Tissue- and isoform-specific effects of aging in rats on protein kinase C in insulin-sensitive tissues

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ABSTRACT

The mechanisms responsible for the age-related decline in insulin sensitivity have not been clearly identified, but activation of the diacylglycerol/protein kinase C (PKC) signalling pathway (often confined to individual isoforms of PKC) has recently been implicated in the pathogenesis of other insulin-resistant states in both humans and animal models. Fasting serum glucose, insulin and triacylglycerol (triglyceride) concentrations, and results of oral glucose tolerance tests, were compared in groups of 6-week-old (n = 8) and 6-month-old (n = 8) Sprague–Dawley rats. Insulin-responsive tissues (liver, soleus muscle and epididymal fat pad) were collected to compare levels of diacylglycerol, PKC enzyme activity and protein expression of individual PKC isoforms in cytosol and membrane fractions. The older group were heavier (556 ± 14 g, compared with 188 ± 7 g) and relatively insulin-resistant and hyperinsulinaemic (477 ± 73 pM compared with 293 ± 51 pM; P < 0.05) compared with young rats; they also had greater areas under the serum glucose (old, 20.3 ± 1.1; young, 17.3 ± 0.7 mmol [l]−1 [h]−1) and insulin (old, 1254 ± 76; young, 721 ± 113 mmol [l]−1 [h]−1) profiles following an oral glucose tolerance test, and significantly higher fasting triacylglycerol levels (old, 1.24 ± 0.06 mM; young, 0.92 ± 0.07 mM; P < 0.01). There were no age-related differences in diacylglycerol levels or PKC activity in muscle and liver, but membrane-associated PKC activity was 2.5-fold higher in the adipose tissue of older rats (101 ± 19 compared with 40 ± 5 pmol min−1 mg−1 protein; P < 0.05) due to increased translocation of PKC-βI, -βII and -ε. Thus insulin resistance due to normal aging is associated with tissue- and isoform-specific changes in diacylglycerol/PKC signalling. In contrast with diabetes and dietary-induced insulin resistance, there were no changes in diacylglycerol/PKC signalling in skeletal muscle and liver, but isoform-specific translocation and higher PKC activity in adipose tissue may blunt the insulin-mediated inhibition of lipolysis and contribute to the increased triacylglycerol levels observed in older animals.

INTRODUCTION

Advancing age is associated with a progressive decline in the rate of insulin-stimulated glucose disposal [1,2], and clinical disorders characterized by insulin resistance (e.g. type II diabetes, hypertension and coronary heart disease) are much more common in the elderly. The biochemical mechanisms responsible for the age-related reduction in insulin sensitivity have not been clearly established, but most of the available evidence points to defects down-
stream of the insulin receptor. For example, Reed et al. [3] showed that a 3-fold decrease in hind-limb glucose disposal in 6-month-old rats compared with that in young controls was independent of any differences in insulin receptor number, autophosphorylation or tyrosine kinase activity.

Activation of the serine/threonine-specific kinase, protein kinase C (PKC), has been implicated as an important post-receptor mechanism of insulin resistance [4,5]. There is evidence that PKC-mediated phosphorylation down-regulates several components in the insulin signalling cascade, e.g. insulin receptor substrate-1 (IRS-1) [6], Akt1 and Akt3 [7], and the rate-limiting enzyme glycogen synthase [8]. PKC activation may also increase insulin receptor degradation [9], and overexpression of PKC in transfected liver cells reduces insulin-stimulated phosphatidylinositol 3-kinase activity [10].

Activation and translocation of PKC from the cytosol to the plasma membrane occurs in response to a transient increase in diacylglycerol (DAG), a product of phosphatidylinositol turnover. It has been shown that, in fact, PKC is a family of multiple isoenzymes with different substrates and cofactor requirements, and different patterns of tissue distribution [11]. The various isoforms of PKC have been classified into three groups: (1) group A, comprising the conventional calcium-dependent isoforms PKC-α, -β1, -β2 and -γ; (2) group B, the novel, calcium-independent isoforms, PKC-δ, -ζ, -η and -θ; and (3) group C, the atypical isoforms, PKC-μ, -ε/λ and -ζ. We have shown previously that expression of the skeletal-muscle-specific isoform, PKC-θ, varies between normal muscles of different fibre-type composition and insulin sensitivity, with higher amounts of PKC-θ found in white insulin-resistant muscles [12]. There is also evidence that the expression of PKC-θ and PKC-α is increased in muscle tissue from rodent models of dietary-induced insulin resistance [12,13], and that tissue-specific increases in PKC activity and expression of novel PKC isoforms are accentuated and influenced by food intake in an experimental model of obesity and diabetes [14]. In addition to affecting glucose transport and metabolism, PKC-dependent pathways may also play a role in regulating lipolysis [15].

The purpose of the present study was to characterize DAG/PKC signalling, and PKC isoform expression, in insulin-responsive tissues of old (6-month-old) and young (6-week-old) Sprague–Dawley rats, and to relate these changes to the adaptations in glucose, insulin and lipid metabolism associated with normal aging.

**MATERIALS AND METHODS**

**Animals**

Normal male Sprague–Dawley rats (n = 16) were obtained at 5 weeks of age and housed under controlled conditions of temperature (21 °C) and lighting (12 h light/dark cycle; lights on 06.00 hours) with free access to water and standard laboratory chow. ‘Young’ rats (n = 8) were studied at 6 weeks of age, and ‘old’ rats (n = 8) were studied at 24 weeks of age. The experimental protocol was approved by the University of Sydney Animal Care and Ethics Committee.

**Metabolic measurements**

Blood samples were drawn from the tail veins of unanaesthetized rats for measurements of fasting serum glucose, insulin and triacylglycerol (triglyceride) concentrations; 2 days later, following a 5 h fast, an oral glucose tolerance test (OGTT) was performed. Tail vein blood samples were collected at baseline and at 30, 60, 90, 120 and 180 min after an oral glucose load (3 g/kg) administered by gavage.

**Collection of tissue samples**

At 2 days after the OGTT, the rats were exsanguinated by decapitation prior to collection of liver, soleus muscle and epididymal fat pad tissues. Tissue samples were quickly excised, snap-frozen in liquid N₂ and stored at −70 °C for subsequent biochemical assays of PKC activity and DAG levels, and Western blotting of cytosol and membrane (particulate) fractions. Tissue samples were homogenized in a Polytron instrument (Brinkman Instruments, Westbury, NY, U.S.A.) in 5 ml of buffer A (containing 20 mM Tris/HCl, pH 7.4, 1 mM EDTA, 0.25 mM EGTA, 2 μM leupeptin, 4 μg/ml pepstatin, 4 μg/ml each of calpain I and II inhibitors, 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 supernatants for liver and muscle, and the infranatant below the fat-cake for adipose tissue, were 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 of buffer A, then mixed with 0.5 ml of buffer B (buffer A without sucrose) containing 2% Triton X-100 and solubilized for 30 s in a sonicator at 4°C. An aliquot of each fraction was saved for protein quantification, and cytosol and particulate samples were then used for biochemical assays of PKC activity and DAG content, and for Western blotting.

**PKC activity**

PKC activity is virtually undetectable in crude subcellular fractions, but is expressed following DEAE-cellulose chromatography to remove inhibitors, phosphates and other protein kinases. Thus PKC activity was measured in partially purified preparations of cytosolic and particulate (membrane) fractions, as described previously [12,16]. The supernatant and particulate fractions were...
applied to DEAE-Sepharcl columns (Pharmacia). After washing with 5 ml of 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 step by measuring the incorporation of $^{32}$P from $[\gamma^{32}\text{P}]{\text{ATP}}$ into GS peptide (HOOC-Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys-NH$_2$). The standard incubation mixture consisted of the following components in a final volume of 100 $\mu$l: 20 $\mu$l aliquots of chromatography fractions, 25 mM Pipes (pH 6.8), 10 mM magnesium acetate, 20 $\mu$M GS peptide, 5 mM 2-mercaptoethanol, 0.1 mM $[\gamma^{32}\text{P}]{\text{ATP}}$ (200–300 c.p.m./pmol), 1.0 mM CaCl$_2$, 150 $\mu$g/ml phosphatidyserine and 10 $\mu$g/ml diolein. Phosphatidyserine 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$^{2+}$, phosphatidyserine and diolein). The reaction was initiated by the addition of $[\gamma^{32}\text{P}]{\text{ATP}}$ at 30 °C. After incubation for 15 min, the reaction was terminated by spotting 50 $\mu$l of the mixture on to 2 cm x 2 cm phosphocellulose strips (Whatman P81), which were dropped immediately into the mixture on to 2 cm phosphocellulose strips (Whatman P81), which were dropped immediately into 75 mM phosphoric acid and washed and dried before counting of radioactivity after addition 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 phosphorylserine, diolein and calcium. One unit of PKC activity is defined as that amount catalysing the transfer of 1 pmol $[\gamma^{32}\text{P}]{\text{phosphate}}$ from $[\gamma^{32}\text{P}]{\text{ATP}}$ to GS peptide per min at 30 °C.

**Western blotting**

Aliquots of the cytosol and particulate fractions were mixed with equal volumes of 2 x sample-loading buffer [4.6% (w/v) SDS, 10% (v/v) 2-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. The mixture was then centrifuged at 5000 g for 5 min, and the supernatant was subjected to SDS/PAGE. For each sample, 25 $\mu$g of total protein was loaded on to the gel, and one set of protein standards was also loaded on to each gel. Following electrophoretic separation, proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.) in transfer buffer containing 10 mM Caps and 10% (v/v) methanol, pH 11. At the conclusion of the transfer process, the membrane was then incubated for 12 h at 4 °C in PBS/Tween (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 x 10 min) in rinsing solution (PBS/Tween plus 1% dried milk, pH 7.4) and then incubated overnight with affinity-purified polyclonal antibodies specific for individual isoforms of PKC. After further washings, membranes were incubated with the horseradish-peroxidase-conjugated IgG fraction of goat anti-(rabbit IgG), diluted 1:20000 in PBS/Tween. The membranes were then washed several times in PBS/Tween, incubated with alkaline phosphatase/streptavidin-conjugated anti-(rabbit IgG) and developed for 60 s using a commercial enhanced chemiluminescence kit (Zymed Laboratories, San Francisco, CA, U.S.A.). 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 DAG**

DAG was extracted by the method of Bligh and Dyer [17] and measured using a slightly modified version of the DAG kinase procedure of Preiss et al. [18]. Dioleoylglycerol was used for the standard curve. Briefly, after extraction, the dried lipid extracts were solubilized by sonication in 20 $\mu$l of a solution containing octyl $\beta$-glucoside (7.5%, v/v) and cardiolipin (5 mM) in 1 mM diethylenetriaminepenta-acetic acid (DETPAC). After a 15 min incubation, 50 $\mu$l of 2 x reaction buffer (100 mM imidazole/HCl, pH 6.6, 100 mM NaCl, 25 mM MgCl$_2$, 2 mM EGTA) and 10 $\mu$l of 20 mM dithiothreitol were mixed with the solubilized lipid/octyl glucoside solution and purified DAG kinase to give a final volume of 90 $\mu$l. The reaction was started by the addition of 10 $\mu$l of $[\gamma^{32}\text{P}]{\text{ATP}}$ (specific radioactivity 5 x 10$^5$ c.p.m./nmol) in 10 mM imidazole/1 mM DETPAC, 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% (v/v) HClO$_4$. After addition of 1 ml of chloroform and 1 ml of 1% (v/v) HClO$_4$, and brief centrifugation, the lower chloroform phase was washed and then dried under N$_2$. The lipid film was dissolved in 5% (v/v) methanol in chloroform, and 50 $\mu$l was spotted on to a 20 cm Silica Gel 60 TLC plate activated by pre-running in acetone. Samples of $[\gamma^{32}\text{P}]{\text{phosphatidic acid}}$ prepared from dioleoylglycerol were also spotted on to the TLC plates as standards. The plates were then developed with chloroform/methanol/ acetic acid (65:15:5, by vol), air-dried and subjected to autoradiography. The radioactive spot corresponding to phosphatidic acid (R$_f$ 0.35) was scraped into a scintillation vial and mixed with 6 ml of scintillation fluid, and the radioactivity was counted.

**Laboratory assays**

Serum glucose and triacylglycerol levels were measured using enzymic colorimetric methods (Trinder Glucose kit and GPO-Trinder Tryglyceride kit respectively; Sigma). Serum insulin concentrations were measured by
double-antibody radioimmunoassay using rat insulin standards and an anti-(rat insulin) primary antibody.

**Statistical analysis**
Serum glucose and insulin concentration–time profiles for the OGTTs were compared by ANOVA. Measurements of fasting serum glucose, insulin and triacylglycerol concentrations were analysed by one-factor ANOVA. All measurements are expressed as means ± S.E.M., and statistical significance was accepted at the 5% level.

**RESULTS**

**Metabolic characteristics of young and old rats**
Rats at 6 months of age had significantly higher fasting serum triacylglycerol and insulin levels compared with young animals (6 weeks of age) (Table 1). Although fasting and peak serum glucose levels after an OGTT were similar in the two groups, the post-absorptive phases of the OGTT were significantly different, with higher serum glucose levels in older rats at 60–150 min after the oral glucose load (Figure 1). Thus the areas under the serum glucose and insulin concentration–time curves were significantly greater for the older rats (Table 1).

**PKC activity and DAG levels in cytosolic and particulate fractions**
There were no significant differences in PKC enzyme activity or subcellular distribution in muscle and liver from the two groups of rats, but in adipose tissue membrane-associated PKC activity was significantly higher in older animals (Table 2). There were no corresponding differences in DAG levels in either the cytosolic or the particulate fraction, so the PKC activation observed in the adipose tissue of older rats could not be explained simply on the basis of increased membrane-associated DAG (Table 2).

There was no difference in PKC activity in muscle tissue between young and old animals; in particular, further analysis of individual isoforms by immunoblotting showed that expression of PKC-θ and PKC-ε in soleus muscle was similar in the two groups (Figure 2).

**Subcellular distribution of individual PKC isoforms in adipose tissue**
In order to evaluate further the mechanism accounting for the increased PKC activity in the adipose tissue of 6-month-old rats, Western blotting was performed to characterize the PKC isoforms expressed in adipose tissue and their subcellular distribution between the cytosolic and particulate fractions. The isoenzymes identified in adipose tissue were PKC-α, -βI, and -βII of the calcium-dependent (conventional) group, PKC-ε of the novel group, and PKC-ζ of the atypical group. There was evidence of increased translocation of PKC-βI, PKC-βII, and PKC-ε in the older animals, as shown by
Protein kinase C in young and old Sprague–Dawley rats

Table 2  PKC activity and DAG content in insulin-sensitive tissues (cytosol and membrane fractions) from young and old Sprague–Dawley rats
Young and old rats were aged 6 weeks and 6 months respectively. Significance of difference compared with young rats: "P < 0.05.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Young (n = 8)</th>
<th>Old (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytosol</td>
<td>Membrane</td>
</tr>
<tr>
<td>PKC activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soleus muscle</td>
<td>30 ± 7</td>
<td>85 ± 22</td>
</tr>
<tr>
<td>Liver</td>
<td>101 ± 25</td>
<td>193 ± 30</td>
</tr>
<tr>
<td>Epididymal fat</td>
<td>73 ± 15</td>
<td>40 ± 5</td>
</tr>
<tr>
<td>DAG content</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soleus muscle</td>
<td>3.2 ± 0.3</td>
<td>7.0 ± 0.3</td>
</tr>
<tr>
<td>Liver</td>
<td>26.0 ± 3.5</td>
<td>65.8 ± 4.5</td>
</tr>
<tr>
<td>Epididymal fat</td>
<td>9.3 ± 1.6</td>
<td>33.7 ± 2.3</td>
</tr>
</tbody>
</table>

Figure 2  Immunoblot analysis of PKC-θ and PKC-ε expression in red soleus muscle from young (Y; □) and old (O; ■) Sprague–Dawley rats
Expression is given in arbitrary densitometric units.

Table 3  Immunoblot analysis to evaluate the expression and subcellular distribution of individual PKC isoforms in adipose tissue from young and old rats
Young and old rats were aged 6 weeks and 6 months respectively. Other PKC isoforms were not detected in adipose tissue. Significance of differences compared with young rats: "P < 0.05; "*P < 0.01.

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Molecular mass (kDa)</th>
<th>Cytosol/particulate ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKC-α</td>
<td>82</td>
<td>0.36 ± 0.08</td>
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<tr>
<td>PKC-β1</td>
<td>78</td>
<td>1.29 ± 0.45</td>
</tr>
<tr>
<td>PKC-β2</td>
<td>80</td>
<td>0.62 ± 0.14</td>
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<tr>
<td>PKC-ε</td>
<td>95</td>
<td>0.64 ± 0.21</td>
</tr>
<tr>
<td>PKC-ζ</td>
<td>68</td>
<td>1.12 ± 0.18</td>
</tr>
</tbody>
</table>

DISCUSSION

The results of the present study have confirmed that normal non-diabetic Sprague–Dawley rats at 6 months of age are relatively insulin-resistant compared with animals at 6 weeks of age, as demonstrated by higher fasting serum insulin and triacylglycerol levels in the older group and a higher area-under-the-curve for the serum insulin and glucose concentration–time profiles following an oral glucose challenge. Several previous studies in both humans and animals have shown a marked decrease in the rate of whole-body insulin-stimulated glucose disposal with increasing age [1,19], and furthermore that the insulin resistance affects skeletal muscle [20], adipose tissue [21] and the liver [22]. The underlying biochemical mechanisms, however, remain unclear [2]. A number of studies have shown that PKC may be an important post-receptor mechanism responsible for phosphorylation and down-regulation of insulin-mediated pathways. For example, there is evidence of...
PKC activation in the insulin resistance due to obesity [14], type II diabetes [23] and muscle denervation [24], and individual isoforms of PKC have been shown to phosphorylate key substrates involved in insulin signalling, e.g. IRS-1 [6], Akt1 and Akt3 [7], as well as the insulin receptor itself [9,25] and glycogen synthase [8]. Since the majority of glucose uptake occurs in skeletal muscle, and hind-limb glucose disposal is reduced with aging [3], the results of the present study are significant in showing that, in contrast with dietary-induced models of insulin resistance [12,13], there were no differences between young and old animals in PKC activity or PKC subcellular distribution in skeletal muscle. In particular, expression of the two novel PKC isoforms that have been strongly implicated in insulin signal transduction, i.e. PKC-θ and PKC-ε [9,12–14], showed no differences between the two groups. PKC-θ is the major isoenzymic form of PKC in skeletal muscle [26] and there is some evidence of developmental regulation, but increases in PKC-θ expression seem to be confined to the first 3 weeks of life [27].

There were, however, significant differences between the two groups in PKC isoform distribution in adipose tissue, and significantly higher PKC activity in the two groups in PKC isoform distribution in adipose tissue [25]. These isoform-specific differences could not be explained simply on the basis of increased phosphatidylinositol turnover, as indicated by similar DAG levels in the two groups, but the individual isoforms involved have been directly linked with mechanisms of insulin resistance. For example, the β isoenzymes of PKC directly regulate the insulin receptor and inhibit its tyrosine kinase activity [25]. Secondly, PKC-ε (in contrast with PKC-ζ) enhances the inhibitory effect of tumour necrosis factor-α (TNF-α) on insulin receptor autophosphorylation [28], and recent work has shown that higher plasma concentrations of TNF-α in the elderly are a major determinant of the age-related decline in insulin sensitivity [29]. TNF-α induces the translocation and activation of PKC [30], which, in turn, via serine phosphorylation of IRS-1 [31], represents a major pathway of TNF-α-induced insulin resistance.

Although DAG levels in adipose tissue were similar in the two groups, it is likely that age-related differences in tissue fatty acid composition account for the increased translocation of specific PKC isoforms. For example, it has been shown that non-esterified fatty acids (e.g. arachidonic acid, oleic acid and linoleic acid) and their CoA esters activate PKC synergistically with DAG [32]. Although changes in PKC can certainly affect glucose transport in adipocytes [33], increased PKC activity in fat tissue is likely to be more important with respect to the metabolism of non-esterified fatty acids. PKC activation attenuates the inhibitory effect of adenosine on lipolysis [15] via phosphorylation of an inhibitory G-protein in the adipocyte plasma membrane [34]. Thus, in the present study, the biological significance of increased PKC activity in the membrane fraction of adipose tissue may be attenuation of the insulin-mediated inhibition of lipolysis, thereby increasing levels of non-esterified fatty acids and contributing to the higher plasma triacylglycerol concentrations found in older animals.

In conclusion, the insulin resistance associated with normal aging occurs independently of any major changes in DAG/PKC signalling in muscle and liver, but isoform-specific changes in PKC activity and subcellular distribution involving adipose tissue may account for the higher serum levels of triacylglycerols observed in older animals.

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REFERENCES


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