Maternal and postnatal vitamin D ingestion influences rat aortic structure, function and elastin content

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Abstract

Objectives: Subtle impairment of fetal nutrition appears to predict hypertension and atherosclerosis in adults. It has been hypothesised that impaired aortic elastogenesis is the initiating step in adult hypertension and aortic aneurysms. Vitamin D has been shown to inhibit elastin synthesis by cultured smooth muscle cells. Here we have investigated, in rats, the hypothesis that increased exposure to vitamin D during gestation and in the postnatal period alters aortic elastin content and aortic function.

Methods: Nine breeding pairs of Sprague–Dawley rats were allocated to one of three diets containing 3000 (control group), 6000 (low dose) or 12,000 (high dose) IU/kg vitamin D during pregnancy and lactation. Male pups were continued on the same diet until 6 weeks of age. Aortic elastin content was assessed by measuring desmosine + isodesmosine content using capillary zone electrophoresis. Transverse aortic sections were used for quantification of elastic lamellae and morphometric analysis. The contractility of aortic rings was assessed in an organ bath preparation.

Results: The desmosine + isodesmosine content of the abdominal aorta of 6-week-old male pups, was 14.1, 10.0 and 10.1 nmol/mg dry weight in the control (n = 20), low- (n = 23) and high-dose (n = 15) groups, respectively (P = 0.007). The median number of elastic lamellae of the distal thoracic aorta was 8.25, 7.13 and 6.88 in the control, low-dose and high-dose groups, respectively (P = 0.001). There were no significant differences in aortic cross-sectional areas or media/adventitia ratios. The mean peak tension of aortic rings, in response to phenylephrine, was 1.3 g, 1.12 g and 0.87 g in the control, low- and high-dose groups respectively (P = 0.002).

Conclusion: In rats, exposure to increased amounts of vitamin D during gestation and early life results in a reduction of aortic elastin content, number of elastic lamellae in the aorta and force generation in aortic rings.

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1. Introduction

Proponents of the ‘fetal origins’ hypothesis have suggested that cardiovascular health in later life is associated with nutrition in utero and early childhood [1]. It has been suggested that impaired elastin synthesis in the aorta during the neonatal period is the initiating event in the pathogenesis of hypertension [2]. No attention has been paid to the possibility that inadequate deposition of elastin protein in the aortic wall is also a key event in the pathogenesis of aneurysmal disease of the abdominal aorta. Despite a commonality of risk factors (e.g. smoking, hypertension), it remains unclear why some patients develop occlusive atherosclerotic arterial disease and others develop aneurysmal disease [3]. One of the consistent biochemical findings in aneurysmal disease is a reduction in the elastin content of the aortic wall [4,5]. The most
popular current theory is that elastin has been destroyed by activation of proteolytic enzymes [3,6]. However, the presence of proteolytic enzymes and a chronic inflammatory infiltrate may only be an association with advanced aneurysmal disease rather than the primary cause of the condition.

One of the nutritional factors that might alter early elastin deposition in the aortic wall is vitamin D [1]. Vitamin D has been shown to inhibit elastin synthesis by cultured smooth muscle cells [7,8]. The hypothesis has been elaborated that exposure to excess vitamin D during gestation may impair aortic elastogenesis, thereby acting as a predisposition to aneurysmal and possibly hypertensive disease in later life [9]. Here we have investigated, in rats, the hypothesis that increasing exposure to vitamin D during gestation and in the postnatal period alters aortic elastin content and aortic function.

2. Methods

2.1. Animal model

All procedures were performed according to a protocol approved and licensed by the Home Office (PPL70/4814). Nine breeding pairs of closed colony Sprague–Dawley rats were allocated to one of three groups.

1. Control group fed normal chow containing vitamin D (1,25 dihydroxycholecalciferol) 3000 IU/kg, calcium 0.8% and phosphorus 0.64%.
2. Low-dose group fed modified chow containing vitamin D 6000 IU/kg, calcium 0.65% and phosphorus 0.52%.
3. High-dose group fed modified chow containing vitamin D 12,000 IU/kg, calcium 0.5% and phosphorus 0.4%.

Chow was prepared commercially (Special Diet Services, Witham, Essex) and apart from the above differences was indistinguishable. The calcium and phosphorus content in the vitamin D-enriched diets was reduced in order to minimize any hypercalcaemia [10]. Breeding pairs were left undisturbed throughout gestation (21 days) and breastfeeding (21 days). Once weaned, male pups (females were not studied) were continued on the same diet allocated to their parents until sacrifice at 6 weeks of age. Vitamin D intake was estimated by weighing chow consumption of breeding pairs and groups of pups. To conform with ethical guidelines and minimise the number of experimental animals, all male pups of all litters were studied.

Under lethal anaesthesia (O₂ + N₂O + halothane) 3 ml of blood were aspirated by cardiac puncture. The thoracic and abdominal cavities were opened and the aorta flushed with chilled Krebs solution via the ascending aorta. The thoracic aorta was removed; the proximal half was stored in chilled Krebs solution and the distal half in buffered formalin. The abdominal aorta was dissected free of surrounding connective tissue, divided below the renal arteries and at its bifurcation and stored at −70°C until analysis.

2.2. Plasma assays

Plasma from blood samples was divided into two aliquots and stored at −70°C. Plasma 25 hydroxy vitamin D levels were measured (n=12 per group) using a competitive protein binding assay following ether extraction and chromatography on a column of silicic acid [11] in the Endocrine Laboratories at Charing Cross Hospital. Plasma phosphate was analyzed (n=10 per group) in the Department of Chemical Pathology at Charing Cross Hospital.

2.3. Organ bath studies

Smooth muscle contraction, in response to phenylephrine (Sigma, Poole, UK) was studied in segments of thoracic aortas of 6-week-old male rats. Immediately after harvesting a 3-mm ring of thoracic aorta was mounted on wire stirrups, the upper one being attached to a pressure transducer, and placed in an organ bath containing oxygenated Krebs solution at 37°C. After equilibration for 15 min, rings were stretched to 2 g and then allowed a further 15 min to equilibrate. A concentration–response curve was generated using phenylephrine as an agonist. Following maximum contraction, smooth muscle viability was confirmed by observation of relaxation in response to sodium nitroprusside (Sigma) 10 μmol/l. Subsequently, after washing the rings with oxygenated Krebs solution, the rings were re-contracted with 80 mM KCl. Peak tension in response to phenylephrine and the concentration of phenylephrine required to sustain half the maximum tension (EC₅₀) were then calculated.

2.4. Quantification of desmosine and isodesmosine

The elastin content of abdominal aortas was measured by quantification of the elastin-specific cross-linking amino acids, desmosine and isodesmosine, using capillary zone electrophoresis [12]. Abdominal aortas were dried to constant weight. Each aorta was hydrolysed in 300 μl of 6 N HCl at 110°C for 48 h. This was followed by vacuum dessication over NaOH and re-hydration with 30 μl of milliQ water.

The Hewlett-Packard HP3D Capillary Electrophoresis system was run with citrate buffer (pH 2.5) and detector absorption set at 200 nm. Leupeptin (acetyl-Leu-Leu-Arg-al, FW 426.6, Sigma) was used as an internal standard in order to correct for variation in sample loading and
migration times. Peak areas for desmosine+isodesmosine and standard were integrated using online software. These areas were then correlated using a concentration curve based on serial dilutions of purified bovine desmosine+isodesmosine (ICN Pharmaceuticals, Basingstoke). The assay was linear in the range 0.01–0.3 mmol/l and the intra-assay and inter-assay coefficients of variation were 1.2% and 8%, respectively.

2.5. Quantification of elastic lamellae and morphometric measurements

Sections (6 μm, n=10 per group) of distal thoracic aorta were stained with haematoxylin and eosin, elastic van Giessen and von Kossa. The aortas were inspected for calcification, inflammatory cell infiltrates and the number of elastic lamellae in each of four quadrants counted using light microscopy. Similarly, the medial and adventitial thickness were quantified by measurement in each of four quadrants in four separate sections. Since there was no evidence of intimal hyperplasia, the thickness of the intima was not measured. The internal and external circumference of each vessel were measured using a video-based image analysis system with edge tracking (Analysis Imaging C, Munster, Germany). Aortic circumference and cross-sectional area were corrected for animal size by dividing by individual animal weight in grams.

2.6. Quantification of total elastase activity in aortic homogenates

Rings of distal thoracic aorta were freeze-dried to constant weight before homogenization in 0.1 g in 1 ml of 50 mM Tris–Cl− pH 7.6 containing 0.5 M NaCl, 2 M urea and 0.1% Brij 35. The homogenates were clarified by ultracentrifugation (100,000×g for 1 h). Protein content was estimated using a dye-binding assay. Total elastase activity was measured, in duplicate, using insoluble [3H]elastin as a substrate, as described previously (assay range 1–300 μg/mg/day) [13]. Bovine pancreatic elastase was used as a positive control and boiled (5 min) aortic homogenates as negative controls. Results were reported as μg elastin solubilised/mg protein/day.

2.7. Data analysis

All measurements and analyses were performed in a blinded fashion. The primary analyses used data from individual pups, but mean litter data were also compared. The 50% effective concentrations (EC50) of the drugs used were calculated by normalising data to the peak response and fitting the concentration–response curve to a logistic plot incorporating Hill coefficient using MicroCal Origin (MicroCal, Northampton, MA, USA). Evaluation of these data was performed using the Student’s t-test for paired-observations or for concentration–response curves using repeated measures analysis of variance, followed by Bonferroni multiple comparison test (Statview 4.0 for Macintosh). Animal weights, plasma assays, aortic contractility, morphometric measurements and desmosine concentrations were compared using ANOVA. Elastic lamellae numbers were compared using the Kruskal Wallis test. These statistical analyses were performed using either Microsoft Excel or VassarStats (http://faculty.vassar.edu/~lowry/VassarStats.html). An alpha of less than 0.05 was accepted as statistically significant.

3. Results

3.1. Animal nutrition

All animals in all three groups appeared healthy. The median (range) litter sizes were as follows: 11 (10–12) for the control group and 10 (2–15) for the low-dose group and eight (8–12) for the high-dose group (P=0.4). Thirty eight percent of the control, and 53% of the treated litters were male (P=0.06). The average daily intake of vitamin D in pregnant and lactating dams was estimated to be: 126, 174 and 216 IU in the control group, low- and high-dose groups, respectively. The average daily intake of individual male pups aged 3–6 weeks was estimated to be: 48, 55, and 170 IU in the control, low- and high-dose groups, respectively. This is reflected in the increasing concentration of plasma vitamin D in the low- and high-dose group pups, although the plasma phosphate concentrations were very similar (Table 1). The mean weights of male pups at 6 weeks were 171, 185 and 191 g in the control, low- and high-dose groups, respectively (Table 1).

3.2. Contractility of thoracic aortic rings

The EC50 for phenylephrine (∼0.3 μM) was similar in control, low-dose and high-dose groups of rats (Table 1). The mean peak tension showed an inverse relationship with dietary vitamin D supplementation being 1.3 g, 1.12 g and 0.87 g in control, low- and high-dose groups, respectively, P=0.002 (Table 1).

3.3. Desmosine +isodesmosine content and elastase activity

As it was impossible to separate the desmosine and isodesmosine peaks, the area of the combined peaks was used for integration. The mean content of desmosine+isodesmosine in the abdominal aorta was higher in control rats (14.1 nmol/mg dry wt.) than in rats in the low-dose and high-dose groups (each 10 nmol/mg dry wt.), P=0.007 (Table 1). A similar trend was observed when analysis was based on mean litter desmosine+isodesmosine although the difference was not statistically significant (P=0.18) due to the smaller numbers. The total
Table 1
Summary data from male rats from normal chow (control), chow supplemented with low dose or high dose vitamin D groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Low-dose</th>
<th>High-dose</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>170.9 (15.6)</td>
<td>184.5 (21.7)</td>
<td>191.1 (13.2)</td>
<td>0.004</td>
</tr>
<tr>
<td>Thoracic aortic ring, phenylephrine EC₅₀ (µM)</td>
<td>0.30 (0.45)</td>
<td>0.25 (0.16)*</td>
<td>0.40 (0.90)**</td>
<td>0.35</td>
</tr>
<tr>
<td>Thoracic aortic ring, phenylephrine max. tension (g)</td>
<td>1.29 (0.45)</td>
<td>1.02 (0.37)*</td>
<td>0.87 (0.26)**</td>
<td>0.002</td>
</tr>
<tr>
<td>Abdominal aorta desmosine (nmol/mg dry wt.)</td>
<td>14.1 (3.1)</td>
<td>10.0 (4.2)</td>
<td>10.14 (5.4)</td>
<td>0.007</td>
</tr>
<tr>
<td>Aortic elastase activity (µg/mg protein/day)</td>
<td>2 (1)</td>
<td>3 (1)</td>
<td>2 (1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma 25-OH vitamin D (nmol/l)</td>
<td>39.1 (6.2)</td>
<td>59.3 (13.3)</td>
<td>103.0 (24.5)</td>
<td>0.007</td>
</tr>
<tr>
<td>Plasma phosphate (mmol/l)</td>
<td>2.99 (0.19)</td>
<td>3.08 (0.19)</td>
<td>3.12 (0.23)</td>
<td>0.35</td>
</tr>
</tbody>
</table>

All values are means (standard deviations) with all P values obtained using ANOVA. *n=20, **n=13. *All elastase values at lower limit of assay detection, precluding reliable statistical analysis.

elastase activity of aortic homogenates was at the lower limit of assay detection and not different between the three groups (Table 1).

3.4. Elastic lamellae and morphometry of the thoracic aorta

There was no obvious calcification or influx of inflammatory cells in the aortas of rats in the low- and high-dose groups. The median number of elastic lamellae in the distal thoracic aorta was 8.25 in the control group, 7.13 in the low-dose and 6.88 in the high-dose groups, P<0.001 (Table 2). After correcting for animal weight, there was still a statistically significant reduction in the number of elastic lamellae with increasing vitamin D consumption, P=0.01. The trend for the cross-sectional area and the media/adventitia ratio of the thoracic aorta to decrease with vitamin D consumption was not statistically significant (Table 2).

4. Discussion

Over the last decade there has been considerable interest in the influence of fetal factors in adult disease, particularly cardiovascular disease [1]. Here we have shown that, in rats, increased vitamin D exposure during gestation and early development can result in changes in aortic structure and function. These changes include a significant reduction in the elastin concentration of the abdominal aorta, as measured by the concentration of the elastin-specific amino acids desmosine and isodesmosine (Table 1). There was also a significant reduction in the number of elastic lamellae in the distal thoracic aorta. In addition, vitamin D exposure during gestation and early development impaired the contractile properties of the mid-thoracic aorta. The combination of these changes could have an important influence on the response of the aorta to genetic and environmental factors known to cause cardiovascular disease in man. In particular, this could apply to both aortic aneurysmal disease and hypertension, where reduced elastin content and increased wall stiffness may be relevant [2,4].

We used an environmental factor, dietary vitamin D, which reduced the amount of mature elastin cross-links in the aorta. The mechanism underlying this association could be through direct effects on elastin expression, effects on proteins involved in the maturation of elastic fibrils (the elastin receptor or microfibril glycoproteins), effects on elastin catabolism or a combination of these effects. The pathology associated with reduced elastin expression has

Table 2
Morphometric data for the thoracic aorta of male rats (n=10 per group) from normal chow (control), chow supplemented with low dose or high dose vitamin D groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Low-dose</th>
<th>High-dose</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elastic lamellae*</td>
<td>8.25 (7–11)</td>
<td>7.13 (6–8)</td>
<td>6.88 (5–8)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Elastic lamellae (n/g body wt.)*</td>
<td>0.047 (0.039–0.56)</td>
<td>0.042 (0.035–0.048)</td>
<td>0.037 (0.031–0.045)</td>
<td>0.01*</td>
</tr>
<tr>
<td>Circumference (µm/g body wt.)*</td>
<td>182.6 (2.18)</td>
<td>190.2 (1.72)</td>
<td>183.3 (3.04)</td>
<td>0.74</td>
</tr>
<tr>
<td>Cross-sectional area (µm²/g body wt.)*</td>
<td>1.92 (0.22)</td>
<td>1.88 (0.18)</td>
<td>1.77 (0.27)</td>
<td>0.35</td>
</tr>
<tr>
<td>Media:adventitia thickness ratio*</td>
<td>2.36 (0.944)</td>
<td>2.28 (0.154)</td>
<td>2.24 (0.121)</td>
<td>0.15</td>
</tr>
</tbody>
</table>

*Values are medians (range) with P value obtained using Kruskal Wallis test. *Values are means (standard deviations) with P value obtained using ANOVA.
been investigated in mice and men hemizygous for elastin [14]. Hemizygosity for elastin in mice resulting in a 50% reduction in aortic elastin mRNA at birth was associated with an ~30% increase in elastic lamellae in the ascending and descending aorta. In man the number of elastic lamellae seen in cases of supravalvular aortic stenosis was more than double that found in normal individuals. In contrast, in our experiments, there was a modest reduction in elastic lamellae associated with vitamin D ingestion and lower concentrations of elastin cross-links. This would suggest that vitamin D was not exerting a major effect on fetal or neonatal elastin gene expression and effects of vitamin D on elastic fibril synthesis and/or catabolism should be considered.

The experiments in mice also have shown that elastin’s effect on aortic lamellar development probably involves smooth muscle cells sensing wall stress, with aortae from hemizygous mice having reduced extensibility at supraphysiologic pressures. There also are indications that the phenotype of smooth muscle cells alters in the absence of elastin or with alterations in fibrillin [15,16]. In the mouse model of Marfan syndrome, phenotypic alteration of smooth muscle cells preceded elastolysis [16]. We found that the phenotype of smooth muscle cells altered with chronic modest elevation of vitamin D (associated with a reduction of cross-linked elastin), aortic rings having diminished contractile response to phenylephrine. Alternatively, changes in either calcium uptake or altered transcriptional regulation of genes associated with contractile mechanisms, throughout gestation and early development by the vitamin D receptor [17] could underlie the reduced force generation in the aorta of rats chronically exposed to non-toxic doses of vitamin D. Our findings complement those of Weishaar et al. who previously demonstrated increased force generation in the aorta of chronically vitamin-D deficient rats [18].

Although, a graded reduction in aortic force generation with increasing exposure to vitamin D was observed, the desmosine + isodesmosine content of abdominal aorta was similar in rats chronically exposed to low dose and high dose increases in vitamin D. The content of these elastin-specific cross-link amino acids was ~40% higher in the abdominal aorta of control rats. The reduction in number of elastic lamellae in the medial layer of the thoracic aorta, if paralleled by a reduction in the number of elastic lamellae in the abdominal aorta, may contribute to the decreased desmosine + isodesmosine content in vitamin D-fed rats. The reduction in the number of elastic lamellae was not associated with a significant decrease in either the cross-sectional area of the aorta or the medial/adventitial thickness ratio, again potentially suggesting that the smooth muscle cell phenotype was altered in vitamin D-fed animals. The possibility that chronic exposure to vitamin D was associated with increased turnover of insoluble elastin (leading to increased elastase activity in aortic homogenates) was not substantiated, although more sensitive elastase assays could be necessary to detect subtle effects.

It has been hypothesised that exposure to excess vitamin D during early development could be a nutritional cause of reduced aortic elastogenesis and supravalvular aortic stenosis [9,19]. The toxic effect of vitamin D on arteries has been previously documented [20]. In experimental studies, excess vitamin D results in arterial wall calcification and, among a variety of other ‘atherosclerotic’ changes, loss of collagen and disruption of elastic lamellae [21,22]. There is evidence that the arterial effects of vitamin D may occur transplacentally; piglets of sows fed vitamin D developed coronary lesions 6 weeks after birth [23]. The mean vitamin D blood levels in the sows was 30 ng/ml, this is equivalent to that of the American general population [24]. Transportation of vitamin D across the rat placenta is specifically enhanced during the last one third of pregnancy (a period of maximal aortic elastin deposition) [25] and the neonate is dependent on stored vitamin D as mammalian milk contains minimal vitamin D [26]. This suggests that if maternal intake is excessive then fetal and neonatal exposure will also be excessive.

The majority of animal studies have used short courses of potentially toxic doses of vitamin D resulting in significant hypercalcaemia. In contrast, we focused on delivering modest, non-toxic increases in vitamin D throughout gestation, during and after weaning. Even in the high-dose group daily consumption was, at most, 1000 IU/kg—considerably less than a recent study that used 300,000 IU/kg as a single dose [22]. Although there was no consistent relationship between litter size and pup weight, the increased weight of the 6 week-old pups in the treated groups may have been partly attributable to the small litter sizes of the vitamin D-fed dams, rather than the diet itself. Despite being heavier, the treated animals had significantly fewer elastic laminae than the control animals. In comparison with control pups at 6 weeks, there were mean 50% and 250% increases in circulating vitamin D in the low and high-dose groups. This was achieved without alteration of plasma phosphate concentrations. Potential weaknesses of our study are that calcium concentrations could not be determined (since only EDTA plasma was stored), that aortic compliance (or stiffness) was not measured and that vessels were not perfusion–fixed, to study the morphology of smooth muscle cells.

How plausible is it that these experimental observations of a pharmacological effect in an animal model can be used to explain the pathogenesis of clinical conditions? Since cod liver oil was found to prevent rickets in 1919, consumption of vitamin D in most Western countries has probably been excessive [24]. It was not unusual for infants to consume 100 µg/day and an ‘epidemic’ of infantile hypercalcaemia and supravalvular aortic stenosis coincided with the increase in vitamin D supplementation of milk in the 1940s although it was not until 1963 that infant food supplementation was reduced in the United
States [27]. Sensitivity to vitamin D appears to be variable as not all infants developed hypercalcaemia and a reduced susceptibility to rickets may also be associated with an increased susceptibility to vitamin D toxicity [27]. Widespread vitamin D supplementation during pregnancy and early infancy (the period when aortic elastin is being deposited) may have been significant and probably excessive from the 1920s onward. It appears therefore, that for many decades humans have been exposed to pharmacological rather than physiological amounts of vitamin D during gestation and infancy. Although many foodstuffs still contain vitamin D supplements, exposure for those born less than 40 years ago has probably fallen considerably.

In the present study, several different techniques were used to assess aortic structure and function. Each technique resulted in data indicating that exposure to pharmacological amounts of vitamin D, during gestation and early life, alters aortic structure and function. The mechanism by which this occurs remains to be elucidated. For example, it is unclear whether exposure during gestation or in the postnatal period (or both) is responsible for the observed changes. The present study involved exposure to supplementary vitamin D throughout the known period of aortic elastogenesis. Further experiments are required with exposure to vitamin D in discrete periods during pregnancy, breast-feeding and weaning in order to clarify this.

If the observed effects have a parallel in man, in later life, they could influence the development of cardiovascular diseases such as hypertension and abdominal aortic aneurysm. The ‘fetal origins’ hypothesis remains topical and controversial with renewed calls for ‘a move from epidemiology to cellular and molecular processes’ [28]. The possibility that vitamin D is an important nutritional factor in the fetal origins paradigm merits further research.

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