Volume 249, number 2, 234–238 FEB 07186 June 1989

Glucose-induced synthesis of diacylglycerol de novo is associated with translocation (activation) of protein kinase C in rat adipocytes

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Received 24 January 1989; revised version received 10 March 1989

Addition of glucose (5–20 mM) to rat adipocytes provoked dose-related increases in diacylglycerol, without increasing production of [^{3}H]inositol phosphates. Cytosolic protein kinase C enzyme activity and immunoreactivity decreased within 1–5 min of 5 mM glucose addition, and further over 20 min. Membrane protein kinase C increased stoichiometrically during the first 5 min and then decreased. Higher concentrations (10 and 20 mM) of glucose provoked greater and more rapid decreases of cytosolic and membrane protein kinase C. Our findings suggest that glucose stimulates diacylglycerol production by providing substrate for phosphatidic acid synthesis de novo, and this is associated with translocative activation of protein kinase C.

Diacylglycerol synthesis; Protein kinase C; Glucose stimulation; Enzyme activation; Translocation; (Rat adipocyte)

1. INTRODUCTION

Protein kinase C (PKC) is a ubiquitous regulatory enzyme, which is activated by phospholipid, Ca^{2+} and diacylglycerol (DAG) [1,2]. The DAG which derives from phospholipase C-mediated hydrolysis of phosphatidylinositol 4′,5′-bisphosphate (PIP_2) [3] causes PKC to translocate from cytosol to the membrane, and this serves as evidence of PKC activation [4]. Some agonists, such as insulin, do not stimulate PIP_2 hydrolysis significantly, but increase DAG through enhanced synthesis of phosphatidic acid (PA) de novo [5–11], and hydrolysis of a phosphatidylinositol (PI)-glycan [12] and phosphatidylcholine [11,13,14]. Insulin stimulates PKC translocation [15], but it is uncertain whether DAG derived from the de novo pathway contributes to this activation.

Recently, Draznin et al. [16] reported that insulin and glucose increase PKC-dependent histone phosphorylation and phorbol ester binding in crude cytosol of rat adipocytes. However, PKC translocation was not apparent, probably because PKC was not purified to remove activating factors, and binding assays may reflect release of the regulatory portion of PKC to the cytosol during activation. Using methodology described presently, we have observed typical PKC translocation responses to insulin, phorbol esters and glucose in rat adipocytes. We report here that glucose stimulates DAG production through PA synthesis de novo, and that this is associated with PKC translocation.

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Abbreviations: PKC, protein kinase C; DAG, *sn*-1,2-diacylglycerol; IP, IP_2 and IP_3, inositol mono-, bis- and trisphosphate; PA, phosphatidic acid; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. EXPERIMENTAL

Epididymal fat pads were obtained from fed male Holtzmann rats (200 g). Adipocytes were obtained by collagenase (Worthington, 1 mg/ml) digestion [17] of fat pads incubated for

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30 min at 37°C in Krebs-Ringer-Phosphate buffer (KRP) containing 3% BSA and 2.5 mM glucose. Adipocytes or fat pads were washed and equilibrated for 30 min in glucose-free KRP containing 1% BSA, and then treated during 20 or 30 min incubation. (NB: similar conditions are commonly used in insulin studies, and insulin treatment for 30 min in glucose-free KRP provoked 2-4-fold increases in 2-[^{3}H]deoxyglucose uptake.)

To purify PKC, adipocytes or fat pads were washed 3 times and homogenized in ice-cold buffer I [20 mM Tris (pH 7.5), 0.25 M sucrose, 1.2 mM EGTA, 0.1 mM PMSF, 20 μg/ml leupeptin, 20 mM 2-mercaptoethanol] and centrifuged at 100000 × g for 60 min to obtain cytosol and membrane fractions. The latter were suspended in buffer I containing 1% Triton X-100 and centrifuged to obtain solubilized membrane fractions. Cytosolic and solubilized membrane fractions (1 and 0.3 mg protein, respectively) were applied to a Mono Q column (0.5 × 5 cm, Pharmacia HR 5/5) connected to a Pharmacia fast protein liquid chromatography (FPLC) system equilibrated with buffer II [20 mM Tris (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 10 mM 2-mercaptoethanol] [18]. PKC was eluted (1-ml fractions) with a 20 ml linear gradient of NaCl (0–0.7 M) in buffer II. Two overlapping peaks of PKC enzyme activity were regularly observed. PKC enzyme activity was measured by phosphorylation of type III-S histone [19]. The reaction mixture (250 μl) contained 20 mM Tris (pH 7.5), 5 mM magnesium acetate, 0.02% histone, 10 μM [γ-^{32}P]ATP (14 × 10^4 cpm/nmol, ICN), 10 μg phosphatidylserine (PS), 0.1 μg diolein, 0.5 mM $CaCl_2$, and 100 μl of column fractions. After incubation for 5 min at 30°C, 25% trichloroacetic acid was added, and precipitates were collected on nitrocellulose filters and counted for ^{32}P. Basal activity was measured in the presence of 0.5 mM EGTA instead of Ca^{2+}, PS and diolein, and subtracted from the total activity to determine PKC activity.

To measure immunoreactive PKC, 10 μg cytosol and 5 μg membrane protein were fractionated by SDS-PAGE [23], transferred to nitrocellulose membranes [24], and blotted [15], using a polyclonal antiserum (supplied by J. Mehegan and B. Roth, Naval Medical Research Institute, Bethesda, MD) raised to synthetic peptide, specific to type II PKC, which is the only type that we find in adipose tissue after hydroxyapatite chromatography.

DAG production was assessed by incubating 0.5 ml adipocytes with 1 μCi [6-^{3}H]glucose (ICN) and various concentrations of glucose. Lipids were extracted and DAG was purified by thin-layer chromatography [6,8,11], and counted for radioactivity. DAG content of lipid extracts was measured by the DAG kinase method (Lipidex) of Preiss et al. [20].

[^{3}H]Inositol phosphate production was measured by incubating adipocytes for 1 h in glucose-free KRP with 10 μCi [^{3}H]inositol (ARC). LiCl (5 mM) and, after 5 min, varying amounts of glucose were added. After 20 min incubation, reactions were stopped with 5% trichloroacetic acid. Acid-soluble fractions were extracted with ether, neutralized, and chromatographed on Dowex-1 columns to purify inositol phosphates (see [8,21,22]). Incorporation of [^{3}H]inositol into phospholipids was measured by extracting trichloroacetic acid precipitate with $CHCl_3$.

3. RESULTS

Adipocyte cytosolic PKC enzyme activity decreased progressively during a 20 min incubation with 5 mM glucose (fig.1). Membrane PKC enzyme activity increased during the first 5 min of glucose treatment, but subsequently decreased over the next 15 min. Time-dependent changes in cytosolic and membrane immunoreactive PKC were also observed with 5 mM glucose treatment (fig.2): cytosolic PKC decreased progressively, whereas membrane PKC increased during the first 1–5 min (there was some variation in the timing of the peak) and then decreased.

In response to 5, 10, and 20 mM glucose, decreases in cytosolic PKC enzyme activity were apparent at 5 min, and greater after 20 min of incubation (fig.3). Membrane PKC increased during a 5 min incubation with 5 mM glucose, but higher concentrations of glucose (10 and 20 mM) caused

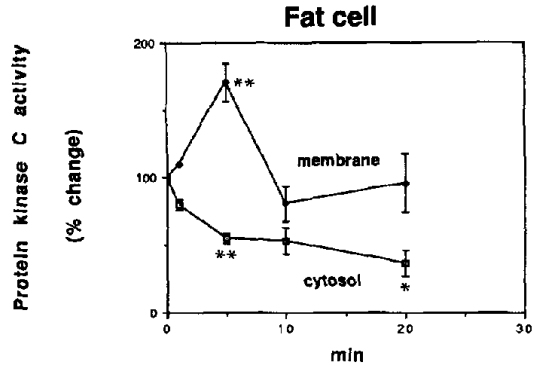

Fig.1. Time-course of effects of 5 mM glucose on PKC activity of cytosol (□—□) and membrane (◆—◆) fractions of rat adipocytes. Equal amounts of cytosolic or membrane protein from each group were applied to the Mono Q column. Total elutable PKC activity was calculated by summing the activities of all fractions, and found to be 80–150 and 70–120 pmol/min per mg protein, in control, cytosol and membrane fractions, respectively. Results are expressed as % of control PKC activity. Mean ± SE of 2–4 determinations from 5 separate experiments. * $P<0.025$; ** $P<0.01$ (paired t-test of 4 comparisons).

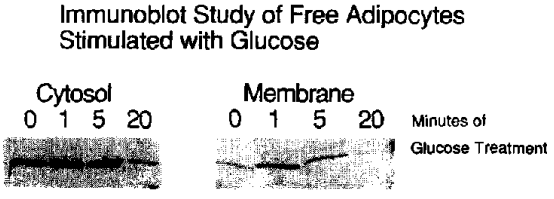

Fig.2. Immunoblot analysis of 5 mM glucose-induced changes in immunoreactive PKC in cytosol and membrane fractions of rat adipocytes.

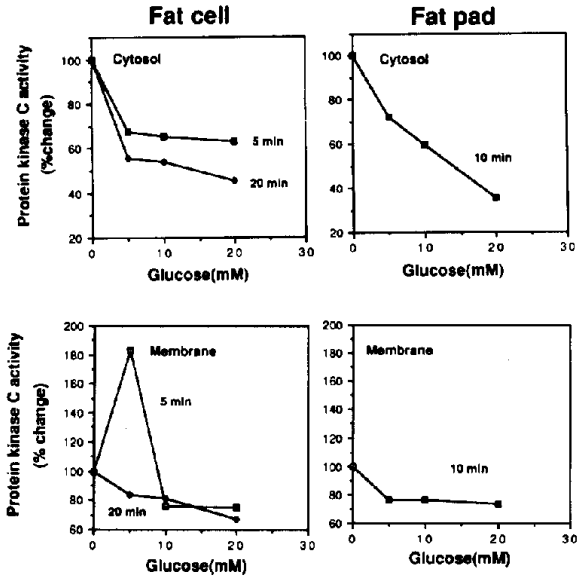

Fig.3. Glucose concentration-dependent activation of PKC in rat adipocytes (left) and fat pads (right). Adipocytes were incubated with 0, 5, 10, and 20 mM glucose for 5 or 20 min. Fat pads were incubated with 0, 5, 10, and 20 mM glucose for 10 min. Total elutable PKC activity from Mono Q columns was calculated as in fig.1.

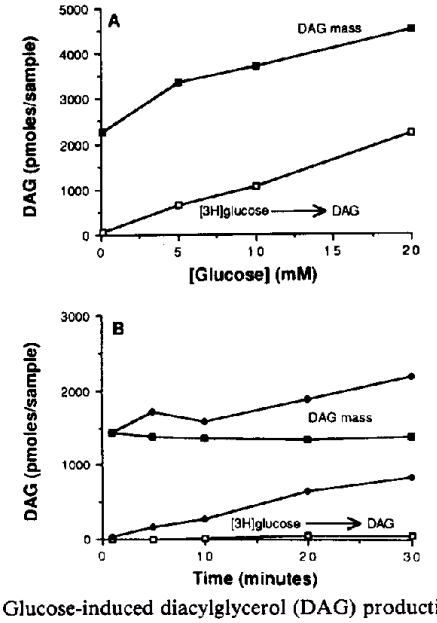

Fig.4. Glucose-induced diacylglycerol (DAG) production in rat adipocytes. (A) Cells were incubated for 20 min with [^{3}H]-glucose in KRP containing 0.1, 5, 10, and 20 mM glucose. (B) Cells were incubated with [^{3}H]glucose in KRP containing 0.1 (□, ■) or 5 mM glucose (◇, ◆) for 1, 5, 10, 20, and 30 min. Open and closed symbols indicate the calculated amounts of [^{3}H]glucose converted to DAG, and DAG mass, respectively. Mean values from 5 experiments. SE values were less than 10%.

PKC activity to decrease within the first 5 min. After 20 min incubation with 5, 10 and 20 mM glucose, membrane PKC activity was decreased in all cases. Glucose concentration-dependent changes in PKC enzyme activity were also demonstrated in fat pads. Cytosolic PKC decreased progressively, and membrane PKC decreased to a plateau during 10 min incubation with 5, 10 and 20 mM glucose, respectively.

Adipocyte DAG production from glucose (calculated from medium glucose specific activity) and the measured mass of DAG increased in parallel as functions of time and medium glucose concentration (fig.4). For example, after 20 min incubation with 0.1 and 20 mM glucose, DAG derived from [^{3}H]glucose was 49 and 2207 pmol/sample, respectively, and DAG content was 2275 and 4470 (difference = 2195) pmol/sample. Glucose had no effect on production of [^{3}H]-inositol phosphates, but did provoke small (15–50%) increases in [^{3}H]inositol labeling of adipocyte phospholipids, presumably reflecting increased synthesis (not shown).

4. DISCUSSION

In this study, we found that glucose provoked rapid increases in DAG production in rat adipocytes, and that this was associated with apparent activation of PKC. The increases in DAG were derived solely or largely from de novo synthesis of PA. The activation of PKC was demonstrated by typical translocation, i.e. redistribution of PKC from cytosol to the membrane fraction, as observed in experiments in which 5 mM glucose was present for 5 min. This translocation pattern was apparent in studies of both PKC enzymatic activity following partial purification by FPLC on Mono Q columns, and immunoreactive PKC. In addition, this translocation pattern was only transiently observed, and was followed by decreases in membrane PKC, presumably reflecting time-dependent proteolytic conversion of PKC to the Ca^{2+}/phospholipid-independent M-form of the enzyme [26,27]. At higher glucose concentrations, decreases in cytosolic PKC were readily apparent, but initial increases in membrane PKC were not observed at 5 min; this suggests that with stronger activation of DAG-PKC signalling, there is more rapid activation of proteolytic enzymes which con-

vert PKC to M-kinase and possibly other related kinases. Along these lines, we observed increases in Ca^{2+}/phospholipid-independent histone phosphorylating activity during glucose treatment, but further studies will be required to determine whether this is M-kinase.

Our results differ from those of Draznin et al. [16], who found that 10 mM glucose provoked increases in crude cytossolic PKC enzyme activity. However, we have found that considerable amounts of lipids (including DAG) are recovered in crude cytosol (probably artifactually), as well as membrane preparations, and this may increase the enzymatic activity of PKC, especially after glucose-stimulated DAG production. In our assays, we used PKC partially purified by chromatography on Mono Q columns, and this largely removes DAG, and possibly other endogenous activators. Thus, our assays, which contained optimal amounts of added diolein, would reflect changes in PKC content more than activity. In keeping with this conclusion is the fact that changes in immunoreactive PKC were similar to changes in histone phosphorylating activity of the partially purified enzyme.

The finding of glucose-stimulated DAG production in [^{3}H]glucose-labeled adipocytes is similar to that observed in pancreatic islets [28]. However, although glucose-stimulated DAG is largely derived from de novo PA synthesis [28], hydrolysis of PIP_2 and PI, and Ca^{2+} mobilization are also stimulated by glucose in islets [29–31]. In adipocytes we did not obtain evidence of inositol phospholipid hydrolysis, and Draznin et al. [16] did not observe changes in cytosolic Ca^{2+}. Thus, it appeared that glucose-dependent increases in adipocyte DAG were derived largely, if not solely, from PA synthesis de novo, and that this DAG was responsible for activation of PKC. Accordingly, DAG derived from the de novo pathway may also contribute to insulin- or other hormone-induced activation of PKC.

It is surprising that a physiological concentration of glucose generates sufficient DAG by the de novo pathway, which, at least in the rat adipocyte, results in the activation of PKC. However, activation of PKC by this mechanism is only partial, and, addition of insulin to adipocytes incubated in 5, 10 and 20 mM glucose results in further PKC activation (unpublished). Thus, in physiological conditions (i.e. et 5 mM glucose), de novo PA synthesis may provide a modest tonic stimulation of rat adipocyte PKC, which may be further activated by agents which stimulate DAG production through PA synthesis de novo and/or phospholipid hydrolysis.

Acknowledgements: This work was supported by funds from the Research Service of the Veteran's Administration and National Institutes of Health Grant no. DK38079.

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