^{2+}-dependent and DAG-sensitive PKC α and β_2 were detected in both the cytosol and membrane fractions of human monocytes. However, only membrane β_2 isoform of PKC was enhanced in monocytes from diabetic patients. Other studies have investigated PKC isoforms in the cell membrane of diabetic patients. Inoguchi et al. (4) and Ishii et al. (30) demonstrated increased membrane localization of PKC β_2 and no subcellular redistribution of PKC α, δ, or ε in aorta, heart, and glomerular cells from STZ diabetic rats, while Babazono et al. (31) found that in glomerular cells from STZ diabetic rats, α, δ, and ε isoforms were increased while β_2 was reduced. In liver from diabetic patients, a significant increase in the amount of α, ε, and ξ isoforms was observed (24); furthermore, the isoforms α, β_2, and ε were increased in hepatocytes from STZ diabetic rats (32). The β_2 isoform of PKC is selectively increased by hyperglycemia (4), and it is preferentially

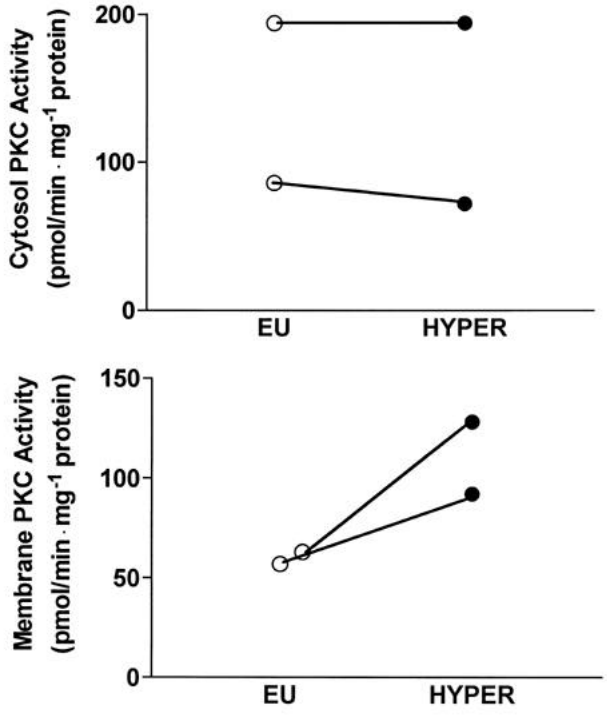

FIG. 7. Individual PKC activities in two normal control subjects at euglycemia (○) and after induction of hyperglycemia (●).

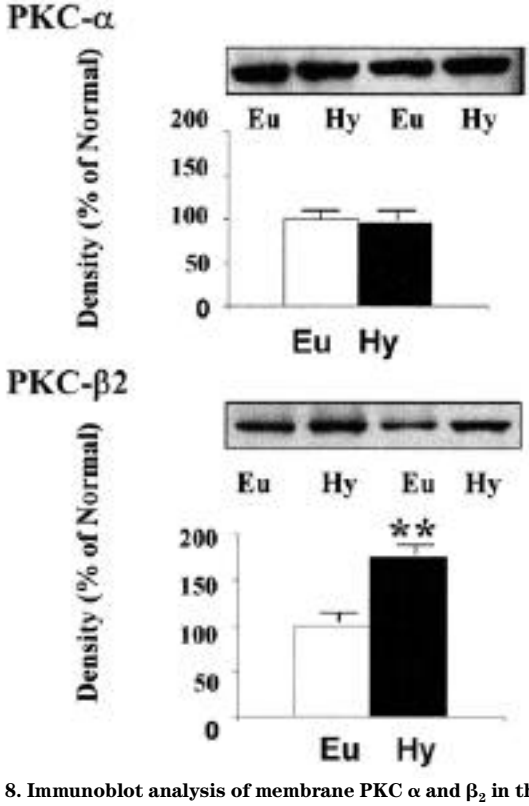

FIG. 8. Immunoblot analysis of membrane PKC α and β_2 in the two normal control subjects at euglycemia (Eu) and at hyperglycemia (Hy). Protein samples (30 µg) were resolved on 10% polyacrylamide gels and transferred onto nitrocellulose membrane. The blots were probed with PKC isozyme-specific antibodies. The graphs illustrate the densitometry measurements (mean ± SE) expressed as a percent of normal patients (taken as 100%) for each PKC isoform. **$P < 0.01$.

activated in large vessels in the presence of diabetes (2). Finally, the oral administration of β isoform–specific inhibitor, LY333531, reduced PKC activity in the retina and kidney of diabetic rats (30). As a whole, these observations suggest that PKC isoform expression is dependent on cell type and animal model.

To verify whether hyperglycemia or impaired insulin function alters PKC β_2 isoform expression and its cellular compartmentalization in monocytes from diabetic patients, we acutely corrected the metabolic control with an exogenous insulin infusion. Euglycemia significantly reduced membrane PKC activity, but it only slightly decreased the content of the membrane PKC β_2 isoform. An explanation for this observed dissociation between protein content and activity after the acute metabolic normalization is not readily apparent. It may indicate that the fall of PKC activity cannot be attributed to a decreased membrane density of PKC molecules but to a reduced turnover number. This phenomenon has also been observed for other membrane proteins such as Na^+/H^+ countertransport (33). It can be argued that insulin infusion may have effects on other unmeasurable isoforms. However, our assay selectively determines the activity of Ca^{2+}- and phospholipid-dependent, conventional, PKC isoforms; among those, only α and β are expressed in monocytes (21). Therefore the latter hypothesis appears unlikely.

The lack of a complete normalization of PKC β_2 may possibly be due to a too-short euglycemic period. This theory is in keeping with the findings of King et al. (34), who showed that in human monocytes, palmitate-labeled diacylglycerol can be normalized only after intensive glycemic control, and with those of Inoguchi et al. (4), who showed that the increased DAG levels and PKC activity were reversed, with the normalization of blood glucose levels only after 3 weeks in STZ-induced diabetic rats in the heart. Moreover, in normal subjects, hyperglycemia markedly increased membrane PKC activity in vivo and enhanced the amount of membrane β_2 isoform of PKC, but not α isoform. Therefore, our data strongly suggest that alterations of PKC activity are not an intrinsic feature of the diabetic state per se but are more likely dependent on the prevailing plasma glucose concentration. It must be borne in mind, however, that in our experimental protocols, the changes in blood glucose levels were always accompanied by inverse changes in insulin level. Therefore it is difficult to indisputably dissect out the individual role of glucose and insulin effect on PKC; further studies are needed in which PKC activity is determined in the presence of hyperinsulinemic euglycemia.

The finding of preferential activation of isoform β_2 by elevated glucose levels is in agreement with previous observations in vascular cells (35,36), while other studies reported that high glucose levels could lead to an increased expression of α, δ, and ε PKC isoforms in VSMC (2) and α and ε in endothelial cells (9). The mechanism through which glucose selectively regulates PKC isoforms is unknown. One speculation could be that α and β_2 are located at different intracellular sites and react differently to the effect of glucose on DAG levels (35).

According to in vitro studies, high glucose levels increase DAG and PKC activity only after 2–3 days of exposure (6), while in vivo studies have shown an acute effect of hyperglycemia on PKC activation (37,38). This difference emphasizes that glucose-mediated PKC activation is not regulated in the same manner in different tissues.

The increased membrane PKC activity suggests that monocytes are a useful cellular model to detect hyperglycemia-induced changes in PKC protein content and activity. The changes in PKC activity in these cells may parallel those in other types of cells, such as vascular cells, and may play a role in increasing monocyte adhesion and differentiation (8). In fact, Carantoni et al. (39) showed that mononuclear cells from diabetic patients present an increased adherence to endothelium.

In conclusion, our findings show that monocyte PKC activity in patients with insulin resistance behaves differently from that in normal patients. Type 2 diabetes is associated with an increase in the membrane, active form of PKC; this activation is strictly dependent on plasma glucose concentration. However, not only the actual metabolic control, but also insulin action appears to affect kinase functions, thus suggesting that these alterations take place over a long time. These glucose-induced alterations in monocyte PKC kinase activity may be relevant to the study of development of diabetic complications and atherosclerosis.

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