

Early growth restriction leads to down regulation of protein kinase C zeta and insulin resistance in skeletal muscle

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Abstract

Epidemiological studies have revealed a relationship between early growth restriction and the subsequent development of type 2 diabetes. A rat model of maternal protein restriction has been used to investigate the mechanistic basis of this relationship. This model causes insulin resistance and diabetes in adult male offspring. The aim of the present study was to determine the effect of early growth restriction on muscle insulin action in late adult life. Rats were fed either a 20% or an isocaloric 8% protein diet during pregnancy and lactation. Offspring were weaned onto a 20% protein diet and studied at 15 months of age. Soleus muscle from growth restricted offspring (LP) (of dams fed 8% protein diet) had similar basal glucose uptakes compared with the control group (mothers fed 20% protein diet). Insulin stimulated glucose uptake into

control muscle but had no effect on LP muscle. This impaired insulin action was not related to changes in expression of either the insulin receptor or glucose transporter 4 (GLUT 4). However, LP muscle expressed significantly less ($P < 0.001$) of the zeta isoform of protein kinase C (PKC ζ) compared with controls. This PKC isoform has been shown to be positively involved in GLUT 4-mediated glucose transport. Expression levels of other isoforms (βI , βII , ϵ , θ) of PKC were similar in both groups. These results suggest that maternal protein restriction leads to muscle insulin resistance. Reduced expression of PKC ζ may contribute to the mechanistic basis of this resistance.

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Introduction

It is widely accepted that there is a relationship between early growth restriction and the subsequent development of type 2 diabetes (reviewed in Hales & Barker 2001). The mechanistic basis of this relationship is not known and the relative importance of genetic and environmental factors remains the subject of much current debate. Wide genome large scans have failed to identify common diabetes susceptibility genes. However, rare genetic mutations have been identified in the glucokinase gene which are associated with both early growth restriction and maturity onset diabetes of the young (Hattersley *et al.* 1998). In favour of an important environmental role, maternal malnutrition during the Dutch Hunger Winter has been shown to be associated with both low birth weight and impaired glucose tolerance in adulthood (Ravelli *et al.* 1998). Studies in Denmark (Poulsen *et al.* 1997) and Italy (Bo

et al. 2000) have also shown that in twins (both monozygotic and dizygotic) who are discordant for diabetes, the diabetic twin had the lower birth weight.

The maternal protein restriction rat model has been used to investigate the mechanistic basis of the relationship between low birth weight and type 2 diabetes. The offspring of rat dams fed a low (8%) protein diet are growth restricted compared with control offspring (of dams fed a 20% protein diet). The early growth-restricted offspring undergo an age-dependent loss of glucose tolerance and have frank diabetes in late adult life (Petry *et al.* 2001). The molecular mechanisms underlying this age-dependent loss of glucose tolerance are not known. However, hyperinsulinaemia suggests that it is related to the development of insulin resistance.

Muscle is considered to be quantitatively the major site of glucose disposal postprandially (DeFronzo *et al.* 1992). The transport of glucose across plasma membranes has

been shown to be the rate-controlling step in skeletal muscle glucose metabolism in both normal subjects and those with type 2 diabetes (Cline *et al.* 1999). These studies have also revealed that impairment of insulin-stimulated glucose transport, not impairment of phosphorylation is responsible for the resistance to insulin-stimulated glycogen synthesis observed in muscle of type 2 diabetics (Cline *et al.* 1999). An understanding of the molecular defects which lead to this impaired glucose transport is therefore important. Muscle insulin resistance has also been shown *in vitro*, as determined by the poor ability of the hormone to stimulate glucose uptake in muscle strips isolated from individuals with type 2 diabetes (Krook *et al.* 2000).

The serine/threonine kinase family of protein kinase C (PKC) plays a key role in regulating a wide range of cellular processes in response to various agonists, including hormones, neurotransmitters and growth factors (Nishizuka 1992, Hug & Sarre 1993). To date, 11 mammalian PKC isoforms have been identified, which according to structure and co-factor requirement are grouped into the classical (α , β I, β II, γ), novel (δ , ϵ , θ , η) and atypical (ζ , λ / ι) PKC isoforms (Newton 1995, Mellor & Parker 1998). PKC's role in insulin action is controversial. Both a direct involvement in insulin-mediated metabolic processes as well as a possible role in the attenuation of the insulin signal and the pathogenesis of insulin resistance in type 2 diabetes have been reported (Considine & Caro 1993). Classical PKC isoforms have been implicated in the degradation of the insulin receptor and inhibition of insulin receptor kinase activity through serine/threonine phosphorylation of the β -subunit of the insulin receptor or insulin receptor substrate-1 and in the development of diabetic late complications (Bollag *et al.* 1986, Takayama *et al.* 1988, Berti *et al.* 1994, Danielson *et al.* 1995, Takayama *et al.* 1995, Koya & King 1998). De-regulation or changes in the expression level of novel PKC isoforms, especially ϵ and θ have been associated with insulin resistance and type 2 diabetes in several diabetic animal models and diabetic patients (Considine *et al.* 1995, Avignon *et al.* 1996, Schmitz-Peiffer *et al.* 1997, Ikeda *et al.* 2001). More recently, focus has also been directed towards the possible role of the atypical PKC isotypes in the insulin signalling pathway involved in the stimulation of glucose uptake. Insulin has been shown to activate the atypical PKCs λ and ζ through a phosphatidylinositol (PI) 3-kinase-dependent pathway. This stimulates their translocation to glucose transporter 4 (GLUT 4) vesicles, which is required to activate GLUT 4 translocation and glucose transport (Kotani *et al.* 1998, Standaert *et al.* 1997, 1999a,b, Braiman *et al.* 2001).

The aim of the present study, therefore, was first to determine the ability of insulin to stimulate glucose uptake into muscle strips isolated from control and early growth-restricted male rats and then to relate any differences to changes in expression of PKC isotypes.

Materials and Methods

Analytical grade biochemicals were obtained from Sigma Chemical Co. or BDH Chemicals (both of Poole, Dorset, UK), unless specified otherwise. Linco rat insulin radioimmunoassay kits were purchased from Biogenesis Ltd (Poole, Dorset, UK) and rabbit antipeptide antibodies against PKC isoforms (α , β I, β II, ϵ , θ , ζ) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). D-[U- 14 C]Glucose was purchased from Amersham (Little Chalfont, Bucks, UK).

Animals

All procedures involving animals were conducted under the British Home Office Animals Act 1986. Virgin female Wistar rats (initial weight 240 to 260 g) used for the study were housed individually and were maintained at 22 °C on a 12 h light/12 h darkness cycle. They were mated and day 0 of gestation was taken as the day on which vaginal plugs were expelled. The rats were fed a diet containing 20% protein ($n=8$ dams) or an isocaloric diet containing 8% protein ($n=8$ dams) throughout pregnancy and lactation. The composition and source of the diets was as described by Snoeck *et al.* (1990).

Spontaneous delivery took place on day 22 of pregnancy after which at 3 days of age litters were reduced randomly to 8 pups, thus ensuring a standard litter size per mother. At 21 days of age all pups were weaned onto a 20% protein diet. For simplicity the two groups of offspring are termed 'control' (C) and 'low protein' (LP), however it is emphasised that only the mothers underwent dietary manipulation. All rats were fasted overnight prior to study and were 15-month-old males.

Glucose tolerance tests

Conscious rats were injected intraperitoneally with 1 ml/100 g body weight of a solution of 10% (w/v) glucose in 0.9% (w/v) saline. Blood was collected from the tail vein and blood glucose was measured using a Hemocue glucose analyser (Hemocue, Sheffield, UK) 0, 15, 30, 60, 120 and 180 min after the glucose injection. Blood samples at these time points were also collected into heparinised tubes for the measurement of plasma insulin.

Protein and plasma assays

Protein content was determined by a modification of the Lowry method (Schacterle & Pollack 1973). Plasma insulin was measured by radioimmunoassay according to the manufacturer's protocol (Biogenesis Ltd). Plasma non-esterified fatty acids (NEFA) were measured colorimetrically using a kit purchased from Boehringer Mannheim

(Germany). Plasma triglycerides were measured spectrophotometrically using a kit purchased from Sigma.

Glucose uptake studies

To determine glucose uptake into muscle tissue an isolated muscle strip technique was used as described by Lund *et al.* (1995). Soleus muscles were removed and cut into 30 mg strips. All incubations described were carried out at 37 °C under continuous gassing with 95% O₂/5% CO₂. The muscle strips were preincubated in Krebs–Ringer buffer containing 2 mM pyruvate, 38 mM mannitol and 0.1% bovine serum albumin for either 10 min or 60 min and then further incubated for 20 min in an identical medium in the presence or absence of insulin (300 pM or 16 nM). The strips were blotted on filter paper, moistened with Krebs–Ringer and incubated for 10 min in 3 ml Krebs–Ringer containing 8 mM [³H]methyl glucose (437 µCi/mmol) and 32 mM [¹⁴C]mannitol (8 µCi/mmol). After incubation, the muscle strips were briefly blotted on filter paper and frozen in liquid nitrogen. Frozen muscle strips were individually weighed, homogenised in 10% (wt/vol) trichloroacetic acid and centrifuged at 1000 g. Radioactivity in the supernatant was determined by liquid scintillation counting (Canberra Packard, Groningen, The Netherlands) with channels for simultaneous quantitation of ³H and ¹⁴C. Specific methyl glucose uptake into the intracellular space was calculated using mannitol as a marker of extracellular space.

Analysis of insulin receptor and GLUT 4 expression

Total muscle membranes were prepared as described previously (Ozanne *et al.* 1996). These membranes (50 µg protein) were then subjected to SDS-PAGE and Western blotting using either an anti-insulin receptor antibody or an anti-GLUT 4 antibody as described previously. The insulin receptor antibody and GLUT 4 antibody were kindly provided by Professor K Siddle (University of Cambridge) and by Professor G Gould (University of Glasgow) respectively. Images were analysed using the NIH Image Analyser programme (Bethesda, USA).

Analysis of PKC

Muscles were extracted in ice-cold solubilisation buffer (20 mM Hepes, 8 mM EDTA, 0.2 mM Na₃VO₄, 10 mM Na₄P₂O₇, 2.5 mM phenylmethylsulphonyl fluoride, 1 mg/ml NaF, 2 mM dichloroacetic acid, 1% Triton X-100, pH 7.4) using a motor-driven Potter homogeniser. After 20 min at 4 °C, the samples were centrifuged at 20 000 g for 60 min to remove insoluble material and the clarified extracts were stored at –80 °C. Equal amounts of cell lysate were dissolved in 2 × Laemmli buffer and subjected to SDS-PAGE. The proteins were transferred to nitrocellulose membranes (Schleicher & Schull, BA85,

Budapest, Hungary). Immunoreactive proteins were made visible using horseradish peroxidase-coupled secondary antibodies and enhanced chemiluminescence reagents according to the manufacturer's instructions (Amersham). The specific bands were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

Hyperinsulinaemic clamps

Animals were anaesthetised by intraperitoneal injection of pentobarbitone sodium (30 mg/kg) plus ketamine hydrochloride (50 mg/kg) and fitted with chronic carotid and jugular cannulas (Kraegen *et al.* 1983, 1985). Hyperinsulinaemic-euglycaemic clamp studies were conducted 5 days after surgery in overnight starved, conscious rats as described previously (Kraegen *et al.* 1983, 1985). Briefly, a continuous infusion of human insulin (Novo Biolabs, Bagsvaerd, Denmark) was given at a dose of 0.25 U/kg/h so as to induce a state of hyperinsulinaemia. The infusion rate of glucose (30% (w/v) glucose solution made with sterile 0.9% (w/v) saline) was adjusted using a peristaltic pump (Watson-Marlow, England) in order to reach and maintain euglycaemia at 4.5 mM. Blood samples were taken at 20-min intervals and blood glucose concentration determined using an automated glucose analyser (Yellow Springs Instrument, USA). Thirty minutes after the establishment of euglycaemia, the rats were killed by an intravenous infusion of pentobarbitone sodium (150 mg/kg). Muscle tissue was excised, freeze-clamped in liquid nitrogen and stored at –80 °C until analysis. PKC ζ expression was determined as described above.

Statistical analyses

All statistical calculations were carried out using Statworks statistical software (Cricket Software, Computer Associates, Slough, UK). For all data sets it was first determined that the data were normally distributed and, where comparisons were to be made, that there was no significant difference in the variances of the two groups. These conditions were met in all cases except the values for plasma insulin. Insulin values were therefore log transformed prior to analysis. The significance of any difference between groups was examined by the Student's *t*-test. Results in all cases except insulin are given as means ± S.E.M., together with the absolute probability (*P*) value where appropriate. Insulin data are presented as geometric means and 95% confidence intervals.

Results

Animal and tissue weights

There was no significant difference in weight of 15-month-old control (779 ± 28 g (*n*=8)) and LP offspring

(745 ± 25 g ($n=8$)). However the weight of the soleus muscle was significantly reduced ($P<0.05$) in the LP group (0.20 ± 0.01 g) compared with controls (0.26 ± 0.01 g).

Plasma lipids

There were no differences in fasting plasma NEFA concentrations between the two groups ($882 \pm 90 \mu\text{M}$ ($n=8$) and $869 \pm 77 \mu\text{M}$ ($n=8$) for C and LP groups respectively). Fasting triglyceride concentrations were also similar between the two groups ($4.44 \pm 0.53 \text{ mM}$ ($n=8$) and $4.75 \pm 0.45 \text{ mM}$ ($n=8$) for C and LP groups respectively).

Glucose tolerance tests

LP offspring had a significantly ($P<0.01$) higher fasting plasma glucose compared with controls (4.8 ± 0.1 ($n=8$) compared with 4.2 ± 0.2 ($n=8$)). In addition they had higher plasma glucose values throughout the glucose tolerance tests (Fig. 1a). By analysing the areas under the curves, LP offspring had a significantly ($P<0.05$) worse glucose tolerance compared with controls (area under the curves of $19.9 \pm 0.6 \text{ mmol/h/l}$ compared with $17.5 \pm 0.7 \text{ mmol/h/l}$).

Plasma insulin during glucose tolerance tests

There were no significant differences in plasma insulin concentrations between the two experimental groups during the glucose tolerance tests (Fig. 1b). When calculated as area under the curves, the value for LP animals (1030 ($825\text{--}1286$) pmol/l/h) tended to be higher than controls (634 ($255\text{--}1575$) pmol/l/h) but this did not reach statistical significance ($P=0.08$) (Fig. 1b).

Glucose uptake

Soleus muscle from LP offspring had similar basal glucose uptakes compared with the C group (43.6 ± 8.9 compared with $44.6 \pm 9.0 \text{ nmol/min/mg protein}$). Insulin stimulated glucose uptake into C muscle ($59.0 \pm 4.0 \text{ nmol/min/mg}$) ($P<0.05$) but had no effect on LP muscle ($41.2 \pm 7.0 \text{ nmol/min/mg}$) (Fig. 2).

Insulin receptor and GLUT 4 expression

There were no detectable differences in expression of either the insulin receptor (Fig. 3a) or the GLUT 4 glucose transporter (Fig. 3b) between the two groups.

PKC expression in soleus muscle

Soleus muscle from LP offspring at 15 months of age showed reduced expression ($P<0.001$) of atypical PKC ζ compared with controls (LP: $71.99 \pm 6.09\%$, C: $100 \pm$

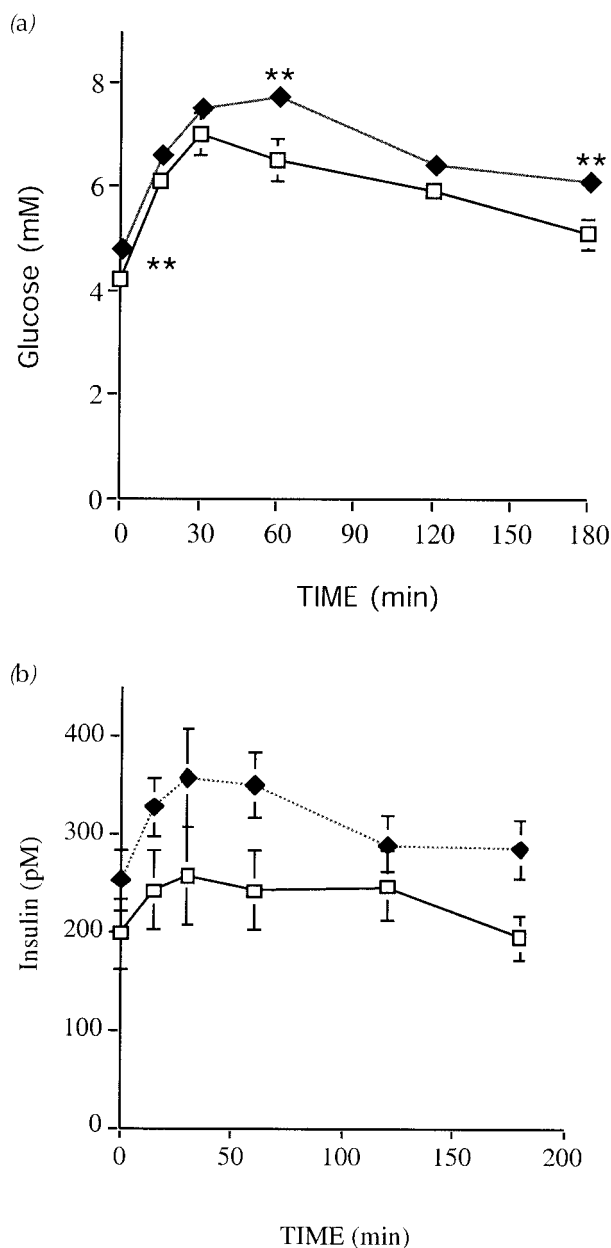


Figure 1 (a) Blood glucose and (b) plasma insulin concentrations following intraperitoneal glucose administration to 15-month-old control (□) and low protein (◆) offspring. Blood glucose and plasma insulin were measured at the indicated times following intraperitoneal injection with 1 ml/100 g body weight of a solution of 10% (w/v) glucose. Results are expressed as means ± S.E.M. of data from 8 control and 8 low protein male offspring. ** $P<0.01$.

4.21%, phosphorimager units expressed as percentage of average in control group ± S.E.M., $n=15$ animals per group) (Fig. 4). No changes in PKC ζ expression were detected in a group of 3-month-old LP offspring. PKC α , β I, β II,

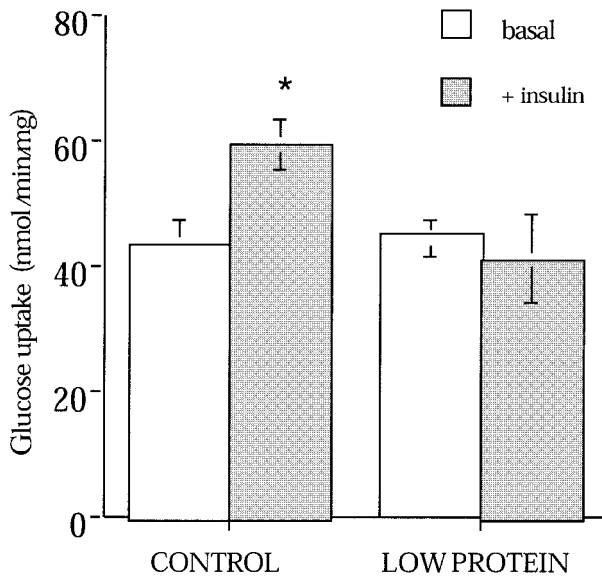


Figure 2 Glucose uptake into soleus muscle of control and low protein offspring. Muscle strips were incubated with a modified Krebs-Ringer buffer in the presence or absence of insulin (16 nM) before addition of [3 H]methyl-glucose. Glucose transport was then determined by liquid scintillation counting, as described in the Materials and Methods section. Results are expressed as means \pm S.E.M. of 8 separate sets of control and low protein experiments each performed in triplicate. * $P < 0.05$ compared with basal.

ϵ and θ expression levels were unaltered in the LP group (data not shown).

PKC ζ expression in basal and hyperinsulinaemic muscle

PKC ζ expression was not altered following *in vivo* exposure to hyperinsulinaemia (36 713 \pm 4173 phosphorimager units and 38 486 \pm 3749 phosphorimager units for basal and clamped muscle respectively).

Discussion

The mechanistic basis of the relationship between early growth restriction and the subsequent development of type 2 diabetes is the focus of much research interest. The LP rat model shows a number of features of human type 2 diabetes and the metabolic syndrome and therefore provides a useful tool for mechanistic studies (Ozanne & Hales 1999).

Soleus muscle weight was significantly reduced in the LP group, both in absolute terms and relative to body weight. This is consistent with the hypothesis that during nutritional deprivation a growing fetus will maintain growth of the brain (the so called 'brain sparing' phenomenon) at the expense of tissues such as muscle (Hales & Barker 1992).

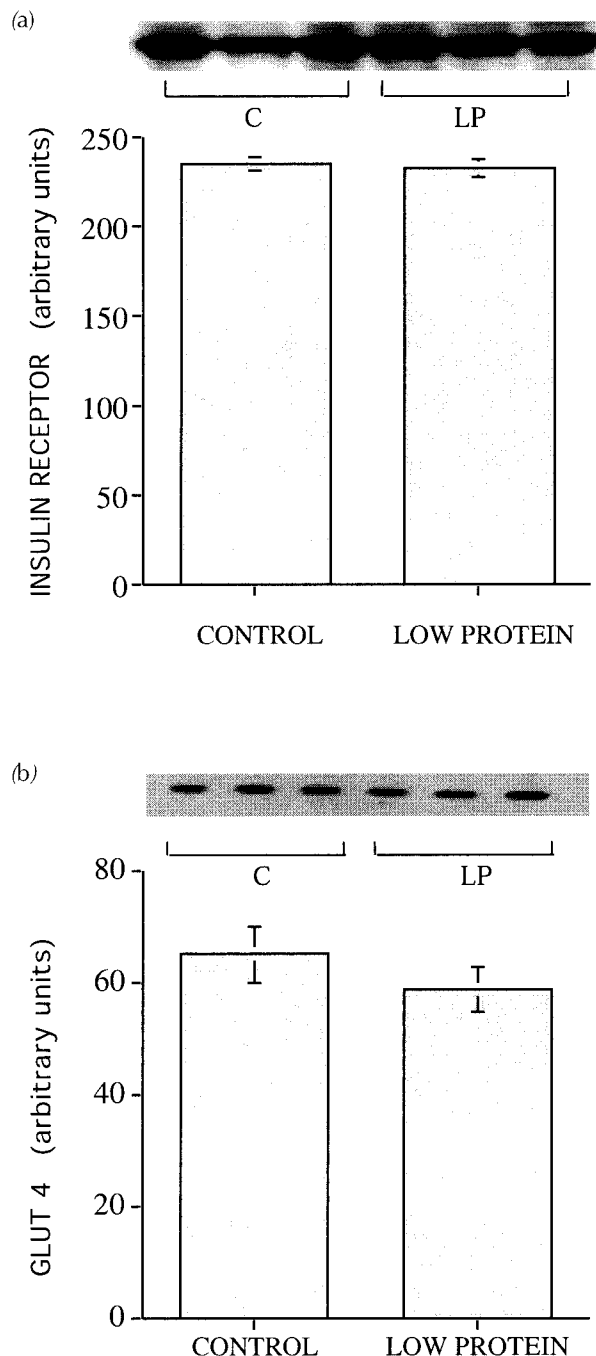


Figure 3 (a) Insulin receptor and (b) GLUT 4 expression in soleus muscle of control (C) and low protein (LP) offspring. Membranes (50 μ g) were electrophoresed and transferred to nitrocellulose that was then reacted with (a) anti-insulin receptor antibody or (b) anti-GLUT 4 and expression was quantified as described in the Materials and Methods section. Results are the means \pm S.E.M. of 8 separate control and low protein samples.

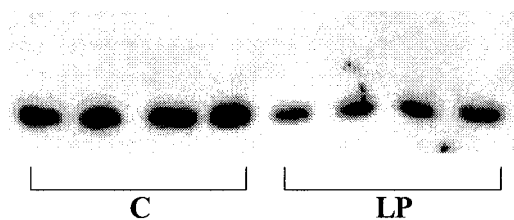


Figure 4 PKC ζ expression in soleus muscle of control (C) and low protein (LP) offspring. Muscle lysates (50 μ g) were electrophoresed and transferred to nitrocellulose that was then reacted with anti-PKC ζ antibody as described in the Materials and Methods section.

In addition to effects on muscle mass, early growth restriction also has long term consequences on muscle metabolism. In young adult life such early growth-restricted offspring have an improved glucose tolerance compared with controls which is associated with increased skeletal muscle insulin sensitivity (Ozanne *et al.* 1996). The underlying mechanisms of this improved glucose tolerance and insulin sensitivity are not completely clear but appear to be related to a generalised increase in insulin receptors in insulin sensitive tissues (Ozanne & Hales 1999). By fifteen months of age the glucose tolerance of the LP offspring has deteriorated markedly such that they have significantly impaired glucose tolerance compared with controls. This appears to be due to insulin resistance as opposed to deficiency, with plasma insulin levels tending to be increased during the glucose tolerance tests. Consistent with findings in human type 2 diabetics, muscle insulin resistance is also apparent. Stimulation of glucose uptake by insulin was apparent in C muscle although to a smaller extent to that observed in younger animals (Ozanne *et al.* 1996). However, stimulation of glucose uptake by insulin was not detectable in muscle from LP offspring. This muscle insulin resistance could not be attributed to any differences in expression of the insulin receptor, perhaps suggesting that the molecular defect resulting in this resistance lies downstream of the insulin receptor. GLUT 4 expression was also comparable between the experimental groups. This is consistent with findings in humans which suggest that GLUT 4 expression in skeletal muscle is not altered in individuals with type 2 diabetes (Krook *et al.* 2000).

A number of proteins have been identified which have been shown to play a major role in pathways that mediate insulin action (Shepherd *et al.* 1998). These include the family of enzymes known as protein kinase C (Newton 1995, Mellor & Parker 1998). These are broadly divided into three subfamilies which are classified as conventional (activated by Ca^{2+} , diacylglycerol (DAG) and phosphatidylserine (PS)), atypical (Ca^{2+} and DAG independent) and novel (activated by DAG and PS but Ca^{2+} independent) (Newton 1995). Atypical PKC isozymes such as PKC ζ and PKC λ have been identified as downstream

targets of PI 3-kinase in insulin-stimulated glucose uptake in adipocytes (Standaert *et al.* 1997). PKC ζ expression was decreased in muscle from early growth-restricted offspring. This effect appeared to be specific to this isoform of PKC as expression levels of the other isoforms including the conventional PKC proteins α , β I and β II and the novel enzymes ϵ and θ were unchanged. This suggests that the observed muscle insulin resistance in the growth-restricted offspring is related to a decrease in expression of PKC ζ . A direct association between PKC ζ expression levels and glucose transport is also suggested by the finding that over expression of PKC ζ in rat skeletal muscle improves both basal and insulin-induced glucose transport (Etgen *et al.* 1999). This reduced expression could be a cause or a consequence of the insulin resistance. It is not apparent in the 3-month-old animals, pointing to a change occurring in later life. A modulation through different factors of the diabetic milieu such as hyperglycaemia and hyperinsulinaemia is conceivable. However, exposure to hyperinsulinaemia *in vivo* did not have any effect of insulin on expression of PKC ζ . This suggests that the reduction in PKC ζ expression may be a cause of the muscle insulin resistance.

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