Early protein restriction and obesity independently induce hypertension in 1-year-old rats

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1. Recent studies have revealed a link between fetal and early post-natal growth retardation and the development of features of the insulin resistance syndrome in later life. Obesity is also a strong risk factor for this syndrome. The aim of this study was to assess whether maternal and early protein restriction, which causes growth retardation, and obesity are risk factors that are independent for the development of certain features of the insulin resistance syndrome, especially hypertension.

2. Pregnant Sprague-Dawley rats were given either 20% or 8% protein isocaloric diets throughout pregnancy and lactation. Female offspring were weaned onto the same diets as their mothers and they remained on these diets until 70 days of age. Half the rats were then given standard laboratory chow, whilst the remainder were fed a highly palatable cafeteria-style diet. Rats were maintained on these diets for the remainder of the study.

3. Rats given the 8% protein diet remained physically lighter than comparable animals fed the 20% protein diet throughout the study. In contrast, cafeteria-fed rats showed excessive weight gain. At 1 year of age the rats had their systolic blood pressures and fasting lipids measured, as well as undergoing an intraperitoneal glucose-tolerance test.

4. Cafeteria-fed rats had worse glucose tolerances than controls and hypertriacylglycerolaemia. The early 8% protein rats had significantly increased blood pressures, as did the cafeteria-fed rats. These increases were additive, suggesting that early protein restriction, and later obesity, are indeed independent risk factors for the development of hypertension.

INTRODUCTION

Results from a number of studies have established an association between low birth weight and the subsequent development of adult degenerative diseases. Firstly, it was found that growth retardation during fetal life and infancy was linked to death rates from cardiovascular disease in adult life [1]. The processes involved in this association may involve hypertension, as it was subsequently found that low birth weight predicts adult hypertension [2, 3]. This alteration in blood pressure appears to be detectable in childhood and is amplified with age [4]. Impaired glucose tolerance and type 2 (non-insulin-dependent) diabetes mellitus (NIDDM) are also risk factors for cardiovascular disease and a strong link was found between their development and reduced weight at birth and at age 1 year [5]. The strongest association of all between low birth weight and adult degenerative disease, however, was found to be with the insulin resistance syndrome [6]. Thus in a study of 64-year-old men in Hertfordshire, U.K. the odds ratio of those born lightest for the presence of the insulin resistance syndrome was 18 in comparison with a value of 1 for those born heaviest [6].

Low birth-weight is a proxy for a variety of intra-uterine influences. The ‘thrifty phenotype hypothesis’ [7] suggests that nutritionally induced fetal growth retardation can lead to changes in cellular structure and function in a developing fetus, thus rendering it more susceptible to the features of the insulin resistance syndrome in later life. The timing of the onset of these features is suggested as depending upon later factors, such as adult obesity, aging and physical inactivity. Obesity itself is strongly associated with the insulin resistance syndrome [8]. Thus in the San Antonio Heart Study, by the fifth decade of life four-fifths of the obese subjects had hypertension and impaired glucose tolerance and more than four-fifths of the subjects with NIDDM were hypertensive and obese [9]. Of those subjects who were hypertensive around three-quarters were obese and one half had abnormal glucose tolerance [10].

Growth-retarded new born infants have been shown to have reduced numbers of pancreatic β-cells and a reduction in insulin secretion [11]. This has been modelled in experimental rats by a maternal or early protein calorie malnutrition [12] and by maternal protein deficiency alone [13]. It was hypothesized that the impairment in insulin secretion may predispose a rat to the development of dia-
betes [12]. Subsequent studies in rats have modelled the findings in humans by showing that maternal protein restriction can lead to fetal growth retardation [13, 14], hypertension [14] and impaired glucose tolerance [15] in the offspring. The aim of the present study was to test the ‘thrifty phenotype hypothesis’ [7], which suggests that intrauterine and early post-natal growth retardation (modelled in this study by maternal and early protein restriction) and adult obesity (modelled by feeding rats a highly palatable cafeteria-style diet) are independent risk factors for the development of features of the insulin resistance syndrome.

METHODOLOGIES

Virgin female Sprague–Dawley rats, weighing 240–255 g, were caged individually and maintained on a 12:12 h light–dark cycle at 22°C. After mating, on the first day of pregnancy (taken as when a vaginal plug was expelled), rats were fed either a 20% (‘control’) protein diet (n = 13) or an isocaloric 8% (‘low protein’) diet (n = 18) (Hope Farms BV, Hoge Rijndijk 14, 3440 HD Woerden, The Netherlands) (Table 1). The rats were maintained on these diets throughout the gestational and suckling periods. When the female offspring reached 28 days of age they were weaned onto the same diets as their mothers. At 70 days of age half of the rats were given Porton Combined Diet (‘pellet’) (Special Diet Services, Witham, Essex, U.K.); this diet contains 17.9% protein, 55.0% carbohydrate and 2.7% fat by weight. The remaining rats were fed a high-fat cafeteria-style diet (‘cafeteria’). This was a modified version of the diet described by Wilding et al. [16] and was designed to be highly palatable so that the rats ate relatively large amounts of it. It contained 330 g/kg ground Porton Combined Diet, 330 g/kg Nestlé's full fat sweetened condensed milk, 70 g/kg sucrose and 270 g/kg water. This cafeteria diet provided 16.7% of its energy content as protein, 67.3% as carbohydrate and 16.0% as fat. In comparison, the pellet diet provided 28.7% of its energy content as protein, 61.7% as carbohydrate and 9.6% as fat. The rats remained on these diets until the study time points and throughout the study were allowed to eat ad libitum. All rats had free access to drinking water throughout. Animal maintenance and experimentation was performed according to the Animals (Scientific Procedures Act) (1986).

At 140 days of age, eight rats from each of the four dietary combination groups (and from different litters) were killed by carbon dioxide inhalation. Body weights and lengths were measured and then the kidneys and hearts were removed and their weights recorded. The remainder of the rats (31 in total) underwent intraperitoneal glucose tolerance tests and had their systolic blood pressures measured at 1 year of age.

Systolic blood pressures were determined by recording tail vein pulsates at 29°C in conscious rats (Blood pressure monitor; Linton Instrumentation, Diss, Norfolk, U.K.) [14]. Before measurement, in order to minimize stress, the rats were trained to enter the Perspex restraining tube (each rat entering the tube three times on three separate occasions was generally found to be sufficient). All measurements were taken in the early evening after staff had left so that extraneous noise was minimized. Due to the reading of the blood pressure traces being somewhat subjective, each blood pressure trace was coded and read by an independent investigator not involved in obtaining the traces. Each of the rats had five blood pressure traces recorded in a single session. The highest and lowest results were discarded and the blood pressure recorded as the mean of the three remaining results. One rat had its blood pressure measured at each of the evening sessions, the recordings having a coefficient of variation of 3.2% (n = 7).

Intraperitoneal glucose-tolerance tests were performed after a 14 h fast. Conscious rats were injected intraperitoneally with 1 ml/100 g of 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 analyzer (Hemocue, Sheffield, U.K.) 0, 15, 30, 60, 120 and 180 min after the glucose injection. Fasting blood samples were also collected into heparinized tubes for the measurement of plasma insulin, corticosterone (samples being collected around 10.00 hours), total cholesterol, free glycerol and triacylglycerols. Insulin and corticosterone were measured by RIA according to the manufacturer’s instructions (using a Linco rat insulin kit purchased from Biogenesis Ltd., Poole, Dorset, U.K.) and a rat corticosterone kit purchased from Amersham International plc, Little Chalfont, Bucks., U.K.). Plasma total cholesterol [17], free glycerol and triacylglycerol concentrations [18] were measured using standard laboratory techniques with kits purchased from Sigma Chemical Co., Poole, Dorset, U.K.

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Table 1. Composition of the diets fed to pregnant female rats throughout their gestational and suckling periods (g/100 g dry weight of diet)

<table>
<thead>
<tr>
<th>Diet</th>
<th>Control</th>
<th>Low protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral and vitamin mixture</td>
<td>5.05</td>
<td>5.45</td>
</tr>
<tr>
<td>Casein (88 g of protein/100 g)</td>
<td>22.00</td>
<td>9.00</td>
</tr>
<tr>
<td>d,l-Methionine</td>
<td>0.20</td>
<td>0.08</td>
</tr>
<tr>
<td>Maize starch</td>
<td>8.00</td>
<td>6.00</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>4.30</td>
<td>4.30</td>
</tr>
<tr>
<td>Cerelose (glucose)</td>
<td>55.15</td>
<td>68.17</td>
</tr>
<tr>
<td>Overall composition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (g/100 g dry weight)</td>
<td>20.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Carbohydrate (g/100 g dry weight)</td>
<td>63.2</td>
<td>76.2</td>
</tr>
<tr>
<td>Fat (g/100 g dry weight)</td>
<td>4.3</td>
<td>4.3</td>
</tr>
<tr>
<td>Energy (kJ/100 g dry weight)</td>
<td>1537</td>
<td>1528</td>
</tr>
</tbody>
</table>
Protein restriction, obesity and hypertension

Statistical analysis

All statistical analyses were performed using the Statistica for Mac (version 4.1) software package (Statsoft, Letchworth, Herts., U.K.). Two-way analysis of variance was used to analyse the data, with early and adult diets being used as the independent variables. If appropriate, the data were log-transformed before analysis to enable the appropriate use of parametric statistical tests. Litter sizes, body weights and food intakes are expressed as means (SD) and data from the different groups are compared using either Student’s t-test or two-way analysis of variance.

RESULTS

Litter sizes from dams given the control or low-protein diets were not significantly different: control 14.2 (2.4) pups and low-protein 14.7 (2.7) pups (P = 0.640). Two days after birth, however, the low-protein pups had significantly lower body weights than the control pups: 5.41 (0.45) g compared with 6.50 (0.95) g (P < 0.001). At weaning (28 days of age) this difference in body weights was more pronounced: low protein 35.84 (7.39) g compared with control 53.87 (14.19) g (P < 0.0001).

Body and organ weight data for the 140 day old rats are shown in Table 2. Between weaning and being given the pellet or cafeteria diets the total food intakes of these low-protein rats were significantly lower than those of the controls: low protein 520.7 (89.3) g compared with control 682.9 (49.4) g (P < 0.0001); both n = 16). Immediately before the pellet or cafeteria diets were given (70 days of age, body weights were: low protein 164.7 (24.8) g and control 255.3 (21.7) g (P < 0.0001; both n = 16). During the period between 70 and 140 days of age the total food intakes of the different groups were: control rats given the pellet diet 1553.8 (88.3) g, control rats given the cafeteria diet 2813.8 (248.9) g, low-protein rats given the pellet diet 1365.5 (128.9) g and low-protein rats given the cafeteria diet 2283.2 (160.9) g (all groups n = 8 from eight different litters). Control animals ate significantly more pellet or cafeteria diet than low-protein animals (P < 0.0001). Cafeteria-fed animals ate significantly more than pellet-fed animals (P < 0.0001). This increased food intake in cafeteria-fed rats was significantly greater in the control than in the low-protein animals (interaction P = 0.007). The early low-protein rats were still significantly lighter than the controls at 140 days of age (P < 0.001) (Table 2). In contrast, the cafeteria-fed rats were significantly heavier than the pellet-fed rats (P < 0.001). This increase in body weight due to the cafeteria feeding was greater in the control animals than in the low-protein animals (interaction between early and later diets P = 0.009). These changes were similar to changes in body length (early low-protein animals being shorter than controls P < 0.0001; cafeteria-fed animals being longer than pellet-fed animals P < 0.001; interaction P = 0.299). Overall body mass indices were therefore significantly higher in the cafeteria-fed rats than in the pellet-fed rats (P < 0.0001). In contrast, the early low-protein rats had significantly lower body mass indices than the control rats (P < 0.0001). The association of cafeteria feeding with higher body mass indices was more pronounced in the control rats than in the low-protein rats (interaction P = 0.014).

Kidney weights were lower in the animals given the early low-protein diet (P < 0.0001). The reduction in kidney weights was still significant when expressed relative to body weights (P = 0.017). Early low-protein rats had significantly smaller heart weights (P < 0.0001), whereas there was a significant increase in heart weights associated with the use of the cafeteria diet (P < 0.001; interaction P = 0.150). Relative to body weights, the heart weights significantly decreased with the use of the cafeteria diet (P < 0.001). A similar trend was seen in low-protein animals (P = 0.055; interaction P = 0.151).

Body weights of the rats at 1 year of age were: control pellet 421.7 (31.5) g (n = 7), control cafeteria 799.9 (131.3) g (n = 6), low-protein pellet 333.7 (23.6) g (n = 9) and low-protein cafeteria 594.1 (127.1) g (n = 9) (early low-protein animals being

Table 2. Body and organ weight data of 140 day old female rats in the four dietary groups. Data are means (SD). Control or low-protein diets were fed to the mothers of these rats during their pregnancy and lactation, and then to these rats until they were 70 days of age. After this the rats were fed either the pellet or cafeteria diets. Each group contained eight animals from eight different litters. *P < 0.05, **P < 0.001 associated with the early diet used (control/low protein). †P < 0.001 associated with the adult diet used (pellet/cafeiteria).

<table>
<thead>
<tr>
<th>Dietary groups</th>
<th>Control pellet</th>
<th>Control cafeteria</th>
<th>Low-protein pellet</th>
<th>Low-protein cafeteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)***††</td>
<td>305.7 (24.9)</td>
<td>428.3 (39.0)</td>
<td>254.6 (34.3)</td>
<td>309.6 (35.9)</td>
</tr>
<tr>
<td>Body length (cm)***††</td>
<td>22.7 (0.6)</td>
<td>23.8 (0.5)</td>
<td>21.4 (0.7)</td>
<td>22.0 (0.7)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)***††</td>
<td>5.9 (0.4)</td>
<td>7.6 (0.4)</td>
<td>5.5 (0.4)</td>
<td>6.4 (0.5)</td>
</tr>
<tr>
<td>Kidney weight (g)***</td>
<td>2.22 (0.21)</td>
<td>2.49 (0.19)</td>
<td>1.71 (0.23)</td>
<td>1.67 (0.15)</td>
</tr>
<tr>
<td>Kidney weight (as % of body weight)††</td>
<td>0.726 (0.047)</td>
<td>0.585 (0.051)</td>
<td>0.672 (0.054)</td>
<td>0.544 (0.057)</td>
</tr>
<tr>
<td>Heart weight (g)***††</td>
<td>1.03 (0.07)</td>
<td>1.23 (0.11)</td>
<td>0.76 (0.10)</td>
<td>0.88 (0.09)</td>
</tr>
<tr>
<td>Heart weight (as % of body weight)††</td>
<td>0.338 (0.038)</td>
<td>0.291 (0.020)</td>
<td>0.307 (0.015)</td>
<td>0.286 (0.019)</td>
</tr>
</tbody>
</table>
The systolic blood pressures of these rats is shown in Figure 1. Mean (SD) systolic blood pressures were: control pellet 128 (25) mmHg, control cafeteria 157 (29) mmHg, low-protein pellet 158 (28) mmHg and low-protein cafeteria 182 (40) mmHg. Early low-protein rats had significantly raised systolic blood pressures (P < 0.001; interaction P = 0.110). The systolic blood pressures of these rats is shown in Figure 1. Mean (SD) systolic blood pressures were: control pellet 128 (25) mmHg, control cafeteria 157 (29) mmHg, low-protein pellet 158 (28) mmHg and low-protein cafeteria 182 (40) mmHg. Early low-protein rats had significantly raised systolic blood pressures (P = 0.026; interaction P = 0.815). These rises were independent and additive, so that the highest systolic blood pressures were generally in the group of early low-protein rats who were subsequently given the cafeteria diet.

The mean blood glucose results from the glucose-tolerance tests is shown in Figure 2. The area under the curves of the different groups [geometric mean (95% confidence interval)] were: control pellet 3257 (3024–3508) units, control cafeteria 3848 (3222–4586) units, low-protein pellet 3505 (3235–3798) units and low-protein cafeteria 3758 (3240–4313) units. The cafeteria-fed rats had significantly worse glucose tolerances than pellet-fed rats (P = 0.027), but there was no significant alteration in glucose tolerance in early low-protein rats in comparison with controls (P = 0.645; interaction P = 0.310).

Fasting plasma hormone and lipid concentrations from the 1-year-old female rats are shown in Table 3. The use of the cafeteria diet was associated with a significant increase in fasting plasma insulin concentration (P = 0.003). Early low-protein animals showed a tendency to have lower fasting plasma insulins (P = 0.061; interaction P = 0.957). Cafeteria-fed rats tended to have lower 10.00 hours plasma corticosterone concentrations than pellet-fed animals, but there was no detectable difference associated with the different early diets used (association with the use of the low-protein diet P = 0.543; association with the use of the cafeteria diet P = 0.146; interaction P = 0.527). There was a tendency for cafeteria-fed animals to have higher plasma total cholesterol concentrations than pellet-fed animals (P = 0.157), but no change could be attributed to alterations in the early diet used (P = 0.871; interaction P = 0.844). The use of the cafeteria diet was associated with a significant increase in fasting plasma free-glycerol concentrations (P < 0.0001). No such detectable alteration was observed with the use of the low-protein diet (P = 0.404; interaction P = 0.108). Cafeteria-fed animals also had significantly increased fasting plasma triacylglycerol concentrations (P < 0.001), but no significant changes could be attributed to the early use of the low-protein diet (P = 0.490; interaction P = 0.330).

**DISCUSSION**

The ‘thrifty phenotype hypothesis’ [7] suggests that intrauterine growth retardation renders a growing fetus more susceptible to the development of the insulin resistance syndrome, and that the time of onset and severity of the condition depends upon factors encountered in adult life, such as obesity. The present study was performed to model these changes and to investigate whether any of the features of the insulin resistance syndrome could be detected in the middle-aged (1-year-old) rat. Intrauterine growth retardation of the rat pups was produced by feeding pregnant rats a diet containing a little under half the protein content of, but iso-caloric with, the control diet. The use of the diet does not affect the fertility of the rats, but the resulting offspring are significantly lighter than offspring from dams fed the control diet [19]. In the present study, obesity was produced by feeding the rats a cafeteria-style diet, such that by 140 days of
age their body mass indices were more than 20% higher than those of rats fed a standard laboratory chow diet. The relative differences in body weights of the rats in the four dietary combination groups at this age were mirrored in the rats studied at 1 year of age.

The early low-protein rats had a marked increase in their systolic blood pressures. In the study carried out by Langley and Jackson [14], maternal protein intake was restricted only during pregnancy, and their rat offspring also developed raised systolic blood pressures, suggesting that this is the critical period in which blood pressure is programmed. Experiments are currently underway to define more precisely the critical period for such programming in our model. The increase in systolic blood pressure in low-protein rats was of a similar magnitude to that produced by making the rats obese. The increases were additive, so that the highest blood pressures were generally found in the early low-protein group who were subsequently cafeteria-fed. This suggests that the two risk factors are independent and may operate through different mechanisms. Glucose tolerance was worse in the cafeteria-fed rats. They also exhibited a marked hypertriglyceridemia. A degree of insulin resistance in these rats is suggested by their higher fasting plasma insulin concentrations. Unlike in a previous study [15], the early low-protein rats did not show a detectable deterioration in glucose tolerance. However, in the present study the rats were considerably younger than those in which impaired glucose tolerance was reported. Interestingly, in a study of individuals where hypertensive and NIDDM coexisted, the diagnosis of hypertension preceded the diagnosis of diabetes eight times more often than in the opposite direction [20]. Thus the development of glucose intolerance in the present model is not precluded from happening at a later age, and this dietary regime followed in the rat may be a useful model of the human situation. This is consistent with the ‘thrifty phenotype hypothesis’, where it has been hypothesized that the exact timing of early growth impairment (combined with effects in adult life, such as physical inactivity and the development of obesity) is important in determining whether hypertension, impaired glucose tolerance or both conditions present subsequently [7].

Findings from human studies also suggest that both intrauterine growth retardation and adult obesity are important independent risk factors for the insulin resistance syndrome. In a study of 370 64-year-old men, those men who had impaired glucose tolerance or newly diagnosed NIDDM were both born lighter and had significantly higher current body mass indices than men with normal glucose tolerance [5]. The glucose intolerant men also had significantly raised systolic and diastolic blood pressures. In a survey of over 3000 36-year-olds, high blood pressure was significantly associated with both low birth weight and high current body mass indices [21]. The finding in the present study, that the effects of early growth retardation and later obesity on blood pressure are additive, suggests that in a low birth weight individual who is already at significant risk of having raised blood pressure, further risk of hypertension can be avoided by keeping body weight down. This may be particularly important, as previous studies have shown that early and adult nutrition may interact to lead to conditions conducive to the deposition of body fat. In one group of normotensive and non-diabetic young adults, birth weight was inversely related to truncal fat deposition [22]. Also, in a study of growth rates of children recovering from a period of protein calorie malnutrition, when they reached their expected weight for their height they had increased body fat in comparison with children who had been adequately nourished throughout [23]. It has been suggested that the period of malnutrition imposes mechanisms of nutritional thrift upon an individual, which become detrimental to health if the individual encounters nutritional abundance [7].

The mechanism by which early low-protein may lead to hypertension is not clearly understood. Maternal low protein has been shown to reduce islet vascularization of the pancreas in rats [13], and it may be that maternal low protein is associated with a generalized alteration in vascularization which predisposes an individual to hypertension. In a recent
study of 210 subjects aged 8–24 years, systolic blood pressures were inversely related to birth weight and left ventricular masses were inversely related to body weights at 9 months and 2 years [24]. This increase in left ventricular mass may have been necessary to overcome the resistance to blood flow caused by an altered vascular tone in growth-retarded individuals. Hypertension would then be the result of a relative over-compensation. In the present study, an increase in left ventricular mass of the rat hearts may have been expected to cause an increase in the heart weights [25]. However the 140-day-old low-protein rats did not have increased heart weights, even when expressed relative to body weights. This suggests that this may not be the mechanism by which early protein restriction led to an increase in blood pressure.

An alternative explanation for early protein restriction leading to hypertension involves growth retardation of the kidneys. Kidney growth has been shown to be particularly compromised in rat models using maternal protein restriction [26]. It has been hypothesized that fetal renal growth retardation causes a deficiency of nephrons at birth, leading to an increased susceptibility to hypertension [27]. Further, it has been suggested that when kidney growth lags behind somatic growth, sodium retention is favoured, predisposing an individual to hypertension [28]. Data from the present study are consistent with these theories. The kidney weights of the low-protein rats were substantially reduced in relation to those of control rats. The low-protein rats also had lower body weights, but when their kidney weights were expressed relative to their body weights they were still lower than those of the controls. If the mechanism of the early protein-restriction-associated hypertension involves a sodium-retentive renal mechanism, then it is predicted that further studies, involving salt-loading the rats, would result in blood pressure differences being exaggerated.

In summary, this study has shown that early protein restriction and later diet-induced obesity are independent risk factors for the development of hypertension in rats. At 1 year of age, the early low-protein animals did not show any detectable changes in glucose tolerance or plasma lipids. However, cafeteria-fed animals had worse glucose tolerances than animals fed a standard laboratory chow and also exhibited hypertriacylglycerolaemia. Thus early low-protein rats who were cafeteria-fed were short and obese, with marked hypertension and mild glucose intolerance and hypertriacylglycerolaemia, key features of the insulin resistance syndrome.

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REFERENCES


