Tissue and isoform-selective activation of protein kinase C in insulin-resistant obese Zucker rats – effects of feeding

X Qu[1], J P Seale[1] and R Donnelly[1,2]

[1]Department of Pharmacology, University of Sydney, Sydney, Australia

[2]School of Medical and Surgical Sciences, University of Nottingham, Derbyshire Royal Infirmary, Derby, UK

(Requests for offprints should be addressed to R Donnelly, School of Medical and Surgical Sciences, University of Nottingham, Derbyshire Royal Infirmary, Derby DE1 2QY, UK)

Abstract

The mechanisms of insulin resistance in the obese Zucker rat have not been clearly established but increased diacylglycerol-protein kinase C (DAG-PKC) signalling has been associated with decreased glucose utilisation in states of insulin resistance and non-insulin-dependent diabetes mellitus. The purpose of this study was to characterise tissue- and isoform-selective differences in DAG-PKC signalling in insulin-sensitive tissues from obese Zucker rats, and to assess the effects of feeding on DAG-PKC pathways. Groups of male obese (*fa/fa*, n=24) and lean (*fa/-*, n=24) Zucker rats were studied after baseline measurements of fasting serum glucose, triglycerides, insulin and oral glucose tolerance tests. Liver, epididymal fat and soleus muscle samples were obtained from fed and overnight-fasted rats for measurements of DAG, PKC activity and individual PKC isoforms in cytosol and membrane fractions. Obese rats were heavier (488 ± 7 vs 315 ± 9 g) with fasting hyperglycaemia (10·5 ± 0·8 vs 7·7 ± 0·1 mM) and hyperinsulinaemia (7167 ± 363 vs 251 ± 62 pM) relative to lean controls. In fasted rats, PKC activity in the membrane fraction of liver was significantly higher in the obese group (174 ± 16 vs 108 ± 12 pmol/min/mg protein, P<0·05) but there were no differences in muscle and fat. The fed state was associated with increased DAG levels and threefold higher PKC activity in muscle tissue of obese rats, and increased expression of the major muscle isoforms, PKC-θ and PKC-ε: e.g. PKC activity in the membrane fraction of muscle from obese animals was 283 ± 42 (fed) vs 107 ± 20 pmol/min/mg protein (fasting) compared with 197 ± 27 (fed) and 154 ± 21 pmol/min/mg protein (fasting) in lean rats. In conclusion, hepatic PKC activity is higher in obese rats under basal fasting conditions and feeding-induced activation of DAG-PKC signalling occurs selectively in muscle of obese (*fa/fa*) rats due to increased DAG-mediated activation and/or synthesis of PKC-θ and PKC-ε. These changes in PKC are likely to exacerbate the hyperglycaemia and hypertriglyceridaemia associated with obesity-induced diabetes.

Journal of Endocrinology (1999) **162,** 207–214

Introduction

There is a negative linear relationship between body mass index and whole-body insulin sensitivity (Ferrannini *et al.* 1997). Obese patients and especially those with a high waist-to-hip ratio are insulin resistant and develop a compensatory increase in circulating plasma insulin concentrations. Type 2 (non-insulin-dependent) diabetes mellitus (NIDDM) often supervenes when the pancreatic insulin response is either defective or insufficient to maintain normal glucose tolerance.

The insulin resistance associated with obesity and diabetes affects multiple pathways of glucose and lipid metabolism in peripheral tissues (i.e. muscle/fat) and the liver. The underlying biochemical mechanisms have not been clearly established (Moule & Denton 1997), but most of the available evidence points to defects at or beyond the insulin receptor and in muscle tissue there is a strong link between insulin resistance and increased lipid availability (i.e. triglyceride and diglyceride levels) (Pan *et al.* 1997). Boneh (1995) has proposed that accumulation of lipid metabolites, via activation of protein kinases such as protein kinase C (PKC), leads to dysregulated substrate phosphorylation and cellular function. Thus, since the reversible addition of phosphate groups by intracellular kinases and phosphatases is an important mechanism for regulating cell-surface receptors and metabolising enzymes, it has been further suggested that changes in the phosphorylation state of key elements in the insulin signalling cascade may contribute to the down-regulation of insulin-mediated responses observed in obesity and other insulin-resistant states (Cortright *et al.* 1997).

The serine/threonine-specific protein kinase, PKC, has been particularly implicated in the pathogenesis of insulin resistance (Considine & Caro 1993, Shmueli *et al.* 1993). For example, increased diacylglycerol (DAG)-mediated

0022–0795/99/0162–207

PKC activation has been associated with insulin resistance in humans and animals with diabetes (Considine *et al.* 1995), and there is evidence of PKC activation in models of obesity (Avignon *et al.* 1996), ageing (Ishizuka *et al.* 1993), muscle denervation (Heydrick *et al.* 1991) and dietary-induced insulin resistance (Donnelly *et al.* 1994, Schmitz-Peiffer *et al.* 1997). There is also evidence that hyperglycaemia-induced insulin receptor inhibition is mediated by PKC (Berti *et al.* 1994). The mechanisms by which PKC activation may down-regulate one or more steps involved in glucose transport and metabolism include decreased autophosphorylation and tyrosine kinase activity of the insulin receptor (Karisik *et al.* 1990, Kellerer *et al.* 1997), inactivation of glycogen synthase (Blackmore *et al.* 1986), inhibition of insulin-stimulated Akt1 and Akt3 activity (Barthel *et al.* 1998), increased insulin receptor degradation (Mosthaf *et al.* 1998) and increased phosphorylation of insulin receptor substrate-1 (Busch *et al.* 1998).

PKC represents a family of 12 isoenzymes with different biochemical characteristics, substrates and cofactor (calcium and/or phospholipid) requirements (Hug & Sarre 1993), and the novel (group B) PKCs (nPKC-δ, -ε. θ and η) have been particularly implicated in insulin resistance (Donnelly *et al.* 1994, Kellerer *et al.* 1997, Schmitz-Peiffer *et al.* 1997). The obese Zucker rat is an excellent model of early-stage NIDDM induced by insulin resistance, overeating and overweight. The model is homozygous for the fatty (*fa*) mutation in the leptin receptor (the same gene is mutated in the diabetes (*db*) mouse (Chua *et al.* 1996)), and the insulin resistance affects primarily muscle with failure of glucose transporter function (King *et al.* 1992). The purpose of this study was to characterise changes in DAG-PKC signalling in tissues from the obese Zucker rat, and to evaluate whether PKC activity and isoform distribution is influenced by feeding.

Materials and Methods

Animals

Male obese *fa/fa* Zucker rats (n=24) and lean non-obese *fa/-* controls (n=24) were obtained from Monash University Animal Supplies (Victoria, Australia) at 9 weeks of age and housed under controlled conditions of temperature (21 °C) and lighting (12 h light-darkness cycle, lights on 0600 h) with free access to water and standard laboratory chow. The experimental protocol was approved by the University of Sydney Animal Care and Ethics Committee.

Metabolic measurements

Blood samples were drawn from the tail vein of unanaesthetised rats for measurements of fasting serum glucose, insulin and triglyceride (TG) concentrations. Fasting glucose levels were measured at weekly intervals until the obese Zucker rats became diabetic at approximately 13 weeks of age. Thereafter, following a 5-h fast, oral glucose tolerance tests (OGTT) were performed on groups of obese (*fa/fa*) (n=24) and lean (*fa/-*) (n=24) animals. Tail vein blood samples were collected at baseline and at 30, 60, 90, 120 and 180 min after an oral glucose load (2 g/kg) administered by gavage.

Collection of tissue samples in fed and fasted conditions

Two days after the OGTT, obese and lean Zucker rats were subdivided into two further groups, overnight-fasted (n=12) and fed (n=12) animals, prior to exsanguination at 0900 h. Tissue samples (liver, epididymal fat pad and soleus muscle) were quickly excised following decapitation, snap-frozen in liquid N_2 and stored at −70 °C for subsequent biochemical assays of PKC activity, DAG levels and Western blotting in cytosol and membrane (particulate) fractions. Tissue samples were homogenised in a Polytron (Brinkman Instruments, Westbury, NY, USA) in 100 mg/ml buffer A (containing 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 inhibitors, 0·2 mM phenylmethylsulphonyl fluoride and 0·25 M sucrose). For subfractionation of the crude homogenate into cytosol and particulate fractions, the samples were first spun at $400 \times g$ for 15 min. The supernatant for liver and muscle, and the infranatant below the fat cake for adipose tissue, were then ultracentrifuged at $105\,000 \times g$ for 60 min. The supernatant was removed as the cytosol fraction; the sediment (particulate fraction) was resuspended in 0·5 ml buffer A, then mixed with 0·5 ml buffer B (buffer A without sucrose) containing 2% Triton X-100 and solubilised for 30 s in a sonic dismembrator at 4 °C. An aliquot of each fraction was saved for protein quantification (Bradford 1976), and cytosol and particulate samples were then used for biochemical assays of PKC activity, DAG content and Western blotting.

Protein kinase C activity

PKC activity is virtually undetectable in crude subcellular fractions but is expressed following DEAE-cellulose chromatography to remove inhibitors, phosphatases and other protein kinases. Thus, PKC activity was measured in partially purified preparations of cytosolic and particulate (membrane) fractions, as described previously (Azhar 1991, Donnelly *et al.* 1994). In brief, the supernatant and particulate fractions were applied to DEAE-Sephacryl columns (Pharmacia, NSW, Australia) and 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 step by following the incorporation of [^{32}P] from [γ-^{32}P]ATP into glycogen synthase (GS). 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 c.p.m./pmol), 1·0 mM $CaCl_2$, 150 μg/ml phosphatidylserine, and 10 μg/ml diolein. 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 and terminated after 15 min 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 in 75 mM phosphoric acid, dried and counted in a scintillation counter (Beckman LS No. 3801). 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 catalysing the transfer of 1 pmol [^{32}P]phosphate from [γ-^{32}P]ATP to GS peptide per min at 30 °C.

Western blotting

Aliquots of the cytosol and particulate fractions were mixed with equal volumes of 2 × sample-loading buffer (4·6% (w/v) sodium dodecyl sulphate (SDS), 10% (v/v) β-mercaptoethanol, 16% (w/v) sucrose, and 0·1 M Tris–HC1, pH 6·8), heated at 95 °C for 5 min, and cooled to room temperature. The mixture was then centrifuged at 5000 × ***g*** for 5 min, and the supernatant subjected to sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli (1970). For each sample, 25 μg total protein were loaded onto the gel together with one lane of protein standards per gel (Bio-Rad Laboratories, Richmond, CA, USA). Following electrophoretic separation, proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA) in transfer buffer containing 10 mM CAPS (3-[cyclohexylamino]-1-propane sulphonic acid) and 10% methanol, pH 11. The membrane was then incubated for 12 h at 4 °C in phosphate-buffered saline-Tween (PBS-T) containing 5% (w/v) non-fat dried milk, pH 7·4. After the blocking step, membranes were washed in rinsing solution (PBS-T with 1% dried milk, pH 7·4) and then incubated overnight with an affinity-purified polyclonal antibody specific for the θ (Donnelly *et al.* 1994) and ε (Transduction Laboratories, MA, USA) isoenzymic forms of PKC. After further washings, membranes were incubated with horseradish peroxidase-conjugated immunoglobulin G (IgG) 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-streptavidin-conjugated antirabbit IgG and developed for 60 s using a commercial enhanced chemiluminescence kit (Zymed Laboratories, San Francisco, CA, USA). Each film was exposed for 60 s. The bands obtained from immunoblotting were scanned by one-dimensional laser densitometry and the areas under the peaks were 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 slightly 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 solubilised 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 2 × reaction buffer (100 mM imidazole-HC1, pH 6·6, 100 mM NaCl, 25 mM $MgCl_2$, 2 mM EGTA) and 10 μl 20 mM dithiothreitol were mixed with the solubilised lipid/octylglucoside solution and purified DAG kinase to give a final volume of 90 μl. The reaction was started by addition of 10 μl [γ-^{32}P]ATP (specific activity 5 × 10^5 c.p.m./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 chloroform/methanol (1:2, v/v) and 0·7 ml 1% $HCIO_4$. After addition of 1 ml $CHCl_3$ and 1 ml 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 scintillation fluid, and counted.

Other assays

Serum glucose and triglyceride levels were measured using enzymatic colorimetric methods (Trinder Glucose kit and GPO-Trinder Tryglyceride kit, Sigma, St Louis, MO, USA). Serum insulin concentrations were measured by double-antibody radioimmunoassay using rat insulin standards (0–8 ng/ml range) and a guinea pig anti-rat insulin primary antibody (Linco Research Inc., St Louis, MO, USA).

Statistical analysis

Serum glucose concentration- and insulin concentration-time profiles for the oral glucose tolerance tests were compared by ANOVA. Measurements of fasting serum glucose, insulin and triglyceride concentrations were analysed by one factor ANOVA. All measurements are

Table 1 Comparison of fasting serum glucose, triglyceride and insulin concentrations in obese *fa/fa* ($n=24$) and lean *fa/-* ($n=24$) rats at 10 and 13 weeks of age. Values are means ± S.E.M.

	10 weeks		13 weeks	
	Lean	Obese	Lean	Obese
Parameters				
Body weight (g)	266 ± 11	315 ± 9	315 ± 9	488 ± 7*
Glucose (mmol/l)	7·1 ± 0·2	7·7 ± 0·1	7·7 ± 0·1	10·5 ± 0·81**
Triglyceride (mmol/l)	1·57 ± 0·04	1·43 ± 0·05	1·43 ± 0·05	4·08 ± 0·16***
Insulin (pmol/l)	208 ± 85	251 ± 62	251 ± 62	7167 ± 363***

*$P<0·05$; **$P<0·01$; ***$P<0·001$ vs corresponding data from lean control rats.

expressed as means ± standard error (S.E.M.) and statistical significance was accepted at the 5% level.

Results

Metabolic characteristics of obese fa/fa *Zucker rats*

Obese rats at 10 weeks of age had fasting serum glucose concentrations within the normal range but fasting triglyceride and insulin levels were significantly higher than in lean (*fa/-*) controls. At 13 weeks, the genetically obese *fa/fa* Zucker rats were significantly overweight (488 ± 7 vs 315 ± 9 g) with marked hyperinsulinaemia and hypertriglyceridaemia compared with their lean counterparts (Table 1). Furthermore, by 13 weeks of age the obese rats had become diabetic (Fig. 1). Following an oral glucose tolerance test, the area-under-the-curve (AUC) of the serum glucose concentration-time profile was significantly higher in obese rats: AUC[glu] was 47·4 ± 13·3 mmol.h/l in obese rats vs 25·0 ± 1·6 mmol.h/l in lean controls ($P<0·001$, Fig. 1). The insulin response to the oral glucose stimulus was also significantly higher: AUC[insulin] was 20 644 ± 1128 mmol.h/l for obese rats compared with 2392 ± 327 pmol.h/l for lean rats ($P<0·001$, Fig. 1).

PKC activity in insulin target tissues in fed and fasted conditions

PKC activity was generally lower in the cytosol fraction compared with the membrane fraction of all three tissues (liver, soleus muscle and fat) in both obese and lean rats (Table 2). In fasted animals, PKC activity in the membrane fraction of liver was significantly higher in obese compared with lean rats but there were no significant differences in muscle and fat (Table 2).

In obese Zucker rats, feeding was associated with almost a threefold increase in PKC activity in the membrane fraction of skeletal muscle but no increase in liver or fat tissue (Table 2). In lean rats, however, PKC activity in the cytosol and membrane fractions of soleus muscle was similar in fed and fasted conditions.

DAG levels and immunoblot analysis of major PKC isoforms in soleus muscle

In order to investigate the mechanism underlying the observed increase in PKC activity in the soleus muscle of obese Zucker rats under fed conditions, Western blotting was performed to evaluate the expression of the two major isoenzymic forms of PKC in muscle, PKC-θ and PKC-ε, both of which have been implicated in the insulin resistance of skeletal muscle (Donnelly *et al.* 1994, Schmitz-Peiffer *et al.* 1997). The results show that in obese rats there is increased expression of PKC-θ and especially PKC-ε in the membrane fraction of soleus muscle in the fed compared with the fasting state (Figs 2 and 3, and Table 3). Under fasted conditions, there were no differences in isoform expression in muscle between obese and lean animals.

To assess whether changes in DAG may be responsible for the increased activation of PKC, and the increased expression of nPKCs in the membrane fraction associated with feeding, DAG content was measured in the cytosol and membrane fractions of soleus muscle from obese and lean rats. In the fasting state, DAG levels were significantly higher in the cytosol fraction of muscle from obese rats, but absolute levels of DAG were higher in the membrane fraction, especially in the fed state (Table 4).

Discussion

These results show that there are tissue- and isoform-specific differences in PKC signalling in obese (*fa/fa*) compared with lean (*fa/-*) Zucker rats under fed and fasted conditions. Obese diabetic animals in the basal fasting state had higher PKC activity in liver but not muscle or fat. Similar 40% increases in membrane-associated PKC were reported in liver biopsy specimens from obese patients with NIDDM (Considine *et al.* 1995), and in parallel experiments in rats these authors showed that the increase in liver PKC in NIDDM is not secondary to hyperglycaemia. Given that PKC activation in hepatocytes reduces insulin-stimulated glycogen and lipid synthesis (Caro *et al.*

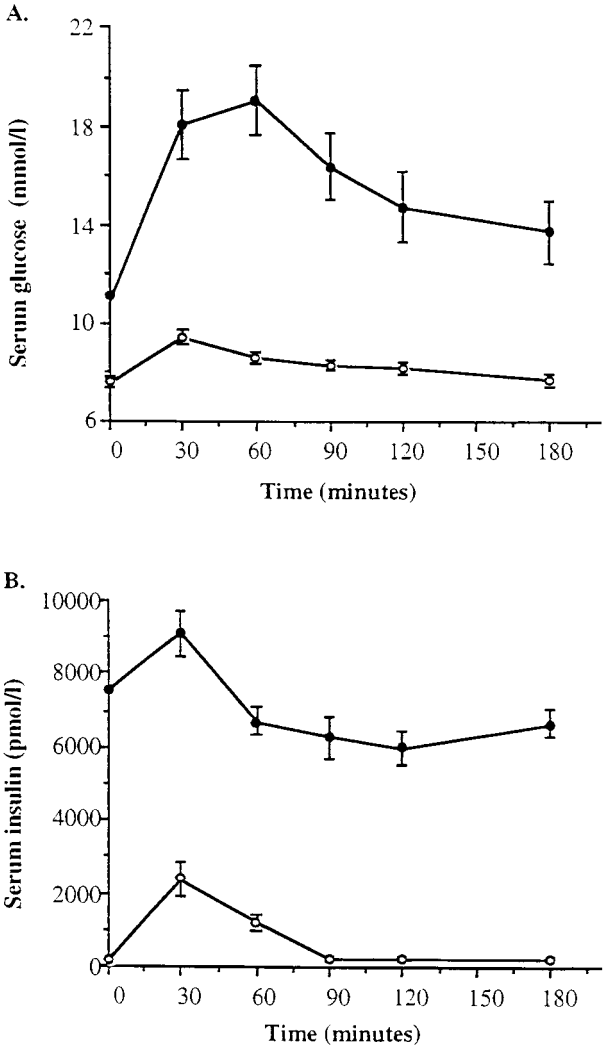

Figure 1 Serum glucose (A) and insulin (B) responses to an oral glucose load (2 g/kg body weight) by gavage in fasted obese (*fa/fa*) (●, *n*=12) and lean *fa/-* (○, *n*=12) Zucker rats. Values are means ± S.E.M.

1992), and overexpression of PKC decreases phosphatidylinositol (PI) 3-kinase activity (Chin *et al.* 1993), the results of the present study suggest that the increase in PKC activity in liver may be exacerbating the metabolic abnormalities of fasting hyperglycaemia and hypertriglyceridaemia observed in obese Zucker rats. It has been shown that activation of hepatic PI 3-kinase is markedly reduced in obese Zucker rats (Anai *et al.* 1998), via down-regulation of insulin receptor substrate (IRS)-1 and IRS-2, and this may involve PKC-mediated phosphorylation of insulin receptor substrates at the serine 612 position (De Fea & Roth 1997).

Rats feed mainly at night, and in the obese group following an overnight feed there was a nearly threefold increase in PKC activity in the membrane fraction of red muscle. In contrast, feeding had no effect on muscle PKC activity in lean animals, and there were no changes in PKC activity in liver or fat in response to food intake. The explanation for the tissue-selective increase in PKC activity after overnight feeding is not entirely clear, and of course this study did not measure PKC activity *in situ*, but there was a parallel increase in DAG levels and evidence of DAG-mediated activation of the two major isoenzymic forms of PKC in skeletal muscle, PKC-θ and PKC-ε. There was no direct evidence of translocation, since cytosolic PKC enzyme activity was unchanged. However, PKC activation in the membrane fraction can occur without translocation, and these data would also be consistent with increased protein synthesis of PKC-θ and PKC-ε in the fed state.

PKC-θ is the major isoenzyme of PKC in muscle (Osada *et al.* 1992); we have previously shown that expression of PKC-θ varies between muscles of different fibre-type composition and insulin sensitivity, and that expression of this isoform is increased in muscle tissue from rats with fructose-induced insulin resistance and dyslipidaemia (Donnelly *et al.* 1994). It has recently been shown that PKC-θ has an especially strong inhibitory effect on insulin receptor signalling which involves phosphorylation of IRS-1 (Kellerer *et al.* 1998). Increased expression of PKC-ε and PKC-θ in muscle has also been reported in the high-fat-fed rat (Schmitz-Peiffer *et al.* 1997), which is another (nonobese) dietary model of insulin resistance associated with increased lipid levels in muscle tissue which parallel the decline in whole-body insulin sensitivity (Storlien *et al.* 1991). The work by Schmitz-Pfeiffer *et al.* (1997) showed evidence of chronic activation of PKC-ε in high-fat-fed Wistar rats, whereas their data for PKC-θ suggested a combination of chronic activation and down-regulation because PKC-θ expression was reduced in the cytosol fraction. The present study reports slightly different changes in muscle PKC in response to acute feeding under conditions of a normal dietary intake, whereas Schmitz-Peiffer *et al.* (1997) were concerned with chronic feeding of an experimental high-fat diet.

The significance of increased expression of PKC-ε in obese compared with lean animals in the present study may be relevant to the role of tumour necrosis factor α (TNFα) in obesity. Secretion of TNFα from adipocytes of obese rats inhibits glucose uptake in skeletal muscle (Hotamisligil *et al.* 1993) and there is evidence that PKC-ε enhances the inhibitory effect of TNFα on insulin signalling (Kellerer *et al.* 1997). Thus, enhanced DAG-mediated activation of PKC-θ and PKC-ε in the muscles of obese Zucker rats under postprandial conditions may, via several mechanisms, down-regulate insulin signalling and glucose disposal and worsen the hyperglycaemia observed in these animals.

The notion that DAG-mediated PKC activation contributes to the pathogenesis of obesity-induced insulin

Table 2 PKC activity (pmol/min/mg protein) in cytosol and membrane (particulate) fractions of three insulin target tissues from obese *fa/fa* and lean *fa/-* Zucker rats under fed and overnight-fasted conditions. Values are means ± S.E.M.

		Fasted (n=12)		**Fed** (n=12)	
	Animal	Cytosol	Membrane	Cytosol	Membrane
Tissue					
Soleus muscle	Obese	49 ± 9	107 ± 20	46 ± 13	283 ± 42*†
	Lean	47 ± 6	154 ± 21	57 ± 16	179 ± 27
Liver	Obese	37 ± 5	174 ± 16*	47 ± 4	186 ± 22
	Lean	28 ± 3	108 ± 12	50 ± 5	127 ± 16
Epididymal fat	Obese	47 ± 11	199 ± 48	99 ± 26	255 ± 42
	Lean	42 ± 9	173 ± 45	92 ± 15	234 ± 35

*P<0·05 compared with corresponding value for lean rats; †P<0·02 vs membrane of soleus muscle from fasted rats.

resistance provides an attractive mechanism linking dysregulated lipid metabolism with resistance to insulin-mediated glucose utilisation, especially in muscle (Cortright *et al.* 1997). Accumulation of naturally occurring lipid metabolites, e.g. unesterified free fatty acids and their coenzyme A esters, activates PKC synergistically with DAG (Shimomura *et al.* 1991). The substrates phophorylated at serine and threonine residues in response to PKC activation have largely been identified *in vitro*, but there is evidence that the insulin receptor tyrosine kinase on the β-subunit is an important target (Karisik *et al.* 1990, Kellerer *et al.* 1997), as well as phosphorylation of IRS-1 (De Fea & Roth 1997) and inhibition of insulin-stimulated Akt1 and Akt3 activity (Barthel *et al.* 1998). Several previous studies have demonstrated impaired tyrosine kinase activity in the Zucker fatty rat insulin receptor (Slieker *et al.* 1990), but whether PKC activation accounts for this reduction in the tyrosine kinase activity *in vivo* requires further investigation (Bassenmaier *et al.* 1997).

Because the majority of whole-body glucose disposal in the fed state is accounted for by skeletal muscle (approximately 85%), PKC-mediated phosphorylation and down-regulation of glycogen synthase (Blackmore *et al.*

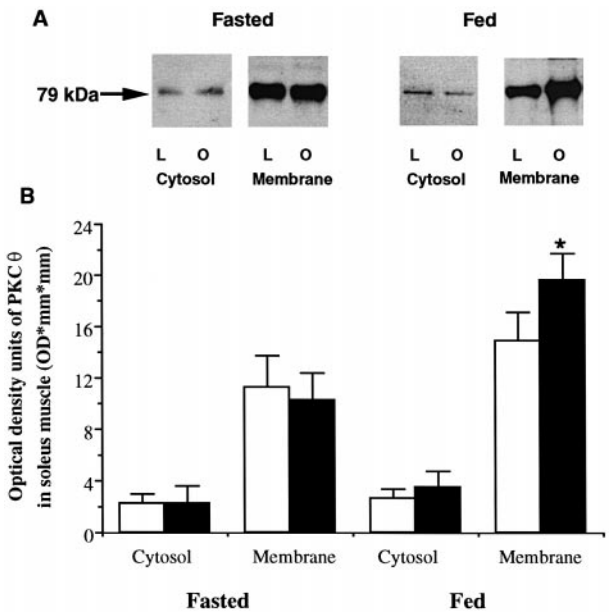

Figure 2 Western blots of PKC-θ (79 kDa) in cytosol and membrane fractions of soleus muscle from obese (O) and lean (L) Zucker rats under fed and fasting conditions. Typical blots (A) and summary of densitometric data (B): lean (open bars, n=12) and obese (solid bars, n=12) groups are shown. *, P<0·01 vs lean.

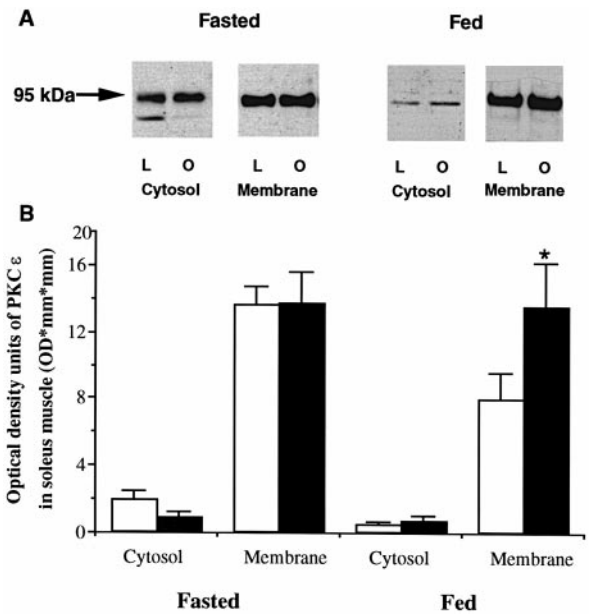

Figure 3 Western blots of PKC-ε (95 kDa) in cystosol and membrane fractions of soleus muscle from obese (O) and lean (L) Zucker rats under fed and fasting conditions. Typical blots (A) and summary of densitometric data (B): lean (open bars, n=12) and obese (solid bars, n=12) groups are shown. *, P<0·01 vs lean.

Table 3 Comparison of densitometric data for two nPKC isoforms, PKC-θ and PKC-ε, from Western blots of cytosol and membrane fractions of soleus muscle from obese *fa/fa* rats relative to lean *fa/-* controls. Data are means ± S.E.M. The ratio is calculated using the lean sample as control for each obese sample. The samples were analysed in parallel and subjected to SDS-PAGE on the same gel

		Fasted ($n=12$)	**Fed** ($n=12$)
	Fraction	Obese/lean ratio	Obese/lean ratio
Isoform			
PKC-θ	Cytosol	1·01 ± 0·18	1·55 ± 0·39
	Membrane	1·03 ± 0·18	1·45 ± 0·17*
PKC-ε	Cytosol	0·89 ± 0·57	1·54 ± 0·61*
	Membrane	1·02 ± 0·10	2·64 ± 0·69**

*$P<0·05$, **$P<0·01$ compared with corresponding data in the fasted state.

Table 4 DAG levels (nmol/mg protein) in cytosol and membrane fractions of soleus muscle tissue from obese *fa/fa* and lean *fa/-* Zucker rats under fed and fasted conditions. Values are means ± S.E.M.

	Fasted rats ($n=12$)		**Fed rats** ($n=12$)	
	Cytosol	Membrane	Cytosol	Membrane
Obese *fa/fa*	2·30 ± 0·20*	10·54 ± 1·55	1·98 ± 0·07	12·55 ± 1·33*
Lean *fa/-*	1·42 ± 0·12	9·07 ± 0·48	1·95 ± 0·23	9·06 ± 0·60

*$P<0·05$ compared with corresponding data from lean control.

1986) may also be an important consequence of the PKC activation observed in muscles of obese rats under fed conditions. In addition, local formation of nitric oxide (NO) stimulates glucose uptake in isolated rat skeletal muscles and there is evidence that NO-mediated glucose oxidation is reduced in muscles from obese Zucker rats (Young & Leighton 1998). Given that PKC activation regulates the activity of nitric oxide synthase (Huang & Yuan 1997), it is also possible that local differences in NO formation contribute to PKC-mediated insulin resistance in skeletal muscle.

In conclusion, the obese Zucker rat is associated with tissue and isoform-specific upregulation of DAG-PKC signalling, which is related to food intake, and there is increasing evidence that these biochemical changes contribute to the insulin resistance and metabolic abnormalities associated with obesity and diabetes.

Acknowledgements

This work was supported by a project grant from the National Health & Medical Research Council of Australia.

References

Anai K, Funaki M, Ogihara T, Terasaki J, Inukai K, Ketagiri H, Fukushima Y, Yazaki Y, Kikuchi M, Oka Y & Asano T 1998 Altered expression levels and impaired steps in the pathway to phosphoinositol 3-kinase activation via insulin receptor substrates 1 and 2 in Zucker fatty rats. *Diabetes* **47** 13–23.

Avignon A, Yamada K, Zhou X, Spencer B, Cardona O, Saba-Siddique S, Galloway L, Standaer TML, & Farese RV 1996 Chronic activation of protein kinase C in soleus muscles and other tissues of insulin-resistant Type II diabetic Goto-Kakizaki (GK), obese/aged and obese/zucker rats. *Diabetes* **45** 1396–1404.

Azhar S 1991 Ca^{2+}-activated and phospholipid-dependent hepatic protein kinase (protein kinase C): a standardised method for the determination of enzyme activity. *Journal of Nutritional Biochemistry* **2** 165–171.

Barthel A, Nakatani K, Dandekar AA & Roth RA 1998 Protein kinase C modulates the insulin-stimulated increase in Akt1 and Akt3 activity in 3T3-L1 adipocytes. *Biochemical and Biophysical Research Communications* **243** 509–513.

Bassenmaier B, Mosthaf L, Mischak H, Ullrich A & Haring HU 1997 Protein kinase C isoforms beta-1 and beta-2 inhibit the tyrosine kinase activity of the insulin receptor. *Diabetologia* **40** 863–866.

Berti L, Mosthaf L, Kroder G, Kellerer M, Tippmer S, Mushack J, Seffer E, Seedorf K & Haring HU 1994 Glucose-induced translocation of protein kinase C isoforms in rat-1 fibroblasts is paralleled by inhibition of the insulin receptor tyrosine kinase. *Journal of Biological Chemistry* **269** 3381–3386.

Blackmore PF, Strickland WG, Bocckino SB & Exton JH 1986 Phosphorylation of glycogen synthase by protein kinase C. *Biochemical Journal* **237** 235–242.

Bligh EG & Dyer WJ 1959 A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37** 911–917.

Boneh A 1995 Possible role of protein kinase C in the pathogenesis of inborn errors of metabolism. *Journal of Cellular Biochemistry* **59** 27–32.

Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry* **72** 248–254.

Busch AK, Castan I, Degerman E, Tornqvist H & Mosthaf L 1998 Protein kinase C mediates hyperglycaemia-induced insulin resistance through serine phosphorylation of IRS-1. *Diabetologia* **41** (Suppl 1) A10.

Caro JF, Jenquin M & Long S 1992 Effects of phorbol esters on insulin receptor function and insulin action in hepatocytes: evidence for heterogeneity. *Molecular and Cellular Biochemistry* **109** 115–118.

Chin JE, Dickens M, Tavare JM & Roth RA 1993 Overexpression of protein kinase C isozymes α, βI, γ, and ε in cells overexpressing the insulin receptor. *Journal of Biological Chemistry* **268** 6338–6347.

Chua SC, Chung WK, Wu-Peng XS, Zhang Y, Liu S-M, Tartaglia L & Leibel RL 1996 Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. *Diabetes* **45** 1141–1143.

Considine RV & Caro JF 1993 Protein kinase C: mediator or inhibitor of insulin action? *Journal of Cellular Biochemistry* **52** 8–13.

Considine RV, Nyce MR, Allen LE, Morales LM, Triester S, Serrano J, Colberg J, Lanza-Jacoby S & Caro JF 1995 Protein kinase C is increased in the liver of humans and rats with non-insulin-dependent diabetes mellitus: an alteration not due to hyperglycaemia. *Journal of Clinical Investigation* **95** 2938–2944.

Cortright RN, Muoio DM & Dohm GL 1997 Skeletal muscle lipid metabolism: a frontier for new insights into fuel homoestasis. *Journal of Nutritional Biochemistry* **8** 228–245.

De Fea K & Roth RA 1997 Protein kinase C modulation of insulin receptor substrate-1 tyrosine phosphorylation requires serine 6_{1Z}. *Biochemistry* **36** 12939–12947.

Donnelly R, Reed M, Azhar S & Reaven GM 1994 Expression of the major isoenzyme of protein kinase C in skeletal muscle, nPKC-θ, varies with fibre-type composition and in response to fructose-induced insulin resistance. *Endocrinology* **135** 2369–2374.

Ferrannini E, Natali A, Bell P, Carallo-Perin P, Lalic N & Mingrone G 1997 Insulin resistance and hypersecretion in obesity. *Journal of Clinical Investigation* **100** 1166–1173.

Heydrick SJ, Ruderman NB, Kurowski TG, Adams HB & Chen KS 1991 Enhanced stimulation of diacylglycerol and lipid synthesis by insulin in denervated muscle. Altered PKC activity and possible link to insulin resistance. *Diabetes* **40** 1707–1711.

Hotamisligil GS, Shargill NS & Spiegelman BM 1993 Adipose expression of tumour necrosis factor-α: direct role in obesity-linked insulin resistance. *Science* **259** 87–91.

Huang Q & Yuan Y 1997 Interaction of PKC and NOS in signal transduction of microvascular permeability. *American Journal of Physiology* **273** H2442–H2451.

Hug H & Sarre TF 1993 Protein kinase C isoenzymes: divergence in signal transduction. *Biochemical Journal* **291** 329–343.

Ishizuka T, Yamamoto M, Kajita K, Yasuda K, Miura K, Hernandez H & Farese RV 1993 Differential effect of aging on protein kinase C activity in rat adipocytes and soleus muscle. *Metabolism* **42** 420–425.

Karisik A, Rothenberg PL, Yamada K, White MF & Kahn CR 1990 Increased protein kinase C activity is linked to reduced insulin receptor autophosphorylation in liver of starved rats. *Journal of Biological Chemistry* **265** 10226–10231.

Kellerer M, Mushack J, Mischak H & Haring HU 1997 Protein kinase C (PKC) epsilon enhances the inhibitory effect of TNFα on insulin signalling in HEK293 cells. *FEBS Letters* **418** 119–122.

Kellerer M, Mushack J, Seffer E, Mischak H, Ullrich A & Haring HU 1998 Protein kinase C isoforms α, δ and θ require insulin receptor substrate-1 to inhibit the tyrosine kinase activity of the insulin receptor in human kidney embryonic cells (HEK 293 cells). *Diabetologia* **41** 833–838.

King PA, Horton ED, Hirshman MF & Horton ES 1992 Insulin resistance in obese Zucker rat (*fa/fa*) skeletal muscle is associated with a failure of glucose transporter translocation. *Journal of Clinical Investigation* **90** 1568–1575.

Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680–685.

Mosthaf L, Busch AK, Juhl LF, Nielsen L, Olsen GS, Ikeda Y & Seedorf K 1998 The insulin receptor is degraded by specific protein kinase C isoforms. *Diabetologia* **41** (Suppl 1) A10.

Moule SK & Denton RM 1997 Multiple signaling pathways involved in the metabolic effects of insulin. *American Journal of Cardiology* **80** 41A–49A.

Osada S-I, Mizuno K, Saido TC, Suzuki K, Kuroki T & Ohno S 1992 A new member of the protein kinase C family, nPKC-θ, predominantly expressed in skeletal muscle. *Molecular and Cellular Biology* **12** 3930–3938.

Pan DA, Lillioja S, Kriketos AD, Milner MR, Bauer LA, Bogardus C, Jenkins AB & Storlien LH 1997 Skeletal muscle triglyceride levels are inversely related to insulin action. *Diabetes* **46** 983–988.

Preiss JE, Loomis CR, Bell RM & Niedel JE 1987 Quantitative measurement of sη-1, 2-diacylglycerols. In *Methods in Enzymology, Part B Calcium and Lipids*, pp 294–300. Eds PM Conn & AR Means. Orlando, Florida: Academic Press Inc.

Schmitz-Peiffer C, Browne CL, Oakes ND, Watkinson A, Chisholm DJ, Kraegen EW & Biden TJ 1997 Alterations in the expression and cellular localization of protein kinase C isozymes epsilon and theta are associated with insulin resistance in skeletal muscle of the high-fat-fed rat. *Diabetes* **46** 169–178.

Shimomura T, Asoka Y, Oka M, Yoshida K & Nishizuka Y 1991 Synergistic action of diacylglycerol and unsaturated fatty acid for PKC activation: its possible implication. *Proceedings of the National Academy of Sciences of the USA* **88** 5149–5153.

Shmueli E, Alberti KGEM & Record CO 1993 Diacylglycerol/protein kinase C signalling: a mechanism for insulin resistance? *Journal of Internal Medicine* **234** 397–400.

Slieker LJ, Roberts EF, Shaw WN & Johnson WT 1990 Effect of streptozotocin-induced diabetes on insulin receptor tyrosine kinase activity in obese Zucker rats. *Diabetes* **39** 619–625.

Storlien LH, Jenkins AB, Chisholm DJ, Pascoe WS, Khouri S & Kraegen EW 1991 Influence of dietary fat composition on development of insulin resistance in rats. Relationship to muscle triglyceride and W-3 fatty acids in muscle phospholipids. *Diabetes* **40** 280–289.

Young ME & Leighton B 1998 Evidence for altered sensitivity of the nitric oxide/cGMP signalling cascade in insulin-resistant skeletal muscle. *Biochemical Journal* **329** 73–79.

Received 2 December 1998
Revised manuscript received 10 February 1999
Accepted 1 March 1999