Maternal undernutrition reduces aortic wall thickness and elastin content in offspring rats without altering endothelial function

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ABSTRACT

Epidemiological studies suggest a link between fetal/early infant nutrition and adult coronary artery disease. In the present study, we examined the effects of altering nutrition during gestation, lactation and juvenile life on aortic structure and function in rats. Wistar rat dams were fed either a control or low-protein diet throughout pregnancy, or a low-protein diet for the final 7 days of gestation only. At 21 days post-partum, male pups were weaned on to a control, low-protein or high-fat diet. At 12 weeks, the offspring rats were killed. In 46 rats, aortic sections were mounted and stained to assess media thickness and elastin content. In a further 38 rats, aortic rings were suspended in an organ bath and vascular reactivity was tested with dose–response curves to the endothelium-dependent dilator acetylcholine and the endothelium-independent dilator sodium nitroprusside. Rats exposed to maternal protein restriction while in utero had a significantly decreased aortic wall thickness compared with control rats (P = 0.005). Total elastin content of the aorta was also decreased by both maternal low-protein (P = 0.02) and early postnatal low-protein (P = 0.01) diets. Neither maternal nor postnatal low-protein or high-fat diets, however, resulted in any significant changes in arterial dilator function. In conclusion, fetal undernutrition in rats, induced via a maternal low-protein diet, causes a decrease in aortic wall thickness and elastin content without altering aortic dilator function. These changes in vascular structure may amplify aging-related changes to the vasculature and contribute to the pathophysiology of the putative link between impaired fetal growth and adult cardiovascular disease.

INTRODUCTION

Recent research has suggested a significant association between impaired fetal growth and increased rates of adult cardiovascular disease [1–5]. Although the exact mechanisms that underlie this association remain unclear, changes in sympathetic nervous system activity and kidney structure and function have been implicated [6,7].

Key early contributors to the pathogenesis of atherosclerosis and hypertension include vascular endothelial dysfunction and arterial wall thickening. The first physical signs of atherosclerosis occur in the aorta [8]. These include fatty lesions and increases in wall thickness. Both endothelial function and wall thickness of conduit arteries, such as the aorta and carotid, are used as indicators of total atherosclerotic burden, and predict the risk of future clinical cardiovascular disease events in humans [9–11].

Indeed, we have recently demonstrated that reduced fetal growth is associated with increased aortic wall thickness...
thickness in newborn infants [12]. Post-mortem studies in children by Napoli et al. [13] have described a corresponding inverse relationship between the extent of fatty lesions in the abdominal aorta and birthweight, such that the extent of lesions was greater in those children with lower birthweights. Furthermore, arterial endothelial function is impaired in children and young adults with low birthweights [14,15].

In addition to traditional atherosclerosis progression, it has been proposed that reduced arterial elastin, which is primarily produced while in utero and during early postnatal life, may link impaired fetal growth with adult hypertension and subsequent cardiovascular disease [16,17].

The role that postnatal growth plays in contributing to adult disease is also a potential confounding factor when studying the effects of fetal growth in adults. There seems to be significant interaction between these factors and the risk of adult cardiovascular disease, such that it is those individuals who have a low birthweight and then rapidly gain weight and develop adulthood obesity who appear to have the greatest risk of cardiovascular events [1,3,18].

Therefore our primary hypothesis was that a maternal low-protein diet would alter aortic structure and function in the offspring. Furthermore, we also examined the influence of postnatal diet, given the epidemiological data linking early postnatal growth with cardiovascular risk. Thus we studied the effects of altering both maternal and early postnatal diet on aortic structure and function in rats.

## MATERIALS AND METHODS

### Experimental animals

Time-mated Wistar rat dams obtained from ARC (Perth, Australia) were placed on either the CON (control) or LP (low-protein) diet on arrival and remained on this diet for the duration of gestation and lactation. A third group received the CON diet for the first 2 weeks of pregnancy, but was then switched to the LP diet for the last week of pregnancy and during lactation (LP in the final trimester group). Both of the maternal diets were isocaloric and dams consumed less than the 40 g (650 kJ) per day offered, thus being fed *ad libitum*.

At birth, litters were culled to a maximum of 14 pups. Litter sizes were similar between the maternal diet groups (aortic structure studies, 13.8 ± 1.0, 13.8 ± 0.4 and 13.8 ± 0.9 pups in the CON, LP throughout pregnancy and LP in the final trimester groups respectively; aortic function studies, 12.6 ± 1.2 and 11.3 ± 0.5 pups in the CON and LP throughout pregnancy groups respectively; values are means ± S.E.M.). At weaning (21 days), the gender of rats was determined and male offspring were then fed one of the experimental diets for the remainder of the study (i.e. only male pups were studied). From weaning, rats were offered up to 650 kJ/day. Monitoring of food intake during the function component of the study indicated that all offspring rats consumed less than this amount, and thus were fed *ad libitum*. All rats were housed in a 12-h light/12-h dark-cycle room maintained at 22 °C.

On the study day at 12–13 weeks of age, the length and weight of rats was measured. Rats were subsequently anaesthetized with an intraperitoneal injection (100 mg/kg of body weight) of pentobarbitone (Nembutal; Merial, Australia).

This study was approved by the Ethics Committee of the University of Sydney.

### Diets

Experimental diet details were as follows: CON, −20 % protein and 4 % fat; LP, −8 % protein and 4 % fat; and HF (high fat), −20 % protein and 25 % fat. Composition of each diet is shown in Table 1.

The HF diet used in the functional component of this study differed slightly from the HF diet used in the structure studies, being lower in total cellulose and higher in both sucrose and dextrose. These minor changes were designed to increase the energy density of the diet and thus increase consumption and maximize effects.

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**Table 1 Dietary composition**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>CON diet</th>
<th>LP diet</th>
<th>Aortic structure study</th>
<th>Aortic function study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>220</td>
<td>90</td>
<td>220</td>
<td>190</td>
</tr>
<tr>
<td>Methionine</td>
<td>3</td>
<td>0.8</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Oil*</td>
<td>43</td>
<td>43</td>
<td>252</td>
<td>220</td>
</tr>
<tr>
<td>Dextrose monohydrate</td>
<td>557</td>
<td>689.2</td>
<td>97</td>
<td>210</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>200</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td>301</td>
<td>50</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Minerals†</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamins‡</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

* sunflower oil was used in the HF diet for the aortic function component of study. Soybean oil used for all other diets.

† AIN-76 mineral mixture containing per kg: 500 g of dibasic calcium phosphate, 74 g of NaCl, 220 g of potassium citrate monohydrate, 52 g of potassium sulfate, 24 g of magnesium oxide, 3.5 g of magnesium carbonate, 6 g of ferric citrate, 1.6 g of zinc carbonate, 0.3 g of cupric carbonate, 0.01 g of potassium iodate, 0.01 g of sodium selenite, 0.35 g of chromium potassium sulfate and 118 g of sucrose.

‡ AIN-76A vitamin mixture containing per kg: 0.6 g of thiamine HCl, 0.6 g of riboflavin, 0.7 g of pyridoxine HCl, 3 g of niacin, 1.6 g of calcium pantothenate, 0.2 g of folic acid, 0.02 g of biotin, 1 g of vitamin B12 (0.1 %), 0.8 g of vitamin A palmitate (500000 IU [international units]/g), 0.25 g of vitamin D3 (400 IU/g), 10 g of vitamin E acetate (500 IU/g), 0.08 g of menadione sodium bisulfite and 981.15 g of sucrose.
After the rats were killed, the thoracic aorta was excised and stored in ice-cold oxygenated Krebs solution (118.3 mmol/l NaCl, 4.7 mmol/l KCl, 2.3 mmol/l CaCl₂, 1.2 mmol/l MgSO₄, 1.2 mmol/l KH₂PO₄, 25 mmol/l NaHCO₃, 11.3 mmol/l glucose and 0.03 mmol/l EDTA, pH 7.2). The thoracic aorta was then cleared of fat and connective tissue, and cut into 2-3-mm-long rings with care taken to minimize damage to the endothelial layer. Between two and four aortic rings were obtained from each rat. Each ring was connected to a force transducer and suspended in an organ bath containing 25 ml of Krebs solution. During the entire study the organ bath was continuously gassed with 95% oxygen/5% carbon dioxide, and kept at a constant temperature of 37°C. After 15 min, rings were gradually stretched to a tension of 2 g. The rings were then allowed to equilibrate for a further 45 min, with a minimum of three washes during this period.

Vascular reactivity was then assessed as described previously [19]. Briefly, incremental doses of noradrenaline (1 × 10⁻⁹–1 × 10⁻⁵ mol/l) were added to the bath to obtain a dose–response curve. The rings were again allowed to equilibrate for a further 45 min with washes every 15 min, before being precontracted with a dose of noradrenaline, equivalent to the EC₆₀–EC₈₀ from the previous dose–response curve. Cumulative doses of acetylcholine (1 × 10⁻⁹–1 × 10⁻⁵ mol/l), an endothelium-dependent vasorelaxing agent, were then added to produce a dose–response curve. After another equilibration period, the vessels were precontracted with noradrenaline, and a dose–response curve to sodium nitroprusside (1 × 10⁻⁹–1 × 10⁻⁴ mol/l), an endothelium-independent, smooth-muscle-dependent, dilator, was obtained. At the end of the study, each of the aortic rings was blotted dry and weighed.

Tracings of the responses were recorded using a PowerLab system (ADInstruments). A single observer, blinded to all specific details of the rat, including the dietary permutations, measured all of the aortic responses.

Statistics
We studied the influence of maternal and postnatal diet on aortic structure and function using two-way ANOVA. The prospectively defined primary study comparisons were between the maternal CON and LP dietary groups, examining media thickness in the structural studies and changes in acetylcholine-induced vasorelaxation in the functional studies. These groups were then separately analysed using Student’s independent samples t tests. Further analyses comparing aortic parameters between the other dietary groups were undertaken using Student’s independent samples t tests and then adjusted for multiple comparisons using Holm’s method [20]. Statistical significance was inferred at two-tailed P ≤ 0.05. Data are means ± S.D. Statistical analyses were performed using SPSS Software (version 9.0).

Table 2  Number of offspring per dietary group for the aortic structure and aortic function studies

<table>
<thead>
<tr>
<th>Maternal diet</th>
<th>Aortic structure study</th>
<th>Aortic function study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Postnatal diet</td>
<td>LP</td>
</tr>
<tr>
<td></td>
<td>throughout</td>
<td>throughout</td>
</tr>
<tr>
<td>CON</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>LP</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>HF</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Aortic structure studies
Forty-six male rats (Table 2) were studied to determine aortic structure characteristics. The aortic structure study included litters from five maternal CON, four maternal LP throughout pregnancy and four maternal LP in the final trimester dams.

The thoracic aorta was perfusion-fixed at physiological pressure in situ using paraformaldehyde. The aorta was then excised and stored in paraformaldehyde for 24 h prior to being embedded in paraffin. Cross-sections (6-μm thick) were then mounted on glass slides and stained with resorcin-fuschin to highlight elastin fibres.

Media thickness and elastin measurement
Magnified digital images of the stained aortic slices were obtained using a digital colour video camera (Exwave HAD; Sony) attached to an optical microscope (BH-2; Olympus). These digital images were calibrated using an objective micrometer (Meiji Techno), and analysed using NIH Image software (version 1.62) by an observer blinded to rat dietary group. Measurements of media thickness were obtained from 18 sections of aortic wall from each rat. A density threshold selection tool was used to select the areas of aortic wall stained for elastin, which was then expressed as a percentage of total wall area. Measurement of elastin was obtained from six sections of aortic wall from each rat.

The total amount of elastin was obtained assuming a spherical aortic cross-section, with the following equation:

\[ \text{Total elastin (mm}^2\text{)} = \left( \pi \times \left( \text{lumen diameter/2 + media thickness}^2\right) \right) - \left( \pi \times \left( \text{lumen diameter/2}^2\right) \right) \times \text{elastin/100} \]

Arterial function studies
After analysing the results of aortic structure, we then proceeded to assess aortic function in an additional 38 male offspring (see Table 2) from five maternal CON and nine maternal LP litters. Only five dietary groups were studied based on the aortic structure results.
Table 3 Effects of maternal undernutrition on aortic structure and composition in offspring

Data are means ± S.D. from offspring fed a postnatal CON diet. *P < 0.05 compared with CON diet (as determined by Student's independent-samples t tests adjusted for multiple comparisons, where necessary).

<table>
<thead>
<tr>
<th>Maternal diet</th>
<th>Final trimester LP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>424 ± 11</td>
<td>350 ± 9*</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>45.5 ± 0.4</td>
<td>43.3 ± 0.4*</td>
</tr>
<tr>
<td>Lumen diameter (mm)</td>
<td>2.03 ± 0.2</td>
<td>1.80 ± 0.1*</td>
</tr>
<tr>
<td>Aortic media thickness (μm)</td>
<td>142.6 ± 29</td>
<td>127.6 ± 28*</td>
</tr>
<tr>
<td>Aortic elastin proportion (%)</td>
<td>62.2 ± 6</td>
<td>61.9 ± 6</td>
</tr>
<tr>
<td>Calculated total aortic elastin content (mm²)</td>
<td>0.597 ± 0.09</td>
<td>0.491 ± 0.07*</td>
</tr>
</tbody>
</table>

RESULTS

Maternal nutrition

The influence of maternal nutrition on offspring anthropometry and aortic structure is shown in Table 3. Two-way ANOVA indicated that maternal diet altered the total quantity of aortic elastin and the lumen diameter (P = 0.06 and P = 0.01 respectively). Furthermore, there was no evidence of an interaction between maternal and postnatal diet. To characterize further which maternal dietary groups altered aortic structure, we undertook individual analyses. Briefly, aortic media thickness was significantly decreased (P = 0.005) in the offspring of dams fed the LP diet throughout pregnancy when compared with the offspring of dams fed the CON diet. The offspring of dams fed the LP diet in the final trimester had a significantly increased (P = 0.009) proportion of elastin in the aortic wall; however, the total amount of elastin present was similar (P = 1.00) to the offspring of dams fed the CON diet. Conversely, the LP diet throughout pregnancy did not alter (P = 0.13) the proportion of elastin in the offspring, but resulted in a significant decrease (P = 0.02) in the calculated total amount of elastin in each aortic section.

The two-way ANOVA indicated that the maternal LP diet decreased noradrenaline-induced vasoconstriction (P = 0.006; Figure 1). However, this was attenuated after adjusting for aortic ring weight (P = 0.31). There were no differences in vasorelaxation due to acetylcholine or sodium nitroprusside (P = 0.12 and P = 0.86 respectively; Figure 2). Furthermore there was no evidence of interaction between maternal and postnatal diets for functional endpoints (P > 0.3 for all).

Postnatal nutrition

The influence of postnatal nutrition is shown in Table 4. Two-way ANOVA indicated that postnatal diet altered aortic media thickness, elastin and lumen diameter (P = 0.02, P = 0.001 and P = 0.003 respectively). Further analysis revealed that the postnatal LP diet in both maternal CON and maternal LP dietary groups decreased media thickness compared with the corresponding postnatal CON diet animals (both P = 0.05). Additionally, aortic media thickness was also decreased in the HF postnatal diet group compared with the corresponding postnatal CON diet animals (maternal CON diet; P = 0.001). The postnatal LP diet also resulted in a significant decrease (P = 0.01) in the total elastin content of the aorta.

We found no evidence for an influence of postnatal diet on either aortic vasoconstriction (P < 0.4 both before and after adjustment for aortic ring weight; Figure 1) or relaxation (P = 0.14 for acetylcholine and P = 0.10 for sodium nitroprusside; Figure 2).

DISCUSSION

Many epidemiological studies have now shown an inverse association between birthweight and risk of adult cardiovascular and metabolic disease. Maternal undernutrition has been proposed as a mechanistic link for this observation. In the present study, we demonstrate that in utero undernutrition in rats, induced via a maternal LP diet, causes a decrease in aortic wall thickness and elastin content, without altering aortic dilator function.

In adults, increased arterial wall thickness is an indication of total atherosclerotic burden and is associated with hypertension and risk of future clinical cardiac events. We and our collaborators [21] have demonstrated
In utero nutrition alters aortic structure

Figure 2  Effects of maternal and early postnatal nutrition on aortic response to (a) acetylcholine and (b) sodium nitroprusside

Table 4  Effects of postnatal diet on aortic structure and composition from offspring of dams fed (a) the CON diet and (b) the LP diet

That in children aortic intima-media thickness is the best non-invasive anatomical marker of atherosclerosis disease progression. Furthermore, we have recently shown [12] that reduced fetal growth (a marker of intrauterine nutrition) is associated with increased aortic wall thickness in newborn infants. Thus, for the present study, we hypothesized that maternal undernutrition would be associated with an increase in aortic wall thickness. However, we observed a decrease in aortic wall thickness in those rats exposed to in utero protein restriction. These contrasting findings are most probably attributable to the different causes of fetal undernutrition, suggesting that a broad-spectrum intrauterine undernutrition due to placental insufficiency (the most likely mechanism in the human cohort) is involved in the pathophysiology of increased aortic wall thickness, rather than the maternal dietary protein restriction used in the rat model.

It is possible, however, that a thinner aortic wall may predispose to future hypertension and cardiovascular disease, via alterations to both arterial compliance and elastic properties. Indeed, in the present study, we observed reductions in aortic elastin content accompanying in utero protein restriction. The impact of a reduction in aortic elastin on future cardiovascular events was beyond the scope of the present study, but is a potential area for future research.

The reduced vasoconstrictor response to noradrenaline in offspring of dams fed the LP diet is most probably attributable to the reduced size of the aorta in this group. Indeed, these differences were no longer apparent after adjusting for aortic ring weight.

The observed lack of an effect on aortic function due to maternal protein restriction is consistent with a previous study [22]. Torrens et al. [22] demonstrated that in the pregnant offspring of dams fed an LP diet during pregnancy, vascular function in the thoracic aorta was
unaffected, although a resistance artery (the mesenteric) displayed altered endothelial function. Unfortunately, arterial structure was not examined. However, this model has been demonstrated previously to increase the incidence of Type II diabetes and the metabolic syndrome in the growth-restricted offspring [23], and may represent better the aetiology of undernutrition exhibited in developing nations, where dietary protein is frequently limited or deficient. Our present results indicate that this form of undernutrition is not associated with increases in arterial wall thickness, but possibly with a reduction in the total quantity of arterial elastin, which may predispose towards heightened risk of age-related arterial stiffness and hypertension.

Contrary to these findings with a maternal protein deficiency model, severe total nutritional deficiency in rats aggravates renal and vascular dysfunction as well as promoting hypertension in offspring [24,25], and may produce more serious consequences than isolated protein deficiency in pregnancy.

Study limitations

Increases in blood pressure have been implicated in playing an important role in the relationship between impaired fetal growth and adult cardiovascular disease [7]. Non-invasive assessment of blood pressure in rats is frequently undertaken using a tail-cuff method. This technique, however, is methodologically difficult and highly operator-dependent [26,27]. We attempted to assess blood pressure in the present study and obtained reliable readings in only approx. one-third of animals (results not shown) and, thus, we had insufficient data on blood pressure changes to allow inclusion of this parameter in our analysis.

Conclusions

In summary, we have shown that maternal protein restriction results in altered aortic structure, with decreased wall thickness and elastin content, in male rat offspring. However, these changes are not associated with alterations in aortic functional characteristics, with preserved endothelium- and smooth-muscle-dependent dilator responses.

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