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ORIGINAL ARTICLE

Impaired Insulin Secretion in Perfused Pancreases Isolated from Offspring of Female Rats Fed a Low Protein Whey-Based Diet

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ABSTRACT

Context Insufficient maternal protein intake has been postulated to cause impaired fuel metabolism and diabetes mellitus in adult mammalian progeny, but the mechanism remains unclear.

Objective To investigate the effect of a maternal low protein whey-based diet during pregnancy and lactation on pancreatic function and skeletal muscle glucose metabolism in the offspring.

Animals Sprague-Dawley rats: 8 mothers and 46 offspring.

Design Female rats were fed throughout pregnancy and lactation with otherwise-complete isoenergetic diets sufficient (20% whey protein; control: n=3) or insufficient (5% whey protein; low-protein: n=5) in whey protein. From weaning all offspring ate control diet.

Main outcome measures Food intake and weight gain were measured for both mothers and offspring, and *in vitro* functional studies of endocrine pancreas and skeletal muscle were performed on offspring at 40 and 50 days of age, respectively.

Results Food intake (P=0.004) and weight gain (P=0.006) were lower in low protein than control mothers during early gestation. Offspring of low protein mothers had significant lower body weight from 5 to 15 days of age, although there was no significant difference in food consumption. Glucose, arginine- and glucose/arginine-stimulated insulin secretion from perfused pancreases isolated from low protein offspring were decreased by between 55 and 65% compared with control values. Studies in skeletal muscle demonstrated no difference in insulin sensitivity between the two groups.

Conclusions Dietary whey protein insufficiency in female rats during pregnancy and lactation can evoke major changes in insulin secretion in progeny, and these changes represent a persistent functional abnormality in the endocrine pancreas.

INTRODUCTION

Programming [1, 2] is a process whereby poor nutrition at an early age leads to permanent changes in an organism [2]. Early adaptations to nutritional stress permanently alter the physiology and metabolism of an organ, such that these changes continue to be expressed in the absence of the original causative events [3]. In humans, such changes are postulated to be causative of later disease, including coronary heart disease [4, 5], depression [6], and the so-called "metabolic syndrome" [1], which refers to the grouping of type 2 diabetes mellitus [1, 2], cardiovascular disease [7] and hypertension [8].

Timing of the nutritional stress appears to be critical, with earlier events more likely to cause changes which persist throughout the life of the organism [9]. Therefore, foetal and early neonatal life represent developmental stages of particular interest. In rodents, it has been well established that low maternal protein intake during gestation can result in low birth weight and subsequently leads to various metabolic disturbances in adulthood, including high blood pressure, impaired glucose tolerance, insulin resistance and altered pancreatic morphology and function [10, 11, 12, 13, 14]. Additional studies are warranted to explore the relationships linking protein nutrition in early life to the postnatal development of disease in animals, as a model for the human condition [11]. Protein is important in foetal growth and pancreatic development [15], and is often scarce and expensive in communities with a high prevalence of diabetes and the metabolic syndrome [16].

A number of comparable studies have been published in which casein-based diets are used, but there is very little literature investigating a whey-based diet. Whey protein is considered to be of higher nutritional quality than casein [17].

Our hypothesis was that a decreased whey protein intake in the maternal diet (maternal low protein) during pregnancy and lactation results in a metabolic insult in the offspring during these periods sufficient to cause impaired pancreatic function and altered glucose metabolism in the offspring once adult, and that the higher quality of the whey protein (compared with casein) would be insufficient to mitigate the low-protein insult. Female rats were fed diets either sufficient or insufficient in whey protein through gestation and lactation, and pancreatic function (the secretory response to stimuli in isolated perfused pancreases) and glucose metabolism (insulin-stimulated uptake of glucose into glycogen in the isolated *soleus* muscle) were studied in the offspring.

Table 1. Composition and analysis of experimental diets (g/100 g diet).

	Maternal diet	
	Control	Low-protein
Ingredient		
- Cellulose	5.0	5.0
- Cornflour	47.8	62.8
- DL-methionine	0.2	0.2
- Mineral mix	3.9	3.9
- Whey protein	20.0	5.0
- Vegetable oil	7.0	7.0
- Sucrose	15.0	15.0
- Vitamin mix	1.1	1.1
Analysis		
- Nitrogen	3.0	0.9
- Total protein	19.1	5.7
- Lipid	7.7	7.3
- Carbohydrate	73.4	87.0
- Net metabolisable energy (MJ/kg diet)	16.9	17.1

Diets were prepared by the authors using commercially available ingredients listed in the table.

MATERIALS AND METHODS

Animals and Diet

Virgin female Sprague-Dawley rats were kept at 21±1ºC, subjected to a 12:12 h light:dark cycle. Diet was standard laboratory chow (18.5% crude protein, 5% fat; NRM diet 86, NRM New Zealand Ltd., Auckland, New Zealand) supplied *ad libitum* prior to mating. Females in oestrus were randomly selected and mated with one of three males. Dams were assigned such that offspring of each male would be represented in both experimental diet groups, to ensure any observed results were not due to a simple genetic effect. A positive pregnancy was determined by the appearance of a vaginal plug (defined as day 1 of gestation), at which stage the rats were transferred to individual cages. Throughout gestation and lactation rats were fed isoenergetic semi-synthetic diets containing either 20% whey protein (control), or 5% whey protein (low-protein) (Table 1). Diets were prepared in the laboratory from commercially available ingredients, and compositional analyses were performed on the diets (Table 1). A control diet of similar

composition has been previously reported [18]. During gestation, animals were not fed *ad libitum*. Instead, the amount fed daily to each pregnant rat was calculated from the body weight of the rat and the particular day of gestation. This calculation was based on food intake data from a previous feeding trial for mothers fed standard laboratory chow (data not shown), with the food restriction being applied to prevent low protein mothers consuming more than control mothers during gestation, as has been previously observed [19]. All animals had free access to water.

Following spontaneous delivery of pups (day 22-23 of gestation), mothers were maintained on the same diet as had been supplied during gestation, without restriction on intake. No restriction was applied during lactation because observations suggest that higher food intake in low protein mothers does not occur during this period [19, 20]. At 21 days of age, pups from all litters were weaned onto the control diet, and this was supplied *ad libitum* for the remainder of the trial.

Dietary Analysis

Nitrogen determination was by the instrumental combustion method (Nitrogen Analyser Series 1500, Carlo Erba, Serono, Italy), lipid determination by the Soxhlet method [21], and carbohydrate determination by an enzymatic and spectrophotometric method [22, 23]. Acid stable amino acids were analyzed by acid hydrolysis, sulfur amino acids by performic acid oxidation followed by acid hydrolysis [24]. Protein was calculated from the nitrogen content (Nx6.38) [25, 26], and total energy as net metabolisable energy was calculated from the individual components as has previously been described [27].

Food Intake, Body Weight and Feeding Efficiency

Maternal food intake and body weight were measured daily from the first day of gestation until 21 days post-partum (weaning). Gestation was divided into two equal periods (equivalent to the first and second trimesters of human pregnancy [28, 29]) in order to determine if any observations in the offspring were due to differences in maternal intake or body weight during a specific part of gestation.

Offspring body weight was measured daily from birth until 50 days of age, while food intake (determined as mean intake per rat within a litter) was measured daily from weaning until 50 days of age. Feeding efficiency for both mothers and offspring was calculated as the efficiency of energy utilization (EEU; total weight gain / total energy intake, g/kJ) as previously reported [30].

Isolation and Perfusion of the Pancreas

Non-fasted offspring (both male and female) were used as pancreas donors (age at experimentation 42±2 days). A total of 5 (2 litters) and 8 (3 litters) animals were used from the control and low protein groups, respectively. After initial anaesthesia by halothane induction (5% plus 2 L/min O_2) an intra-peritonal injection of sodium phenobarbitone (Nembutal®, Sigma, St Louis, MO, USA) 50 mg/kg body weight) was performed and animals maintained on oxygen during subsequent removal of the pancreas. Pancreases were prepared according to the method of Grodsky and Fanska [31], with minor modifications. Briefly, the pancreas, stomach, and duodenal remnant were isolated *en bloc*, the aorta and portal vein cannulated, and the preparation transferred to a perfusion chamber. Perfusion was performed simultaneously through the coeliac and superior mesenteric arteries (flow rate 1.2 mL/min) and effluent was collected without recycling from the cannulated portal vein. The perfusion medium was modified Krebs-Henseleit buffer (final concentrations: NaCl, 112.8 mmol/L; KCl, 4.4 mmol/L; KH_2PO_4, 1.5 mmol/L; $MgSO_4$, 1.2 mmol/L; $CaCl_2$, 2.3 mmol/L; $NaHCO_3$, 2.93 mmol/L; D-glucose, 3 mmol/L), supplemented with dextran (4% (w/v), molar weight 71,400, industrial grade; Sigma, St Louis, MO, USA) and albumin (0.5% (w/v), bovine fraction V; A-6793, Sigma, St Louis, MO, USA). Buffer was sterile filtered (0.2 µm; Vacucap, Gelman,

Ann Arbor, MI, USA) before equilibration against a gas phase of 95% O_2/5% CO_2 (BOC Gases NZ Ltd., Auckland, New Zealand), and had a final pH of 7.4 at 37°C.

Stimulation of the Pancreas and Sample Collection

Organs were equilibrated for 20 min. For 90 min thereafter portal vein effluent fractions were collected at 1 min intervals and stored on ice. Each gland was stimulated during the experiment by means of side arm infusions (0.05 mL/min) with either glucose (final concentration 21.7 mmol/L), arginine (final concentration 10.85 mmol/L), or a mixture of the two. Both glucose and lactate were measured simultaneously on every second sample with a glucose/lactate analyser (YSI 2300 Stat Plus, Yellow Springs Instrument Co., Yellow Springs, OH, USA); glucose to ensure that the final perfusate concentration was as expected, and lactate to confirm pancreas viability [32]. Samples were stored at -80°C until analysis for insulin and amylin content.

Insulin Radioimmunoassay

Perfusate insulin concentration was determined in duplicate at each point using a double antibody RIA. Briefly, antibodies were raised against bovine insulin in guinea pigs. Radiolabelled tracer (^{125}I-insulin) was prepared by iodination of bovine insulin using the chloramine-T method [33], and purified by gel filtration chromatography. The standard curve was generated using serial dilutions of soluble human insulin (Actrapid, Novo Nordisk, Copenhagen, Denmark) as a standard. The minimum detectable concentration was 16 pmol/L, the linear range 140-1,800 pmol/L, and the mean within-assay coefficient of variation 19% at the EC_{50}.
Duplicate 50 µL samples of standards or unknowns were incubated with 200 µL of antiserum (diluted 1/100,000 in RIA buffer (0.05 mol/L phosphate buffer, 0.1% (v/v) sodium azide, 0.5% (w/v) bovine serum albumin, pH 7.4)). After 5 h at room temperature, 100 µL of tracer (diluted in insulin RIA buffer to give a reading of approximately 9,000 cpm/100 µL) was added and incubated for 48 h. One hundred µL of goat anti-guinea pig IgG (diluted 5/100 (v/v) in RIA buffer), and 100 µL of normal guinea pig serum (1/100 (v/v) in RIA buffer) were added and incubated for 1 h at room temperature. One mL of polyethylene glycol 6000 (6% (w/v) in 0.05 mol/L phosphate buffer, pH 7.4) was added, followed by further incubation for 90 min at 4°C. Tubes were spun at 1500 *g* for 30 min, decanted and counted for 1 min in a gamma counter (Wizard^{TM}, Wallac, Turku, Finland).

Amylin Radioimmunoassay

Perfusate amylin concentration was measured in duplicate at each point using an in-house RIA (M. Stokjovic, unpublished results). Amylin antibodies were raised in rabbits using synthetic rat amylin (Bachem AG, Bubendorf, Switzerland). Radiolabelled tracer (^{125}I-rat amylin) was prepared by iodination of purified synthetic rat amylin (Bachem AG, Bubendorf, Switzerland) using the chloramine-T method [33], and purified by gel filtration chromatography. The assay was standardised using lyophilised rat amylin standards (Peninsula Laboratories, Belmont, CA, USA). The minimum detectable concentration was 25 pmol/L, the linear range 25-250 pmol/L, and the mean within-assay coefficient of variation 22% at the EC_{50}.
Duplicate 100 µL samples of standards or unknowns were incubated with 100 µL of antiserum (diluted 1/100,000 in RIA buffer (0.05 M phosphate buffer, 3.1 mmol/L sodium azide, 0.25% (w/v) bovine serum albumin, 0.1% (v/v) Triton X-100, 25 mmol/L EDTA, 1% aprotonin, pH 7.4). After 24 h at 4°C, 100 µL of tracer (diluted in amylin RIA buffer to give a reading of approximately 9,000 cpm/100 µL) was added and incubated for 48 h (4°C). One hundred µL of goat anti-rabbit IgG diluted 1/15 (v/v) in buffer and 100 µL of normal rabbit serum diluted 1/100 (v/v) were added and incubated for 1 h at room temperature. Polyethylene glycol 6000 (0.5 mL, 8% (w/v) in 0.05 mol/L phosphate buffer) was then added followed by a further incubation for 90 min at 4°C. Samples were

centrifuged at 1,500 *g* at 4°C for 30 min, decanted and counted for 1 min in a gamma counter. Data analysis for both assays used Multicalc™ software version 2.0 (Wallac, Turku, Finland).

Insulin-Stimulated Uptake of Glucose into Muscle Glycogen

Male and female offspring (13 animals from three litters for control group, and 20 animals from four litters for low protein group; age: 50±2 days) were starved overnight (16-18 h) with free access to water. An intra-peritoneal injection of sodium phenobarbitone (80 mg/kg body weight; Nembutal®, Sigma, St Louis, MO, USA) was administered, and the animals sacrificed by cervical dislocation once the anaesthetic had taken effect. The *soleus* muscle was removed from each leg under Krebs-Henseleit buffer (final concentrations (mmol/L): NaCl, 118.5 mmol/L; KCl, 4.75 mmol/L; $MgSO_4$, 1.18 mmol/L; $NaHCO_3$, 24.8 mmol/L; KH_2PO_4, 1.18 mmol/L; $CaCl_2$, 2.54 mmol/L; D-glucose, 10 mmol/L) saturated with 95% O_2/5% CO_2. Each *soleus* muscle was then split longitudinally into two or three strips, depending on the muscle diameter. After 20 min equilibration in a separate container of 95% O_2/5% CO_2-saturated Krebs-Henseleit buffer, up to five strips were transferred to a 50 mL Erlenmeyer flask in a 30°C shaking waterbath. Each flask contained 10 mL of normal Dulbecco's Modified Eagle Medium with one of the following concentrations of insulin: 0, 0.71, 2.37, 7.1, 23.7, 71.0, 237.0 nM. Exactly 2 min after the transfer of muscle strips to the flask, 10 µL of a 1.85 MBq/mL solution of U-^{14}C-D-glucose (American Radiolabeled Chemicals Inc., St Louis, MO, USA) were added, and the flask incubated for a further 2 h at 30°C under a constant stream of 95% O_2/5% CO_2. Each muscle strip was then snap-frozen in liquid nitrogen and freeze-dried for a minimum of 24 h.

Glycogen Extraction from *Soleus* Muscle and Scintillation Counting

Freeze-dried muscle was digested in 250 µL of 60% (w/v) KOH for 45 min at 70°C and the glycogen precipitated by addition of 750 µL cold 95% ethanol. Samples were kept at -20°C overnight then centrifuged (10,000 *g*, 15 min, 4°C) and the supernatant aspirated. The pellet was washed in 750 µL cold 95% ethanol, centrifuged and the supernatant aspirated. The wash step was repeated once more and the glycogen pellet dried at 60°C for 2 h. Glycogen was redissolved in 200 µL deionized water and transferred to a scintillation tube. StarScint® (Canberra Packard, Pangbourne, Berkshire, UK) scintillation fluid (1.8 mL) was added and the sample counted for 5 min (LS 3801 beta-counter, Beckman Coulter Inc., Fullerton, CA, USA).

Collection of Blood and Plasma Measurements

Blood was collected into lithium heparin vacutainers immediately *post mortem* from the vena cava of animals sacrificed for the removal of *soleus* muscle. Plasma was separated from the red cells and stored at -85°C until measurements were performed. Glucose and lactate concentration (control: 13 animals from three litters; low protein: 13 animals from four litters) was measured using a glucose/lactate analyzer (Yellow Springs Instrument Corp., Yellow Springs, OH, USA), while cholesterol and triacylglycerol (control: 9 animals from three litters; low protein: 3 animals from four litters) were determined using a Cobas Mira analyzer (Roche, Basel, Switzerland).

ETHICS

All experiments performed were approved by the University of Auckland Animal Ethics Committee.

STATISTICS

All statistical analyses were performed using GenStat® for Windows 5th edition or 6th edition (GenStat® Release 4.22, PC/Windows XP, Lawes Agricultural Trust, Rothamsted Research, Harpenden, Hertfordshire, United Kingdom, Version 6.1.0.210). Unless otherwise stated, analysis was a linear mixed model residual maximum likelihood (REML),

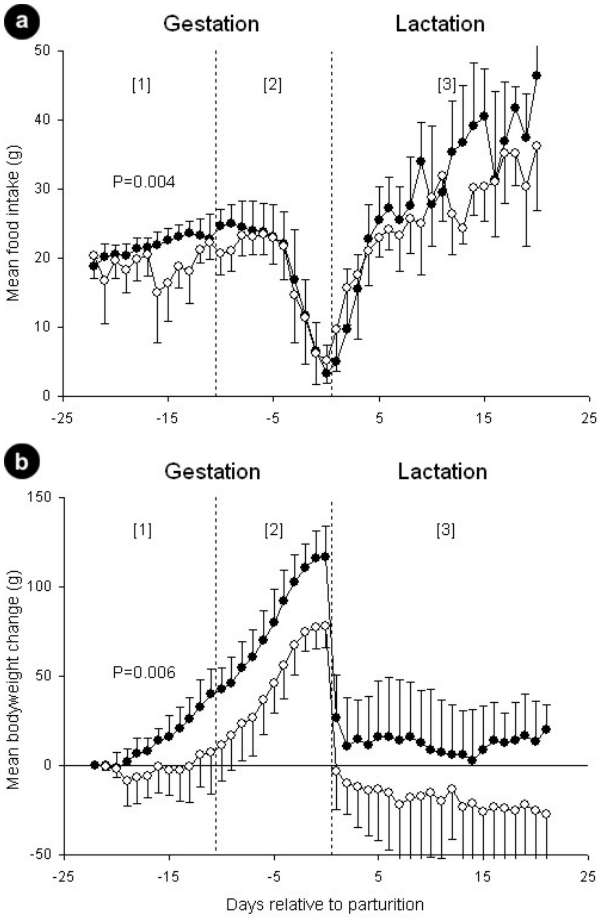

Figure 1. Food intake and body weight of female Sprague-Dawley rats fed through gestation and lactation with diets varying in protein content. Data represent mean values±SD for daily food intake (**a.**), and for change in body weight from the first day of gestation (**b.**). Mothers were fed iso-energetic diets (prepared in our laboratory) containing either 20% (control: filled circle) or 5% (low-protein: open circle) whey protein through gestation and lactation. The numbers within brackets above each figure represent the trimester of human gestation to which each indicated period is equivalent. The total number of observations was 5 and 6 mothers for the control and low-protein groups, respectively. P values indicate the trimester equivalents during which there was a significant difference between the two diet groups.

with diet being the fixed effect and litter the random effect. Unless otherwise stated, data are presented as the mean value for each of the control and low protein groups, with the mean and standard error of the difference (difference±SE) for the comparison between the two groups. Graphs show mean values and standard deviations (SD).

For the insulin-stimulated uptake of glucose into glycogen, a four-parameter logistic curve was fitted to the data with the equation:

$$y = (a - b) / (1 + (insulin/c)^{d}) + b$$

where the parameters were defined as follows:

a is the asymptote as x tends to 0 for all d greater than 0 (*i.e.* the minimum value);

b is the asymptote as x diverges (*i.e.* the maximum value);

c is the predicted response midway between the asymptotes (*i.e.* the effective insulin concentration at which the half-maximal response occurs (EC_{50}));

d is a function of the rate of change (or slope) of the fitted curve at the point of inflection.

Because the maternal diet was altered, the experimental unit in all cases was the litter; thus, the effective number of observations for each experiment was determined by the number of litters rather than the number of individual offspring. A two-tailed probability lower than 0.05 indicated a significant difference between treatments.

RESULTS

Maternal Food Intake, Body Weight and Feeding Efficiency

Food intake in the low protein group was significantly lower than the control group during the first half of gestation (control: 248 g; low protein: 219 g; difference±SE: -29±11 g; P=0.004) (Figure 1a). As with food intake, energy intake (net metabolisable energy) during the first period of gestation was significantly lower in the low protein mothers compared to those in the control group (control: 4,197 kJ; low protein: 3,708 kJ; difference±SE: -489±186 kJ; P=0.008).

Total weight gain of the mothers through gestation in the low protein group was lower than that in the control group (control: 117 g; low protein: 78 g; difference±SE: -39±9 g; P<0.001), primarily due to a difference during the first half of gestation (control: 40 g; low protein: 10 g; difference±SE: -30±11 g; P=0.006) (Figure 1b). Weight change showed no difference during the second half of gestation (control: 77 g; low protein: 68 g; difference±SE: -9±8 g; P=0.285) or through lactation (control: -23 g; low protein: -18 g; difference±SE: 5±15 g; P=0.766).

Table 2. Insulin secretion by perfused pancreases isolated from offspring of female rats fed through gestation and lactation with diets varying in protein content.

		Maternal diet		Difference±SE	P value [a]
		Control	Low-protein		
Stimulus	Glucose	57 pmol	20 pmol	-37±9 pmol	<0.001
	Arginine	13 pmol	5 pmol	-8±4 pmol	0.044
	Glucose + arginine	87 pmol	38 pmol	-49±20 pmol	0.013
Total insulin secretion		162 pmol	64 pmol	-98±28 pmol	<0.001
Mean basal insulin secretion		0.16 nmol/L	0.05 nmol/L	-0.11±0.04 nmol/L	0.005

[a] Linear mixed model residual maximum likelihood (REML)

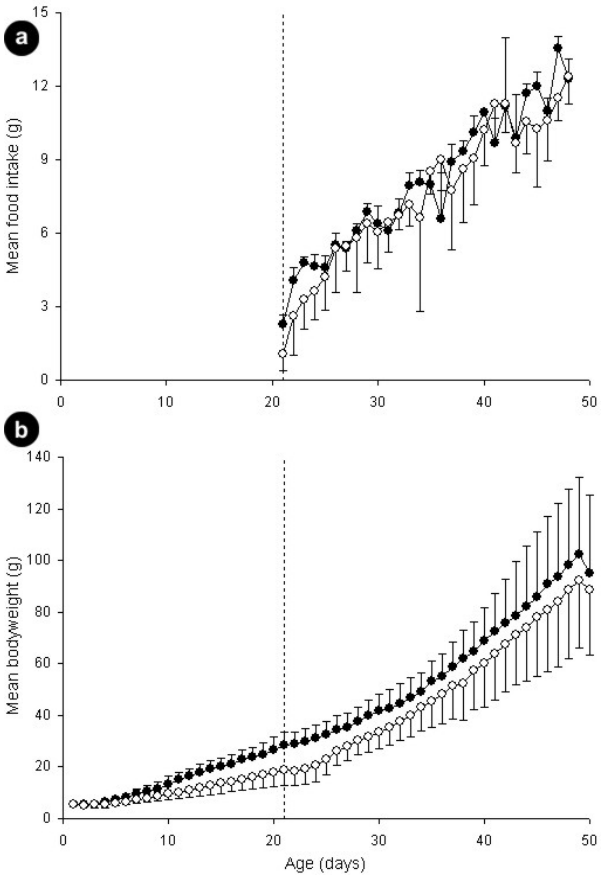

Figure 2. The effect of protein level in the maternal diet during gestation and lactation on food intake and body weight of offspring. Data represent mean values±SD for daily food intake (**a.**) and body weight (**b.**) of offspring of mothers fed isoenergetic diets (prepared in-house) containing either 20% (control: filled circle) or 5% (low-protein: open circle) whey protein through gestation and lactation. All offspring were weaned (indicated by the dashed vertical line) onto diet control. The total number of litters was 5 for control and 6 for low protein groups. There was no significant difference in pre-weaning weight gain, or post-weaning weight gain or food intake, between the two diet groups.

Feeding efficiency (EEU: efficiency of energy utilization) was significantly lower in the low protein group than the control group during gestation (control: 0.015 g/kJ; low protein: 0.011 g/kJ; difference±SE: -0.004±0.001 g/kJ; P<0.001) and lactation (control: 0.015 g/kJ; low protein: 0.008 g/kJ; difference±SE: -0.007±0.002 g/kJ; P<0.001). As with weight gain and food intake, the overall difference during gestation was mainly due to a significant difference during the first half of gestation (control: 0.009 g/kJ; low protein: 0.003 g/kJ; difference±SE: -0.006±0.003 g/kJ; P=0.016). The calculation for lactation included the weight gain by offspring during this period, as offspring growth was dependent on maternal food intake.

Offspring Food Intake and Body Weight

There was a small but significant (ranging from P=0.003 to P=0.049 for the comparison on each day) difference in body weight of the offspring between day 5 and day 15 inclusive, with the low protein offspring weighing less than those in the control group (Figure 2b). There was no significant difference with respect to birth weight (control: 5.4 g; low protein: 5.4 g; difference±SE: 0.0±0.4 g; P=0.990), total weight gain (control: 97 g; low protein: 92 g; difference±SE: -5±11 g; P=0.641), or rate of weight gain either pre-weaning (control: 1.2 g/day; low protein: 1.0 g/day; difference±SE: -0.2±0.2 g/day; P=0.257) or post-weaning (control: 2.7 g/day; low protein: 2.8 g/day; difference±SE: 0.1±0.8 g/day; P=0.760) between the two groups (Figure 2b). There was no difference between the two groups in terms of offspring

food intake (Figure 2a; control: 225 g; low protein: 203 g; difference±SE: -22±19 g;P=0.257).

Pancreatic Hormone Secretion

Table 2 shows the effect of maternal diet on the secretion of insulin from the isolated perfused pancreas. Total insulin secretion, mean basal insulin secretion and secretion in response to each of the three stimulations (glucose, arginine, and glucose plus arginine) were significantly reduced in low protein offspring. In the case of the response to glucose, both first-phase (control: 27 pmol; low protein: 9 pmol; difference±SE: -18±5 pmol; P<0.001) and second-phase (control: 30 pmol; low protein: 11 pmol; difference±SE: -19±11 pmol; P=0.001) secretion were reduced in the low protein group, while in the response to arginine, only the second-phase secretion was reduced (control: 5.3 pmol; low protein: 2.2 pmol; difference±SE: -3.1±1.4 pmol; P=0.025). In the case of the stimulation by the combined glucose plus arginine solution, the low protein offspring showed a loss of first-phase insulin secretion, both as a significant decrease compared to the control group (control: 34 pmol; low protein: 11 pmol; difference±SE: -23±7 pmol; P<0.001) and first-phase as a percentage of total secretion resulting from this stimulus (control: 40%; low protein: 30%; difference±SE: -10±2%; P<0.001).
Neither amylin secretion, nor the ratio of amylin/insulin, showed any significant difference between the two diet groups (data not shown).

Insulin-Stimulated Uptake of Glucose into Muscle Glycogen

Glucose uptake into *soleus* muscle glycogen was not significantly different in low protein animals compared to those in the control group at any point of the dose-response curve (Figure 3). There was no significant difference between the two groups with respect to minimum (P=0.459), maximum (P=0.842), EC_{50} (P=0.302) or slope (P=0.362) as determined by four-parameter logistic curve fitting.

Plasma Measurements

There was no difference between offspring of the two diet groups in terms of plasma levels of glucose (control: 4.3 mmol/L; low protein 6.7 mmol/L; difference±SE: 2.4±2.0 mmol/L; P=0.241), lactate (control: 4.1 mmol/L; low protein: 4.9 mmol/L; difference±SE: 0.8±0.8 mmol/L; P=0.301) or cholesterol (control: 2.1 mmol/L; low protein: 2.2 mmol/L; difference±SE: 0.1±2.3 mmol/L; P=0.808), but low protein offspring had a significantly lower plasma triacylglycerol concentration (control: 0.93 mmol/L; low protein: 0.50 mmol/L; difference±SE: -0.43±0.13 mmol/L; P=0.001) than control offspring.

DISCUSSION

In general, the pattern of food intake and body weight observed in the low protein mothers was as has been reported. A sharp decline in intake over the last days of gestation in the low protein group [34] was reflected in the slowing of weight gain towards the end of gestation [19], while the low intake during lactation [19, 35] caused a loss of weight

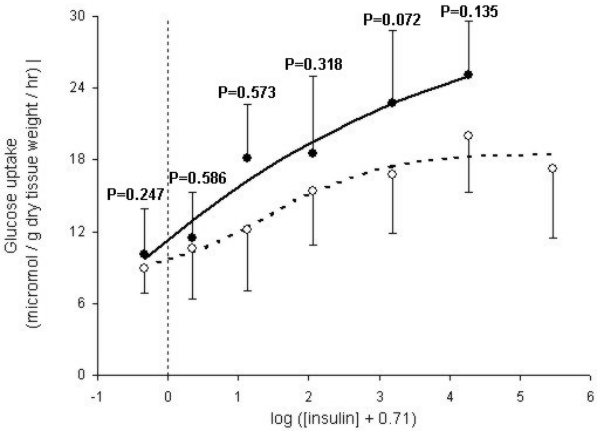

Figure 3. Incorporation of ^{14}C-glucose into *soleus* muscle glycogen of offspring of Sprague-Dawley females fed diets differing in protein content during gestation and lactation. Rates of ^{14}C glucose uptake into glycogen were determined in rat *soleus* muscle isolated from the offspring of mothers fed diets sufficient (20% whey protein; control: filled circle) or insufficient (5% whey protein; low-protein: open circle) in protein. Data represent the mean value±SD for 13 animals from three litters for control group, and 20 animals from four litters for low-protein group. There was no significant difference between the two groups at any insulin concentration.

through lactation [19]. Food intake in the low protein group during gestation was unusual in that it tended to be less than in the control group; therefore, there was no evidence of compensatory feeding [19, 20]; the restriction on intake imposed during gestation seems to have been unnecessary in this case. In fact, the lower maternal food (and therefore energy) intake in the low protein group means that the offspring of these dams were exposed to both caloric and protein restriction during early gestation. Although the caloric restriction was much less than the protein restriction, we cannot exclude the possibility that this has influenced the outcomes reported here. In future studies we would employ a more strict dietary regime such that caloric intake was the same for both diet groups.

The observed food intake of the control group mothers was unusual. While the decline in food intake for control rats before parturition (similar to that seen in the low protein group) is routinely observed, the magnitude of this decline, and the low intake throughout lactation, are at variance with our own studies using standard laboratory chow (data not shown), and previously reported data [36, 37]. This suggests that the protein content of the diet is not the only factor controlling food intake. The pattern of maternal body weight in the control group reflected the pattern of food intake, with a slowing in growth rate at the end of gestation and an average weight loss of over 20 g per dam through lactation, neither of which have been observed in previous studies using a similar diet [20, 36].

Offspring in both diet groups (all of which were weaned onto control diet) consumed similar amounts of food through the duration of the trial, but this amount was significantly less than we observed in animals fed standard laboratory chow (data not shown). Body weight in both groups was similar, with the slightly lower weight observed in the low protein group from day 5 to day 15 presumably a reflection of the tendency to lower food consumption in the low protein mothers through lactation. In both cases, body weight was significantly less than previously reported values [20].

In the isolated perfused pancreas, low protein offspring showed evidence of impaired pancreatic function in response to the main insulin secretagogue (glucose) and a membrane depolarizing agent (arginine). In this particular experiment, there was a significant difference in each of the stimulations applied to the pancreas. The loss of glucose-induced insulin secretion is in agreement with previous studies showing a decreased insulin secretion both *in vivo* and using an *in vitro* isolated islet system [38]. There was also an impairment of first-phase insulin secretion in response to a combined glucose/arginine stimulation. A loss of first phase glucose-induced insulin secretion is a feature of the early stages of type 2 diabetes [39, 40] and could be due to either exhaustion of the labile pool of insulin granules or a time-dependent inhibition of the secretion response [39]. There is evidence that the islet size, proliferative capacity and vascularization [15], as well as the amount of beta cells within each islet and the percentage of islet tissue and beta cells within the pancreas [41] are all reduced in the pancreases of offspring of rat dams fed a low protein diet during gestation [15, 41] and/or lactation [41]. The lower insulin secretion observed in low protein offspring may be due to one or more of these factors.

Previous studies show an inhibitory effect by amylin on insulin secretion [42, 43] at a concentration as low as 75 pmol/L, and it was for this reason that amylin secretion was measured. Secretion levels higher than 100 pmol/L were observed in low protein offspring during the basal phase, although there was considerable variation between animals. Because of this variability, it is unclear whether or not amylin is impairing the insulin secretion from low protein offspring. Additional studies employing, for example, a specific amylin antagonist such as the amylin$^{8-37}$ molecule (a truncated peptide of native rat amylin in which the first seven amino acids are absent) [44] would be helpful in clarifying this.

A number of studies have been performed which suggest that an increase in peripheral

insulin sensitivity in the offspring of protein-restricted rats may be an adaptive response to a reduction of insulin secretion. These include both low protein [45] and protein-free [46] diets during early lactation only, and low protein during both gestation and lactation [38]. However, in the current study, there was no evidence of an increase in insulin sensitivity in the low protein offspring. The reasons for this observation are unclear; it would be expected that decreased insulin secretion would lead to a higher plasma glucose concentration in the absence of any change in insulin sensitivity. No such change in plasma glucose was observed, although the insulin sensitivity of tissues such as liver and adipose was not measured in the experiments reported here. Further studies incorporating such measurements are required to clarify this issue.

Finally, the observation that fasting plasma triacylglycerol levels were lower in low protein offspring is in agreement with previous studies [47], suggesting that in this instance a maternal low protein diet during gestation and lactation may program risk for diabetes, but not for vascular disease, as may be the case for early human undernutrition. The possibility cannot be excluded that changes relevant to vascular disease may occur after 50 days of age, the point at which these experiments ended.

CONCLUSION

Overall, these results show a decrease in pancreatic insulin secretion in the offspring of female rats fed a low whey protein diet during gestation and lactation; such a decrease is associated with the early development of type 2 diabetes in humans and suggestive of an increased susceptibility to the development of this disease in adult life, as has been observed in similar studies using casein as a protein source. This observation is in accordance with the hypothesis that the fetal/neonatal environment plays a role in the programming of type 2 diabetes.

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Abbreviations EC_{50}: concentration of agonist required to provoke a response halfway between the baseline and maximum responses; EEU: efficiency of energy utilization

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