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Tissue-dependent activation of protein kinase C in fructose-induced insulin resistance

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Rats fed a fructose-enriched diet develop increases in blood pressure and resistance to insulin-mediated glucose disposal, but the underlying biochemical alterations have not been clearly defined. Since protein kinase C (PKC) has been implicated in the pathogenesis of insulin resistance, as well as blood pressure (BP) regulation, the present study was initiated to see whether changes in PKC signaling are present in rats with fructose-induced insulin resistance and hypertension. Consequently, liver, muscle, and adipose tissues were collected from fructose ($n = 13$) and chow ($n = 12$) fed Sprague-Dawley rats. PKC enzyme activity, and expression of classical PKC isozymes, were measured in cytosol and membrane fractions, and 1,2-diacylglycerol (DAG), an endogenous stimulator of PKC, was measured by radio-enzymatic assay. Fructose feeding was associated with significant increases in fasting plasma insulin (140%) and triglyceride (400%) levels, and increased BP (20 mmHg). PKC activity was increased in the membrane fraction of adipose tissue (234 ± 38 (SE) *vs* 85 ± 30 pmol/min/mg protein, $P < 0.007$), without evidence of increased translocation or activation by DAG. Thus, fructose-induced insulin resistance has no effect on conventional PKC activity and subcellular distribution in liver and muscle, but the 3-fold increase in membrane-associated kinase activity in fat may be relevant to the mechanism of hypertriglyceridemia associated with fructose feeding.

Keywords: diacylglycerol; triglyceride; blood pressure; membranes; immunoblotting

Introduction

Protein kinase C (PKC) encompasses a family of multifunctional, serine/threonine protein kinases with widespread tissue distribution (Nishizuka, 1992; Hug & Sarre, 1993; Selbie *et al.*, 1993; Akimoto *et al.*, 1994). There is evidence (Farese, 1990) that exposure of various tissues to insulin leads to rapid formation of diacylglycerol (DAG), followed by activation of PKC (Ishizuka *et al.*, 1989), which then appears to exert negative feedback control of the intracellular response, e.g. by decreasing the tyrosine kinase activity of the insulin receptor (Bollag *et al.*, 1986; Berti *et al.*, 1994) and/or inactivating glycogen synthase (Ahmad *et al.*, 1984). Increased DAG-PKC signaling has been associated with insulin resistance due to aging (Ishizuka *et al.*, 1993), obesity (Cooper *et al.*, 1993), and denervation (Heydrick *et al.*, 1991), which has led to recent suggestions that activation of PKC might negatively regulate insulin action (Considue & Caro, 1993; Shmueli *et al.*, 1993).

In an effort to further explore the role of PKC regulation in insulin action, we felt it would be informative to examine DAG-PKC signaling in liver, muscle and adipose tissues from a rodent model of insulin resistance. We chose the fructose-fed Sprague-Dawley rat as a non-obese, non-diabetic model of dietary-induced insulin resistance (Zavaroni *et al.*, 1980; Tobey *et al.*, 1982; Hwang *et al.*, 1987; Storlien *et al.*, 1993; Bhanot *et al.*, 1994). In addition, this model is hypertensive (Hwang *et al.*, 1987; Storlien *et al.*, 1993; Bhanot *et al.*, 1994) and PKC-dependent mechanisms have also been implicated in blood pressure regulation (Andrea & Walsh, 1992).

Results

The effects of the fructose-enriched diet on body weight, plasma glucose, insulin and triglyceride concentrations, and blood pressure, are summarized in Table 1. Weight gain was similar in both groups of rats, but fructose feeding was associated with significant increases in fasting plasma insulin and triglyceride concentrations and blood pressure (Table 1). There was no significant difference in plasma glucose concentrations.

PKC activity in liver, soleus and TFL tissues (expressed per mg of protein) was significantly higher in the membrane fraction (1B) as compared with the cytosol (Figure 1A). PKC activity in liver and muscle tissues was not significantly different between the two groups. Also, there was no difference in total protein yield from liver and muscle tissues between chow (control) and fructose fed animals (Table 2). In contrast, large differences in PKC activity were observed in the membrane fraction of adipose tissue ($P < 0.007$, Figure 1B). More specifically, PKC activity in the membrane fraction of fat was 2–3 times higher in fructose-fed rats, but there was no evidence of increased translocation; PKC activity in the cytosol fraction was similar in chow versus fructose-fed animals (Figure 1A).

In order to evaluate whether the increase in PKC activity observed in adipose tissue of fructose-fed rats was due to increased activation by DAG, or an increase in immunoreactive protein, we used a quantitative assay for DAG and performed Western blotting on samples of cytosol and particulate fractions. The results of the DAG measurements are shown in Figure 2. Mean values for DAG, both in cytosol and particulate fractions, were not significantly different between the two groups; e.g., for the membrane fraction, 360

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Table 1 Measurements of body weight, systolic blood pressure, and fasting plasma glucose, insulin and triglyceride concentrations at baseline and after 14 days in chow- and fructose-fed rats (mean±SEM)

	Chow (n = 12)		*Fructose* (n = 13)	
	Baseline	*14 days*	*Baseline*	*14 days*
Body weight (g)	216±4	276±9	223±7	278±12
Plasma glucose (mg/dl)	124±5	132±4	130±3	141±6
Plasma insulin (μU/ml)	27±9	27±7	23±9	55±14*
Plasma triglycerides (mg/dl)	95±16	108±24	113±21	578±124†
Systolic blood pressure (mmHg)	126±2	129±2	124±3	144±3*

*$P<0.01$; †$P<0.001$

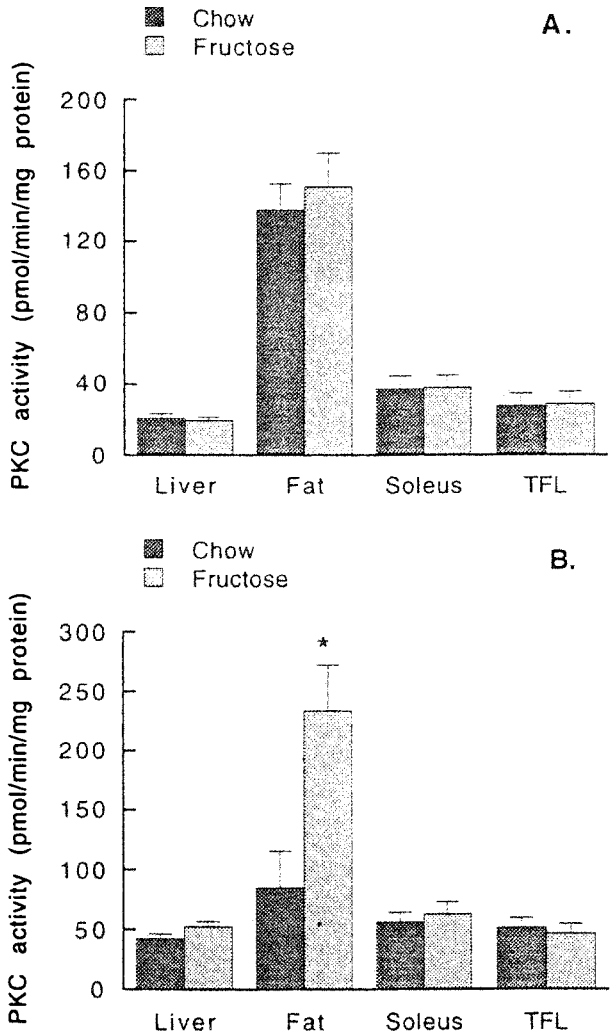

Figure 1 Effects of fructose feeding on protein kinase C activity in cytosol (A) and membrane (B) fractions of liver, epididymal fat, and soleus (red) and tensor fascia latae (TFL, white) skeletal muscles from chow ($n = 12$) and fructose fed ($n = 13$) rats

Table 2 Total protein content of cytosolic and particulate fractions from liver, muscle and adipose tissues of chow (control) and fructose fed animals

	Protein content (μg/mg tissue)	
Tissue	*Cytosolic fraction*	*Particulate (membrane) fraction*
Liver		
Chow fed	64.8±6.8	37.2±2.7
Fructose fed	66.8±2.6	32.5±2.6
Soleus		
Chow fed	20.0±2.3	5.9±0.9
Fructose fed	20.8±0.8	5.0±0.3
TFL		
Chow fed	19.9±1.2	3.8±0.6
Fructose fed	20.7±1.1	3.4±0.6
Adipose tissue		
Chow fed	2.80±0.20	0.30±0.03
Fructose fed	2.90±0.20	0.32±0.05

Results are mean±SE. Control ($n = 12$) and fructose fed ($n = 13$). TFL = tensor fascia latae.

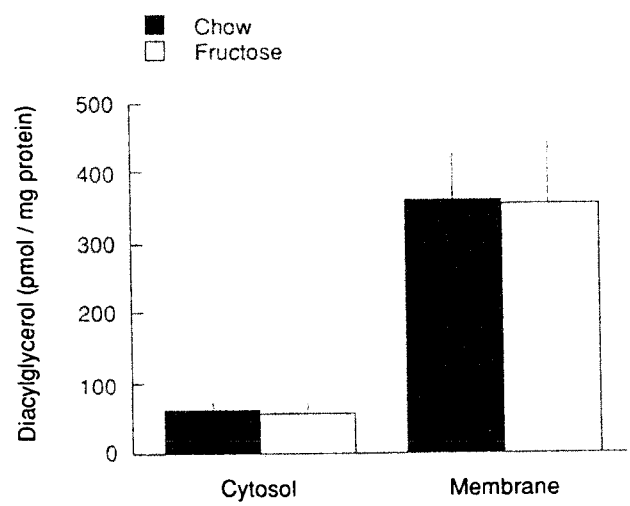

Figure 2 Levels of diacylglycerol (DAG) in cytosol and membrane fractions of adipose tissue from chow ($n = 12$) and fructose fed ($n = 13$) rats. The experimental details were same as described under Materials and methods

pmol/mg protein (chow) as compared with 350 pmol/mg in the fructose-fed group. Similarly, Western blots showed no difference in conventional PKC protein (α,β,γ) between chow-fed and fructose-fed animals (Figure 3).

Discussion

The interpretation and significance of these results can be discussed at several levels. Firstly, as shown previously by our laboratory and others (Zavaroni *et al.*, 1980; Tobey *et al.*, 1982; Hwang *et al.*, 1987; Storlien *et al.*, 1993; Bhanot *et al.*, 1994), fructose feeding was associated with both hemodynamic and metabolic alterations. Specifically, fructose-fed rats developed increases in blood pressure and evidence of insulin resistance, as reflected by the large increases in fasting plasma insulin and triglyceride concentrations. The biochemical mechanism of fructose-induced insulin resistance remains unclear, but most of the available evidence points to molecular changes at or beyond the

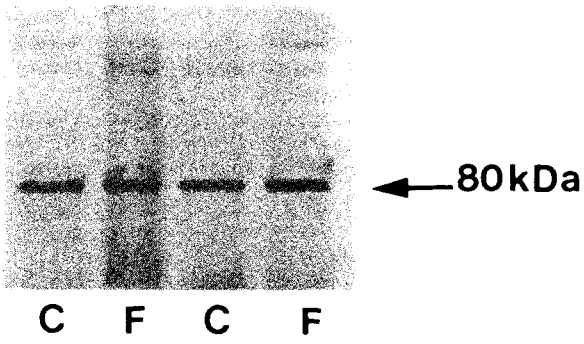

Figure 3 Western blots of PKC protein (α, β, γ) in the membrane fraction of adipose tissue from chow ($n = 12$) and fructose fed ($n = 13$) rats. The Western blotting was performed as described under Materials and methods. The affinity purified polyclonal antibody of human PKC (raised against the conserved C4 catalytic domain, residues 528–538) used here, is specific for α, β and γ isoenzymic forms of PKC

insulin receptor (Mayes, 1993). The results of this study clearly demonstrate that fructose feeding is associated with differential effects on PKC activity in different insulin-sensitive tissues. The major finding was a three-fold increase in PKC activity in the membrane fraction of adipose tissue, whereas, in contrast to other forms of insulin resistance (Heydrick *et al.*, 1991; Cooper *et al.*, 1993; Ishizuka *et al.*, 1993), no differences were observed in skeletal muscle and liver. The mechanism accounting for the increase in PKC activity in fat is not entirely clear, but these data rule out a number of simple explanations. Firstly, since there was no evidence of a corresponding reduction in enzyme activity in the cytosol, the increase in membrane-associated kinase activity cannot simply be attributed to increased translocation of PKC from the intracellular compartment. Secondly, increased DAG-induced activation of PKC seems unlikely since quantitative measurements of DAG in the membrane fraction were similar in both groups of tissues; and thirdly, fructose feeding had no effect on the amount of immunoreactive PKC protein as detected by Western blots. Thus, having excluded several possibilities, we would speculate that the observed increase in PKC activity in fat might be due to a conformational change in the tertiary structure of the enzyme, possibly due to fructose-induced changes in plasma membrane composition.

The biological significance of this increase in PKC activity in adipose tissue is still uncertain, but the results of this study are relevant to an on-going debate about the possible role of PKC in insulin signal transduction. A number of groups have recently suggested that activation of PKC might be an important underlying mechanism in the development of resistance to insulin-mediated glucose disposal (Considue & Caro, 1993; Shmueli *et al.*, 1993). Thus, PKC-mediated phosphorylation appears to down-regulate the insulin receptor (Bollag *et al.*, 1986), as well as glycogen synthase (Ahmad *et al.*, 1984), and recent studies have found specific changes in DAG-PKC signaling in muscle and adipose tissues associated with insulin resistance due to aging (Ishizuka *et al.*, 1993), obesity (Cooper *et al.*, 1993) and muscle denervation (10). In the present study, however, where fructose-induced insulin resistance affects both liver (Tobey *et al.*, 1982) and muscle (Storlien *et al.*, 1993), neither of these tissues showed significant changes in PKC activity or subcellular distribution. Thus, in contrast to other forms of insulin resistance, the metabolic changes induced by fructose feeding have no impact on DAG-mediated activation of conventional PKC isoforms in liver and muscle.

The specific changes observed in fat suggest that PKC activation might be related to the development of fructose-induced hypertriglyceridemia. For example, there is evidence that PKC plays an important regulatory role in lipolysis (Vikman *et al.*, 1993), and that activation of PKC stimulates release of free fatty acids via phosphorylation of inhibitory G-proteins in the adipocyte membrane (Pyne *et al.*, 1988). Thus, by increasing the supply of substrate to the liver, activation of PKC in adipose tissue might be a contributory mechanism in fructose-induced hypertriglyceridemia.

Materials and methods

Materials

[γ-^{32}P]ATP (3000 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA). Dioleoylglycerol, bovine cardiolipin (heart) and phosphatidylserine (brain) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). DAG Kinase was the product of Lipidex (Madison, WI). The following reagents were supplied by Sigma Chemical Co. (St. Louis, MO): ATP, DETAPAC, EGTA, EDTA, perchloric acid, DEAE cellulose, Triton X-100, Tween 20. A synthetic dodecapeptide of glycogen synthase (GS peptide, PLSRT-LSVAAKK) was purchased from St. Vincents Hospital, Melbourne, Australia. An affinity-purified polyclonal antibody raised against the conserved C4 catalytic domain (residues 528–537) of human PKC, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). This antibody specifically recognizes the α, β and γ isoenzymic forms of PKC. All other reagents used were of analytical grade.

General protocol

Male Sprague-Dawley rats (Bantin and Kingman, Fremont, CA), initially weighing approximately 200 g, were used for the study. Prior to dietary manipulation, all rats were fed standard rat chow and maintained on a 12 h light/dark cycle (6 AM to 6 PM). Rats were acclimated to the procedure of blood pressure measurement at 1 PM daily for 2 days prior to each blood pressure measurement. Following baseline blood pressure measurements and blood sampling, rats were randomly separated into two groups: one group was continued on the standard chow diet while a second group was switched to a pelleted high-fructose diet (Teklad diet no. 78463) for 16 days. The composition of the fructose diet was 66% fructose, 22% protein and 12% fat. Blood pressure measurements and blood sampling were repeated at the end of the dietary period. Blood samples were drawn from the tail veins of unanesthetized rats at 1 PM, 5 hrs after food withdrawal, for subsequent assay of fasting plasma glucose, insulin, and triglyceride concentrations, as described previously (Zavaroni *et al.*, 1980; Tobey *et al.*, 1982). At the end of the dietary intervention rats were sacrificed for collection of liver, muscle (soleus) and epididymal fat tissues. Tissues were quickly excised and frozen between blocks of solid CO_2 (enclosed in plastic bags) and stored in a liquid nitrogen freezer until assayed.

Blood pressure measurement

Rats were removed from the animal room and taken to the laboratory at 9 AM; they were allowed free access to diet and

water and kept in a quiet area before the blood pressure was measured at 1 PM, as described previously (Hwang *et al.*, 1987). The tail cuff method, without external preheating, was used to measure the systolic blood pressure (Bhanot *et al.*, 1994). Ambient temperature was kept at 30°C. The equipment used included magnetic animal holders connected with a manual scanner (Model no. 65-12, IIC, Woodland Hills, CA), a pulse amplifier (Model no. 59, IIC) and a dual channel recorder (Model no. 1202, Linear Instruments, Reno, NV). The systolic blood pressure was measured in the conscious state, and it has been shown that measurements obtained with this technique are directly comparable to those obtained by direct arterial cannulation (Buñag, 1973). The mean of five consecutive readings was used as the measurement of the systolic blood pressure of each rat for that day, and the mean blood pressure on the 2 days before starting the diet and on the last 2 days of each diet period were averaged and used for statistical comparisons.

Preparation of tissue samples

Tissue samples were homogenized in a Polytron (Brinkman Instruments, Westbury, NY) in 100 mg/l buffer A (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.25 mM EGTA, 2 μg/ml leupeptin, 4 μg/ml pepstatin, 4 μg/ml each of calpain I and II, 0.2 mM PMSF and 0.25 M sucrose). For subfractionation of the crude homogenate into cytosol and particulate fractions, the samples were first spun at 400*g* for 15 min. The supernatant for liver and muscle, and the infranatant below the fat cake for adipose tissue, was then ultracentrifuged at 105 000*g* for 60 min. The supernatant was removed as the cytosol fraction; the sediment (particulate fraction) was resuspended in 0.5 ml Buffer A. An aliquot of each fraction was saved for protein quantification (Bradford, 1976), and cytosol and particulate samples were then used for biochemical assays of cPKC activity and DAG and for Western blotting.

Measurement of protein kinase C activity

PKC activity was measured in partially purified preparations of cytosolic and particulate (membrane) fractions. In brief, a suitable aliquot of particulate fraction (total volume 0.5 ml) was mixed with 0.5 ml of Buffer B (Buffer A without sucrose) containing 2% Triton X-100 and solubilized for 30 s in a sonic dismembrator at 4°C. Aliquots of each fraction (before addition of Triton X-100) were collected for protein quantitation (Bradford, 1976). The supernatant and particulate fractions were then applied to DEAE-Sephacryl columns (Pharmacia). After washing with 5 ml Buffer B, the enzyme fractions were eluted in 3 ml fractions from the column using Buffer B containing 0.15 M NaCl. Enzyme activity was assayed immediately after completion of the chromatography by following the incorporation of [^{32}P] from [γ-^{32}P]ATP into GS peptide as described previously (Azhar, 1991). The standard incubation mixture consisted of the following components in a final volume of 100 μl: 20 μl aliquots of chromatography fractions, 25 mM PIPES-NaOH (pH 6.8), 10 mM magnesium acetate, 20 μM GS peptide, 5 mM 2-mercaptoethanol, 0.1 mM [γ-^{32}P]ATP (200–300 cpm/pmol), 1.0 mM $CaCl_2$, 150 μg/ml phosphatidylserine, and 10 μg/ml diolein. Phosphatidylserine and diolein were dissolved in chloroform, dried in N_2 and the residues resuspended in 10 mM PIPES, pH 6.8, before addition to the assay. Basal activity was determined in the presence of 0.5 mM EGTA (instead of Ca^{2+}, phosphatidylserine and diolein). The reaction was initiated by the addition of [γ-^{32}P]ATP at 30°C. After incubation for 15 min, the reaction was terminated by spotting 50 μl of the mixture onto 2 × 2 cm phosphocellulose strips (Whatman P81) which were dropped immediately into 75 mM phosphoric acid. The strips were then washed four times, including one overnight wash, in 75 mM phosphoric acid, dried and counted in a Beckman LS no. 3801 scintillation counter after the addition of 6 ml of scintillation fluid. PKC activity was calculated by subtracting the enzyme activity observed in the presence of 0.5 mM EGTA from that measured in the presence of phosphatidylserine, diolein and calcium. One unit of PKC activity is defined as that amount catalyzing the transfer of 1 pmol of [^{32}P]phosphate from [γ-^{32}P]ATP to GS peptide per minute at 30°C.

Western blotting

Samples were mixed with equal volumes of 2× sample-loading buffer [4.6% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) β-mercaptoethanol, 16% (w/v) sucrose, and 0.1 M Tris-HCl, pH 6.8], heated at 95°C for 5 min, and cooled to room temperature. In each case, the mixture was then centrifuged at 5000 *g* for 5 min, and the supernatant subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE), as described by Laemmli (1970), using a 16 × 14 cm slab gel (7% w/v polyacrylamide) system (Hoefer Scientific Instruments, San Francisco, CA). For each sample, 20 μg total protein were loaded onto the gel. Protein standards were also loaded onto each gel [myosin, 200 kilodaltons (kDa); β galactosidase, 116 kDa, phosphorylase b, 97 kDa, albumin 66 kDa, ovalbumin, 45 kDa; Bio-Rad Laboratories, Richmond, CA]. Following electrophoretic separation, proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) in transfer buffer containing 10 mM CAPS (3-[cyclohexylamino]-1-propane sulfonic acid) and 10% methanol, pH 11. At the conclusion of the transfer process, each gel was stained with Coomassie Blue G-250, then destained by serial washings in a solution of 10% acetic acid and 2% dimethylsulfoxide (with 10% glycerol in the final wash), to confirm >98% efficiency of transfer. The membrane was then incubated for 12 h at 4°C in phosphate buffered saline-Tween (PBS-T:10 mM sodium phosphate, 0.15 M NaCl, 25 mM $MgCl_2$ and 0.2% Tween-20) containing 5% (w/v) non-fat dried milk, ph 7.4. After the blocking step, membranes were washed (4 × 10 min) in rinsing solution (PBS-T with 1% dried milk, pH 7.4) and then incubated overnight with an affinity-purified polyclonal antibody (raised against the conserved C4 catalytic domain [residues 528-537] of human PKC), specific for α, β and γ isoenzymic forms of PKC. After further washings in rinsing solution four times, 15 min each time), membranes were incubated with horseradish peroxidase-conjugated immunoglobulin G fraction of goat antirabbit IgG, diluted 1:20 000 in PBS-T. The membranes were then washed several times in PBS-T, incubated with alkaline phosphatase-streptovidin-conjugated antirabbit IgG and developed for 60 s using a commercial kit (Zymed Laboratories, San Francisco, CA). Each film was exposed for 60 s. The bands obtained from immunoblotting were scanned by one-dimensional laser densitometry (Beckman Instruments, Palo Alto, CA). The areas under the peaks were then measured and analysed using the Gelscan XL software package.

Measurement of 1,2-Diacylglycerol (DAG)

DAG was extracted by the method of Bligh and Dyer (1959) and measured by a slight modified version of the DAG kinase procedure of Preiss *et al.* (1987). Dioleoylglycerol was used for the standard curve. Briefly, after extraction the dried lipid extracts were solubilized by sonication in 20 μl of a solution containing octyl-β-D glucoside (7.5%) and cardiolipin (5 mM) in 1 mM diethylenetriamine pentaacetic acid (DETAPAC). After 15 min incubation, 50 μl of 2× reaction buffer (100 mM imidazole-HCl, pH 6.6, 100 mM NaCl, 25 mM $MgCl_2$, 2 mM EGTA) and 10 μl of 20 mM dithiothreitol were mixed with the solubilized lipid/octylglucoside solution and purified diacylglycerol kinase to give a final volume of 90 μl. The reaction was started by addition of 10 μl of [γ-^{32}P]ATP (specific activity 5×10^5 cpm/nmol) in 10 mM imidazole, 1 mM DETAPAC, pH 6.6, and allowed to proceed at 25°C

for 30 min. The reaction was stopped by adding 3 ml of chloroform/methanol (1:2, v/v) and 0.7 ml of 1% $HCLO_4$. After addition of 1 ml $CHCl_3$ and 1 ml of 1% perchloric acid, and brief centrifugation, the lower chloroform phase was washed and then dried under N_2. The lipid film was dissolved in 5% methanol in chloroform and 50 µl spotted on to a 20 cm Silica Gel 60 TLC plate activated by prerunning in acetone. Samples of [γ-^{32}P]phosphatidic acid made from dioleoylglycerol were also spotted onto the TLC plates as standards. The plates were then developed with chloroform:methanol:acetic acid (65:15:5, v/v), air dried and subjected to autoradiography. The radioactive spot corresponding to phosphatidic acid ($R_f = 0.35$) was scraped into a scintillation vial, mixed with 6 ml of scintillation fluid, and counted. The amount of diacylglycerol present in the samples was calculated from the amount of [^{32}P]phosphatidic acid produced and the specific activity of ATP, and corrected for the volume of aliquots removed from the organic phase and spotted for TLC.

Statistical analysis

All biochemical and blood pressure measurements, and the areas under peaks derived from scanning the immunoblots of PKC, were compared for cytosol and membrane fractions between chow and fructose-fed rats using one-way analysis of variance, with probability values $<5\%$ taken as significant. Results throughout are expressed as mean ± SEM.

References

Ahmad, Z., Lee, F.T., DePaoli-Roach, A. & Roach, P.J. (1984). *J. Biol. Chem.*, **259**, 8743–8747.

Akimoto, K., Mizuno, K., Osada, S.I., Hirai, S.I., Tanuma, S.I., Suzuki, K. & Ohno, S. (1994). *J. Biol. Chem.*, **269**, 12677–12683.

Andrea, J.E. & Walsh, M.P. (1992). *Hypertension*, **20**, 585–595.

Azhar, S. (1991). *J. Nutr. Biochem.*, **2**, 165–171.

Bhanot, S., McNeill, J.H. & Bryer-Ash, M. (1994). *Hypertension*, **23**, 308–312.

Berti, L., Mosthaf, L., Kroder, G., Kellerer, M., Tippmer, S., Mushack, J., Seffer, E., Seedorf, K. & Haring, H. (1994). *J. Biol. Chem.*, **269**, 3381–3386.

Bligh, E.G. & Dyer, W.J. (1959). *Can. J. Biochem. Physiol.*, **37**, 911–917.

Bollag, G.E., Roth, R.A., Beaudoin, J., Mochly-Rosen, D. & Koshland, D.E. (1986). *Proc. Natl. Acad. Sci. USA*, **83**, 5822–5824.

Bradford (1976). *Analytical Biochem.*, **72**, 248–254.

Buñag, R.D. (1973). *J. Appl. Physiol.*, **34**, 279–282.

Considue, R.V. & Caro, J.F. (1993). *J. Cell Biochem.*, **52**, 8–13.

Cooper, D.R., Watson, J.E. & Dao, M.L. (1993). *Endocrinology*, **133**, 2241–2247.

Farese, R.V. (1990). *Proc. Soc. Exp. Biol. Med.*, **195**, 312–324.

Heydrick, S.J., Ruderman, N.B., Kurowski, T.G., Adams, H.B. & Chen, K.S. (1991). *Diabetes*, **40**, 1707–1711.

Hug, H. & Sarre, T.F. (1993). *Biochem. J.*, **291**, 329–343.

Hwang, I.-S., Ho, H., Hoffman, B.B. & Reaven, G.M. (1987). *Hypertension*, **10**, 512–516.

Ishizuka, T., Cooper, D.R. & Farese, R.V. (1989). *FEBS Lett.*, **257**, 337–340.

Ishizuka, T., Yamamoto, M., Kajita, K., Yasuda, K., Miura, K., Hernandez, H. & Farese, R.V. (1993). *Metabolism*, **42**, 420–425.

Laemmli, U.K. (1970). *Nature*, **227**, 680–685.

Mayes, P. (1993). *Am. J. Clin. Nutr.*, **58** (Suppl), 7545–7656.

Nishizuka, Y. (1992). *Science*, **258**, 607–614.

Preiss, J.E., Loomis, C.R., Bell, R.M. & Niedel, J.E. (1987). *Methods Enzymol.*, **141**, 294–300.

Pyne, N.J., Murphy, G., Milligan, G. & Housley, M.D. (1988). *FEBS Lett.*, **243**, 77–82.

Selbie, L.A., Schmitz-Peiffer, C., Sheng, Y. & Biden, T.J. (1993). *J. Biol. Chem.*, **268**, 24296–24302.

Shmueli, E., Alberti, K.G.M.M. & Record, C.O. (1993). *J. Int. Med.*, **234**, 397–400.

Storlien, L.H., Oakes, N.D., Pan, D.A., Kusunoki, M. & Jenkins, A.B. (1993). *Diabetes*, **42**, 457–462.

Tobey, T.A., Mondon, C.E., Zavaroni, I. & Reaven, G.M. (1982). *Metabolism*, **31**, 608–612.

Vikman, H.-L., Kaartinen, J.M., Tuominer, R.K. & Ohisalo, J.J. (1993). *Clin. Sci.*, **85**, 265–268.

Zavaroni, I., Sander, S., Scott, S., & Reaven, G.M. (1980). *Metabolism*, **29**, 970–973.