

Impaired PI 3-kinase activation in adipocytes from early growth-restricted male rats

S. E. OZANNE,¹ M. W. DORLING,¹ C. L. WANG,¹ AND B. T. NAVE²

¹Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Cambridge CB2 2QR; and ²Department of Biochemistry, University College London, London WC1E 6BT, United Kingdom

Received 8 June 2000; accepted in final form 27 November 2000

Ozanne, S. E., M. W. Dorling, C. L. Wang, and B. T. Nave. Impaired PI 3-kinase activation in adipocytes from early growth-restricted male rats. *Am J Physiol Endocrinol Metab* 280: E534–E539, 2001.—Epidemiological studies have established a relationship between early growth restriction and subsequent development of type 2 diabetes. Animal studies have shown that offspring of protein-restricted rats undergo a greater age-related loss of glucose tolerance than controls. The aim of this study was to investigate the possibility that this deterioration of glucose tolerance is associated with changes in adipocyte insulin action. Adipocytes from low-protein offspring had higher basal levels of glucose uptake than controls. Insulin stimulated glucose uptake into control adipocytes but had little effect on low-protein adipocytes. Both groups had similar levels of basal and isoproterenol-stimulated lipolysis. Insulin inhibited lipolysis in control adipocytes but had a reduced effect on low-protein adipocytes. These changes in insulin action were not related to altered expression of insulin receptors or insulin receptor tyrosine phosphorylation; however, they were associated with reduced phosphatidylinositol 3-kinase and protein kinase B activation. These results demonstrate that reduced glucose tolerance observed in late adult life after early growth restriction is associated with adipocyte insulin resistance.

maternal protein restriction; insulin receptor; insulin resistance; insulin action; phosphatidylinositol 3-kinase

A NUMBER OF EPIDEMIOLOGICAL STUDIES have revealed that there is a relationship between fetal and early growth restriction and the subsequent development of type 2 diabetes and the insulin resistance syndrome (see review in Ref. 21). The molecular basis of this relationship is not known; however, a possible mechanism has been proposed in the Thrifty Phenotype Hypothesis (10), which suggests that, during times of nutritional deprivation, the growing fetus adopts at least two strategies to aid survival. First, it diverts nutrients to the brain to preserve brain growth at the expense of organs such as the pancreas, liver, and muscle. Second, metabolic programming occurs in a manner that is beneficial to survival under conditions of poor postnatal nutrition. However, if the organism is born into

conditions of adequate or overnutrition, then this may conflict with the earlier programming and type 2 diabetes may result (10).

The proposal of the Thrifty Phenotype Hypothesis has led to a number of studies that use a reduced-protein rat model. In this model, rat dams are fed a low (8%) protein diet during pregnancy and lactation, which produces growth restriction in the offspring (7). In young adult life, low-protein offspring have an improved glucose tolerance compared with controls (11, 15). This is associated with increased insulin-stimulated glucose uptake into skeletal muscle (18) and adipocytes (17). However, low-protein offspring undergo a greater age-dependent loss of glucose tolerance, such that by 15 mo of age, low-protein offspring have a significantly worse glucose tolerance compared with controls (11). The mechanistic basis of this deterioration in glucose tolerance is not clear.

Skeletal muscle is the major site of insulin-stimulated glucose disposal via the glucose transporter GLUT-4 (6). However, recently the importance of the adipocyte as a site of GLUT-4 expression and insulin-stimulated glucose uptake has been recognized (1, 25). It has been shown that overexpression of GLUT-4 in adipocytes in mice leads to enhanced glucose tolerance and increased glucose uptake into adipocytes (25) and that the selective knocking out of adipocyte GLUT-4 in mice leads to glucose intolerance (1). In addition, it has been shown that, in patients with coronary heart disease, there is a correlation between insulin-stimulated glucose uptake of isolated adipocytes and insulin sensitivity as measured during hyperinsulinemic euglycemic clamps (9). Insulin also inhibits lipolysis and thus regulates the release of free fatty acids from the adipocyte. Thus the effect of insulin on adipose tissue may have a more general metabolic significance, because elevations in plasma free fatty acid concentrations inhibit insulin-stimulated glucose uptake into muscle (3, 22). Therefore, resistance to the anti-lipolytic action of insulin could potentially lead to resistance to insulin-stimulated glucose uptake into muscle (3). The aim of the present study was thus to determine whether

Address for reprint requests and other correspondence: S. E. Ozanne, Dept. of Clinical Biochemistry (Level 4), Addenbrooke's Hospital, Hills Rd., Cambridge CB2 2QR, UK (E-mail: seo10@cam.ac.uk).

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changes in glucose tolerance that resulted from maternal protein restriction were related to changes in insulin action on the adipocyte.

METHODS

Materials. Analytical grade biochemicals were obtained from Sigma Chemical or BDH Chemicals (both of Poole, Dorset, UK), unless specified otherwise. [^{14}C]glucose was obtained from Amersham (Buckinghamshire, UK), and γ -[^{32}P]ATP was from New England BioLabs (Hertfordshire, UK). Anti-PY20 anti-phosphotyrosine antibody was purchased from Signal Transduction Laboratories.

Animals. All procedures involving animals were conducted under the British Home Office Animals Act, 1986. Virgin female Wistar rats (initial weight 240–260 g) used for the study were housed individually and maintained at 22°C on a 12:12-h light-dark 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 or an isocaloric diet containing 8% protein throughout pregnancy and lactation. The composition and source of the diets were as described by Snoeck et al. (27).

Spontaneous delivery took place on *day 22* of pregnancy, after which, at 3 days of age, litters were reduced randomly to eight 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” and “low protein”; however, it is emphasized that only the mothers undergo dietary manipulation. All rats studied were 15-mo-old males and were starved overnight before commencement of procedures.

Blood glucose and plasma insulin determinations. Blood glucose was measured with a glucose meter (Hemocue, Sheffield, UK). Plasma insulin was measured with a Linco radioimmunoassay kit (Biogenesis, Dorset, UK) that provided rat insulin standards.

Adipocyte isolation. Adipocytes were isolated essentially as described previously, by collagenase digestion (5). The method was modified slightly, because cells were not collected after addition of phthalate oil but were allowed to float freely to the surface of the digestion mixture and were collected and washed twice with Krebs-Ringer containing 4% BSA and 5 mM glucose. Adipocyte numbers were determined manually by use of a hemocytometer.

Glucose transport studies. Cells (12% suspension) were incubated at 37°C with constant shaking in an 8% suspension by volume, in Krebs-Ringer-HEPES (30 mM) buffer (pH 7.4), with 2.5% BSA (fraction V) and 200 nM adenosine, either without (basal) or with (stimulated) 80 nM insulin. After an initial 30-min incubation period with or without insulin, [U- ^{14}C]glucose (3 μM) was added for 60 min, and the reaction was terminated by separating cells from media by spinning the suspension through dinonyl phthalate oil (24).

Lipolysis studies. Cells (12% suspension) were incubated at 22°C in an 8% suspension by volume, in Krebs-Ringer-HEPES (30 mM) buffer (pH 7.4), with 2.5% BSA (fraction V), either without additions (basal) or with 0.1 μM isoproterenol (stimulated) containing either 0 or 10 nM insulin. After 1 h, medium was removed, and glycerol release was measured using a kit purchased from Sigma Chemical, Poole.

Western blot analysis of insulin receptor, GLUT-4, and p85 expression and insulin receptor tyrosine phosphorylation. Insulin receptor, GLUT-4, and p85 expression was determined by Western blot analysis, as described previously (17). For insulin receptor tyrosine phosphorylation analysis, isolated adipocytes (12% suspension) were incubated for 5 min in

Krebs-Ringer-HEPES (30 mM) buffer (pH 7.4) with 5 mM glucose and 2.5% BSA (fraction V), either without (basal) or with (stimulated) 80 nM insulin. Cells were removed and lysed in 20 mM Tris, pH 8.0, containing 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 0.5 mM Na_3VO_4 , 10 $\mu\text{g/ml}$ leupeptin, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 10% (wt/vol) glycerol, and 1% (wt/vol) Nonidet-P40 (NP-40). Cleared lysates were immunoprecipitated with an anti-insulin receptor antiserum, and tyrosine phosphorylation was assessed by Western blot analysis.

Measurement of p85 association with p110 α and p110 β . The p110 α and p110 β catalytic subunits of phosphatidylinositol (PI) 3-kinase were immunoprecipitated from cleared adipocyte lysates by use of antibodies purchased from Upstate Biotechnology (Lake Placid, NY). Immunoprecipitated proteins were then subjected to SDS-PAGE and blotted using anti-p85 antibodies, as previously described (17).

Protein assays. Protein content was determined in 12% adipocyte suspensions in Krebs-Ringer-HEPES without BSA by a modification of the Lowry method (23).

Immunoprecipitation of proteins for PI 3-kinase assays. Adipocytes were incubated for 1 h in a modified Krebs-Ringer-phosphate buffer containing 5 mM glucose and 2% BSA with or without 80 nM insulin. Cells were removed and lysed in 20 mM Tris, pH 8.0, containing 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 0.5 mM Na_3VO_4 , 10 $\mu\text{g/ml}$ leupeptin, 0.2 mM PMSF, 10% (wt/vol) glycerol, and 1% (wt/vol) NP-40. Cleared lysates were immunoprecipitated with an anti-phosphotyrosine antiserum as described previously (13). To remove any traces of NP-40, immunoprecipitates were washed extensively as previously described (13).

PI 3-kinase activity assays. PI 3-kinase enzymic activity was assayed at 37°C by measuring the incorporation of ^{32}P from γ -labeled ATP into PI in buffer at pH 7.4 containing 20 mM β -glycerophosphate, 5 mM sodium pyrophosphate, 30 mM NaCl, 1 mM dithiothreitol, 3 mM MgCl_2 , 1.2 μM ATP, 0.6 mg/ml PI, and 0.33% cholate. Experiments confirmed that production of lipid product was linear for up to 20 min under the conditions used (results not shown). The ^{32}P -labeled phospholipid product was resolved by TLC as described previously (13), with a single band being observed in reactions from anti-phosphotyrosine immunoprecipitates. This band was not observed when the incubations were performed in the presence of 0.1% NP-40 or 50 nM wortmannin, indicating that the band was the product of PI 3-kinase activity. Bands were quantified with a Phosphoimager (Fujix, Tokyo, Japan).

Protein kinase B assay. Cells were incubated for 5 min at 37°C in Krebs-Ringer-HEPES in the presence and absence of 100 nM insulin. Reactions were stopped by the addition of 27 μl of stop solution (74 mM EDTA, 37 mM EGTA, 0.037 mM microcystin, 3.7% 2-mercaptoethanol, and 37 mM sodium orthovanadate) and then snap-frozen in liquid nitrogen and stored at -80°C before analysis. Protein kinase B (PKB) activity was determined as described previously (29). In brief, samples were thawed and centrifuged at 20,000 g for 5 min, and the infranatant was collected. PKB- β was immunoprecipitated as previously described (29) and assayed for PKB activity with Crosstide (4).

Statistical analyses. All statistical calculations were carried out using Statworks statistical software (Cricket Software). 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. The significance of any difference between groups was examined by the Student's *t*-test. Results in all cases are given as means \pm SE, together with the absolute probability

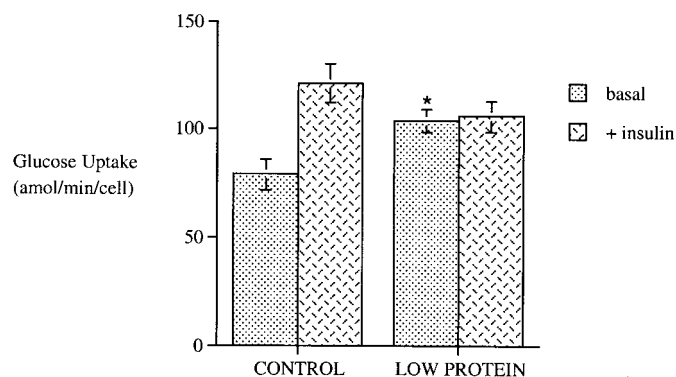


Fig. 1. Effect of insulin on glucose uptake into freshly isolated adipocytes. Adipocytes were incubated with a modified Krebs-Ringer buffer in the presence or absence of insulin (80 nM) before addition of [^{14}C]glucose. Glucose uptake was then determined by liquid scintillation counting, as described in METHODS. Results are expressed as means \pm SE of 8 separate sets of control and low-protein experiments, each performed in triplicate. * $P < 0.05$ vs. control value under identical conditions.

(P) value where appropriate. Insulin data are presented as geometric means and 95% confidence intervals.

RESULTS

Animal and tissue data. There was no significant difference in weight of 15-mo-old control (785 ± 32 g, $n = 8$) and low-protein offspring (728 ± 30 g, $n = 8$). Epididymal fat pad weights (14 ± 1 g for controls and 12 ± 1 g for the low protein) were also similar in the two experimental groups. Low-protein offspring had a significantly ($P < 0.01$) higher fasting plasma glucose compared with controls (4.9 ± 0.1 vs. 4.2 ± 0.1 mM). Fasting plasma insulin concentrations tended to be higher in the low-protein group (272 ± 41 vs. 218 ± 39 pM), but this did not reach statistical significance.

Glucose uptake. Adipocytes from low-protein offspring had a significantly ($P < 0.05$) elevated basal glucose uptake compared with controls (104 ± 5 vs. 79 ± 7 amol \cdot min $^{-1}\cdot$ cell $^{-1}$; Fig. 1). Insulin stimulated ($P < 0.01$) glucose uptake into control adipocytes (to 121 ± 9 amol \cdot min $^{-1}\cdot$ cell $^{-1}$) but had no effect on glucose uptake into low-protein adipocytes (106 ± 7 amol \cdot min $^{-1}\cdot$ cell $^{-1}$ in the presence of insulin; Fig. 1).

Lipolysis. There were no significant differences in basal rates of lipolysis between the two groups ($5.85 \pm$

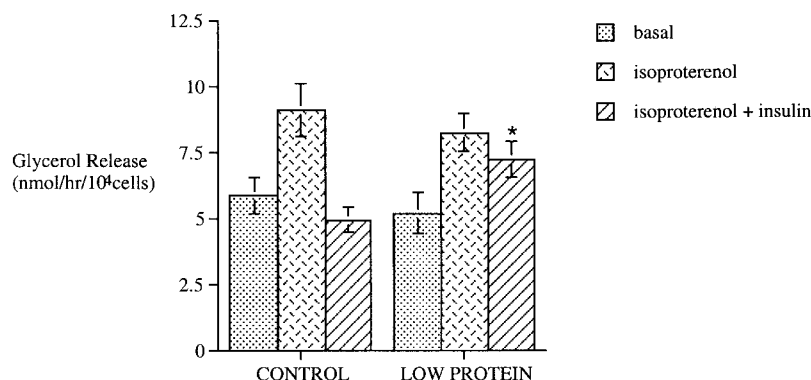
0.67 and 5.18 ± 0.80 nmol glycerol released \cdot h $^{-1}\cdot$ 10 4 cells $^{-1}$ for control and low-protein offspring, respectively; Fig. 2). Isoproterenol stimulated lipolytic rates ($P < 0.001$) to similar levels in both groups (to 9.10 ± 0.99 and 8.25 ± 0.73 nmol glycerol released \cdot h $^{-1}\cdot$ 10 4 cells $^{-1}$ for control and low-protein offspring, respectively). Addition of insulin reduced lipolytic rates to basal levels in the control group (to 4.93 ± 0.46 nmol glycerol released \cdot h $^{-1}\cdot$ 10 4 cells $^{-1}$). However, insulin had a markedly reduced effect in the low-protein group, with lipolytic rates (7.23 ± 0.68 nmol glycerol released \cdot h $^{-1}\cdot$ 10 4 cells $^{-1}$) remaining significantly ($P < 0.01$) higher than basal rates and significantly ($P < 0.05$) higher than rates of controls in the presence of insulin.

GLUT-4, insulin receptor, and p85 expression and tyrosine phosphorylation. Western blot analysis revealed that there were no differences in expression of either the GLUT-4 glucose transporter (70 ± 4 vs. 72 ± 5 arbitrary units for control and low protein, respectively; Fig. 3A), the insulin receptor (35 ± 3 vs. 38 ± 4 arbitrary units for control and low protein, respectively; Fig. 3B), or p85 (48 ± 2 vs. 46 ± 3 arbitrary units for control and low protein, respectively; Fig. 3C) between the two groups. Tyrosine phosphorylation of the insulin receptor was also similar under both basal and insulin-stimulated conditions in each group (Fig. 4).

Association of p85 with p110 α and p110 β . Association of the regulatory subunit (p85) of PI 3-kinase with the p110 α catalytic subunit of PI 3-kinase was similar in the groups (39 ± 4 vs. 37 ± 3 arbitrary units associated in control and low protein, respectively; Fig. 5A). In contrast, there was significantly ($P < 0.001$) less p110 β associated with p85 in the low-protein group (compared with controls \sim 4.0 \pm 0.5-fold reduction; Fig. 5B).

Phosphotyrosine-associated PI 3-kinase activity. Adipocytes from low-protein offspring had significantly elevated basal phosphotyrosine-associated PI 3-kinase activity compared with control offspring (Fig. 6, $P < 0.001$). Insulin stimulated phosphotyrosine-associated activity in both groups ($P < 0.01$) but had a greater effect in the control group compared with the low-protein group. Hence, insulin-stimulated phosphotyrosine activity was significantly reduced in the low-

Fig. 2. Effect of isoproterenol and insulin on lipolysis in freshly isolated adipocytes. Adipocytes were incubated with a modified Krebs-Ringer buffer in the presence or absence of isoproterenol (0.1 μ M) and insulin (10 nM). Glycerol release was measured as described in METHODS. Results are expressed as means \pm SE of 8 separate sets of control and low-protein experiments, each performed in triplicate. * $P < 0.05$ vs. control value under identical conditions.



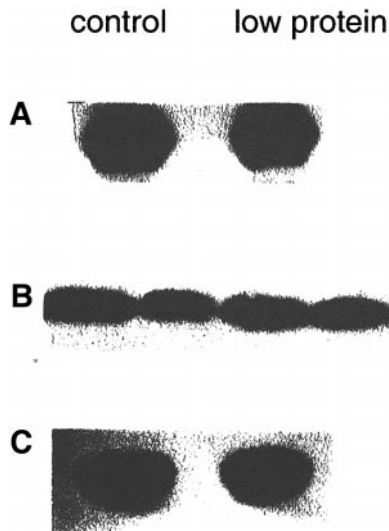


Fig. 3. Immunoblot analysis of GLUT-4 (A), insulin receptor (B), and p85 in adipocyte lysates (C). Lysates (50 μ g) were electrophoresed and transferred to nitrocellulose paper, which was reacted with the appropriate antibody. Similar results were observed on 8 separate occasions.

protein group compared with controls (Fig. 6, $P < 0.01$).

PKB activity. Basal levels of PKB activity were similar in the experimental groups. Insulin stimulated PKB activity in the control group but had a markedly reduced effect ($P < 0.05$) in the low-protein group (Fig. 7).

DISCUSSION

The present study was designed to investigate the possibility that age-dependent changes in glucose tolerance observed in the offspring of maternally protein-restricted rats were associated with changes in insulin action on adipocytes. At 15 mo of age, there were no significant differences in body weight or epididymal fat pad weight between the two groups.

A number of studies have shown that low-protein offspring have a better glucose tolerance than controls

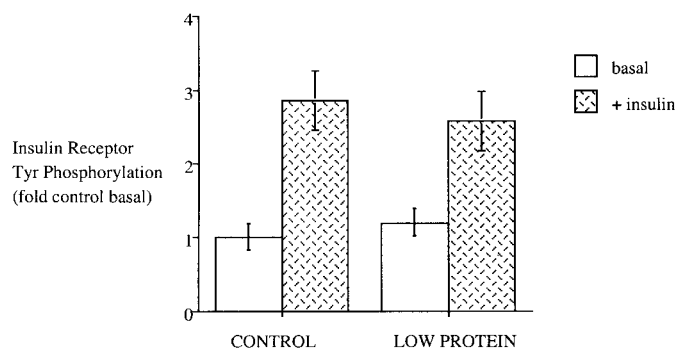


Fig. 4. Effect of insulin on tyrosine phosphorylation of the insulin receptor. Adipocytes were incubated with a modified Krebs-Ringer buffer in the presence or absence of insulin (80 nM). Insulin receptor tyrosine phosphorylation was measured as described in METHODS. Results are expressed as means \pm SE of 8 separate sets of control and low-protein experiments.

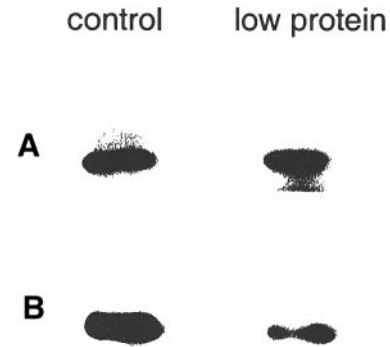


Fig. 5. Association of p85 with p110 α (A) and p110 β (B). p110 α or p110 β was immunoprecipitated from cleared adipocyte lysates and then blotted with p85, as described in METHODS. Similar results were observed on 8 separate occasions.

in young adult life (11, 12, 15). This improved glucose tolerance in young adult life is associated with increased glucose uptake into muscle (18) and adipocytes (17). However, early growth-restricted offspring undergo a greater age-dependent loss of glucose tolerance, such that by 1 yr of age they have a glucose tolerance similar to controls (20), and by 15 mo they have a significantly worse glucose tolerance compared with controls (11). In males, this glucose intolerance appears to result from insulin resistance, the early growth-restricted offspring having elevated plasma insulin concentrations during the glucose tolerance test compared with controls (11).

The present study demonstrates that the insulin resistance observed in male low-protein offspring in late adult life is associated with changes in the insulin sensitivity of adipocytes. Epididymal adipocytes from low-protein animals had an elevated basal glucose uptake compared with controls. This difference in basal glucose uptake is similar to that seen in 3-mo-old offspring (17). The mechanistic basis of this elevated basal glucose uptake is not known. It is unlikely to result from a defect in insulin signaling, because basal

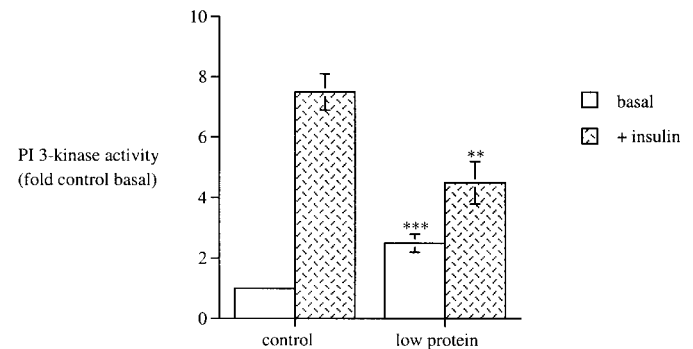


Fig. 6. Effect of insulin on phosphotyrosine-associated phosphatidylinositol (PI) 3-kinase activity in adipocytes. Adipocytes were incubated with a modified Krebs-Ringer buffer in the presence or absence of insulin (80 nM). PI 3-kinase activity was then measured in phosphotyrosine immunoprecipitates, as described in METHODS. Results are expressed as means \pm SE of 6 separate sets of control and low-protein experiments, each performed in triplicate. ** $P < 0.01$ vs. control under identical conditions; *** $P < 0.001$ vs. control under identical conditions.

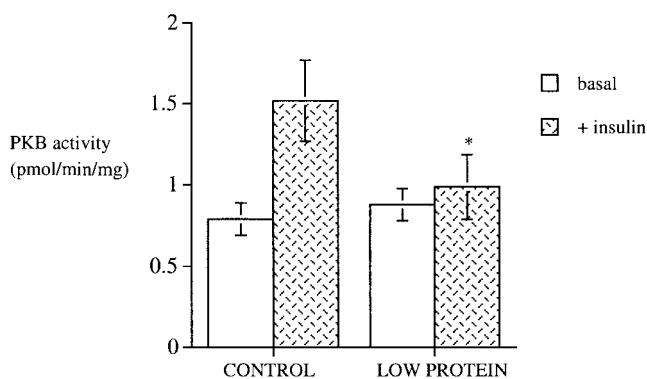


Fig. 7. Effect of insulin on protein kinase B (PKB) activity in adipocytes. Adipocytes were incubated with a modified Krebs-Ringer buffer in the presence or absence of insulin (80 nM). PKB activity was then measured as described in METHODS. Results are expressed as means \pm SE of 6 separate sets of control and low-protein experiments, each performed in triplicate. ** $P < 0.01$ vs. control under identical conditions; *** $P < 0.001$ vs. control under identical conditions.

levels of insulin receptor tyrosine phosphorylation were similar to controls. Insulin was unable to stimulate glucose uptake into low-protein adipocytes, whereas glucose uptake was increased significantly in control adipocytes in the presence of insulin. This was not related to any difference in expression of GLUT-4, the major insulin-sensitive glucose transporter found in adipocytes. It is possible that there may be a defect in the signaling pathway that signals to GLUT-4 translocation to the plasma membrane, or in the activity of the transporter itself. However, this impaired ability of insulin to stimulate glucose uptake into adipocytes of low-protein offspring is consistent with these animals being less glucose tolerant and relatively insulin resistant compared with control animals at this age. A relationship between whole body insulin resistance and in vitro insulin resistance to insulin-stimulated glucose uptake into isolated adipocytes has previously been observed in a number of human studies (8, 9).

The regulation of lipolysis was also altered in adipocytes from the low-protein offspring in the current study. There were no differences in basal rates of lipolysis or in isoproterenol-stimulated lipolysis. This contrasts with results from studies of young offspring, which have shown that adipocytes from 3-mo-old male low-protein offspring have a greater response to isoproterenol compared with controls (19). The major difference in the regulation of lipolysis between the two groups at 15 mo of age was the relative inability of insulin to inhibit the catecholamine-stimulated lipolysis in the low-protein group. Thus 15-mo-old low-protein offspring adipocytes are resistant to both the anti-lipolytic action of insulin and the action of insulin to stimulate glucose uptake.

Insulin receptor expression was similar in both groups. This contrasts with data from younger animals, which have shown that young adult low-protein offspring have increased muscle (18) and adipocyte (17) insulin receptors. This appears to be associated with increased insulin receptor substrate-1-associated PI

3-kinase activity in adipocytes (17), increased muscle insulin sensitivity (18), and an overall improved glucose tolerance. These young animals also tend to have a lower fasting plasma insulin concentration (24), which is again indicative of increased insulin sensitivity. The mechanism leading to the increased expression of insulin receptors in a wide range of tissues (liver, muscle, and adipocytes) in young adult low-protein offspring has not been defined. This, however, has the effect of increasing the insulin sensitivity of glucose uptake into muscle and adipocytes and probably at least contributes to the increased glucose tolerance. At 15 mo of age, insulin receptor expression and its tyrosine phosphorylation under both basal and insulin-stimulated conditions are similar in the early growth-restricted and control groups. This suggests that the molecular alteration that leads to insulin resistance must therefore be a postreceptor defect.

In recent years, a clearer picture of the molecules involved in insulin signaling has emerged (28). PI 3-kinase has been identified as a key enzyme in the signaling of the metabolic actions of insulin (26). PI 3-kinase is a heterodimeric enzyme that consists of a regulatory subunit (termed p85) and a catalytic subunit (termed p110). In adipocytes, there are two major isoforms of the catalytic subunit, p110 α and p110 β (26). The functional significance of the existence of two isoforms remains unclear. It is well established that when insulin binds to its receptor, autophosphorylation of the receptor occurs, thereby activating the receptor and resulting in the tyrosine phosphorylation of a number of insulin receptor substrates (26). PI 3-kinase has been shown to dock with phosphotyrosine residues on insulin receptor substrates via SH2 domains, which results in activation of its kinase activity (26). Both the ability of insulin to stimulate glucose uptake and the anti-lipolytic action of insulin are sensitive to the PI 3-kinase inhibitor wortmanin (16). The present study showed that adipocytes from the 15-mo-old early growth-retarded rats had elevated basal phosphotyrosine-associated PI 3-kinase activity. In contrast, insulin-stimulated phosphotyrosine-associated PI 3-kinase activity was reduced in the low-protein group, despite apparently normal levels of tyrosine phosphorylation of the insulin receptor. This reduced activation of PI 3-kinase was accompanied by a reduction in the level of p110 β associated with the p85 regulatory subunit. This is consistent with findings in adipocytes from 3-mo-old low-protein offspring, which have shown a reduction in expression of the p110 β protein (17).

PKB has been identified as an enzyme downstream of PI 3-kinase that is thought to mediate a number of actions of insulin, including the stimulation of glucose uptake into adipocytes (14). PI 3-kinase activity is thought to be required for PKB activation, because such activation can be prevented by inhibitors of PI 3-kinase and by overexpression of dominant negative forms of PI 3-kinases (see review in Ref. 2). Consistent with the reduction in insulin-stimulated phosphotyrosine-associated PI 3-kinase activity, insulin-stimu-

lated PKB activity was also reduced in the early growth-restricted group. Ineffective PI 3-kinase activation and consequently reduced PKB activation may thus, at least in part, explain the insulin resistance observed in the low-protein adipocytes. The mechanistic basis of this inability of insulin to stimulate PI 3-kinase activity and its relationship to changes in expression of the p110 β isoform of the catalytic subunit of the enzyme remain to be fully understood. However, the nature of this molecular defect will be the focus of future studies.

We thank D. Hutt, A. Flack, A. Wayman, and L. Smith of the Dunn Nutrition Laboratory for their invaluable assistance. We are grateful to Professor C. N. Hales and Dr P. Shepherd for advice and useful discussions.

This work was supported by the British Diabetic Association, the Medical Research Council, the Parthenon Trust, and the Wellcome Trust.

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