Fibrillin-2 Defects Impair Elastic Fiber Assembly in a Homocysteinemic Chick Model

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ABSTRACT Homocysteinemia in humans is associated with vascular complications that increase the risk for atherosclerosis and stroke. Animal studies have shown that the disease is multifactorial and includes lesions associated with the elastin component of the extracellular matrix. In the following experiments we have used the aortas from rapidly growing chicks to assess the cause of the elastin defects resulting from homocysteinemia. Day-old chicks were fed diets containing varying amounts of DL-methionine, DL-homocysteine, homocysteine thiolactone or DL-cysteine for periods up to 9 wk. Three weeks after feeding 2% DL-methionine the plasma methionine was elevated >20-fold, whereas plasma homocysteine was more than 3-fold normal plasma values. The aortas showed severe histopathology, evidenced by the pronounced separation of elastic lamellae with marked smooth muscle proliferation and, in some instances, aneurysms. There was no evidence of decreased desmosine content or a significant reduction in lysyl oxidase in the aortas from the treated groups compared to those from controls. Increasing other dietary factors such as the vitamins required for methionine metabolism had no effect on the development of the vascular lesions. Twenty to 30% of the chicks fed the high methionine diets exhibited severe neurological problems, expressed as tonic contractions or seizures. Electron microscopy revealed disordered aortic elastic fibrils, associated with either an absence of or disrupted assembly of microfibrils. Immunohistochemical studies demonstrated a loss of fibrillin-2 immunoreactivity in the aortas of chicks fed 2% methionine. The studies suggest that elevated plasma methionine or its metabolites disrupt normal microfibril configuration, leading to the assembly of aberrant elastic fibers. J. Nutr. 132: 2143–2150, 2002.

KEY WORDS: • microfibrils • homocysteinemia • elastin • fibrillin • desmosine

Cardiovascular disease is still the major cause of morbidity and death in developed countries and has long been associated with major risk factors such as serum cholesterol, diabetes, smoking, age and family history. Other factors may also increase the probability of developing cardiovascular disease and contribute to atherosclerosis. Among these, elevated plasma homocysteine concentration has been considered a major independent risk factor for vascular disease and is associated with premature cardiovascular complications induced by accelerated atherosclerosis and thromboembolism (1–3). The mechanism remains unknown and is considered multifactorial.

Homocysteine is normally metabolized by remethylation or trans-sulfuration (4,5). Under conditions of low methionine intake, homocysteine is metabolized primarily by methionine-conserving remethylation pathways. With high dietary intake of methionine, homocysteine is converted to cystathionine by trans-sulfuration and then to cysteine, which is further metabolized to sulfate and excreted in the urine. If the levels of methionine are excessive, or if specific metabolic enzymes or cofactors are deficient, there is an accumulation of homocysteine. In addition, some methionine can be converted to homocysteine thiolactone. It is the elevated levels of homocysteine or homocysteine thiolactone that are thought to be responsible for vascular damage.

The adverse consequences of feeding high levels of methionine to chicks and other animals have long been observed and early studies indicated that high levels of methionine depressed chick growth (6–8). Rats fed high levels of methionine also exhibited weight loss and retarded growth (9–11). Many investigators have examined other metabolic manifestations associated with high levels of methionine feeding. Some of these studies have addressed the possibility of overcoming methionine toxicity by the addition of other metabolites to the diet. For instance, it was shown that dietary glycine ameliorated the growth-depressing effect of methionine (12,13). Klavens and Peacocke (14) studied the interaction of both glycine and arginine on methionine toxicity and Benevenga and Harper (15) reported that both serine and glycine alleviated but did not prevent methionine- or homocysteine-induced growth depression. In addition to growth depression, pathological lesions of spleen, pancreas, liver, small intestine and kidney have been reported as a result of feeding high levels of methionine and homocysteine to rats (16–18), although changes in spleen, pancreas and liver, which were apparent after 1 mo of excessive methionine feeding, were reversed by continuation of the diets for 3 mo (17).

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Investigations addressing the biochemical consequences of excessive methionine intake have reported that excess dietary methionine decreased hepatic levels of several enzymes involved in homocysteine metabolism (19,20). Elevated serum homocysteine was shown to modulate vascular smooth muscle cell growth and cause other extracellular matrix defects (21,22). Hyperhomocysteinemia in minipigs resulted in fibroelastic disorders and pronounced smooth muscle proliferation (23). Zulli et al. (24) found that rats fed 2% methionine for 15 wk presented histological evidence of a loss of elastic fibers in the aortic wall. Rats injected intraperitoneally with homocysteine and methionine showed evidence of decreased crosslinks in collagen and elastin (25).

Elastin is secreted from the cell as a 72-kDa soluble precursor, tropoelastin, which is then stabilized in the extracellular matrix through the action of lysyl oxidase. This enzyme oxidizes the epsilon amino groups of lysine to form intermolecular crosslinks, primarily desmosine and isodesmosine (26). For fiber assembly to proceed, the tropoelastin must first be arranged on a scaffold of microfibrils, composed of at least three proteins: fibrillin-1, fibrillin-2 and microfibril-associated glycoprotein (27–29). Impedance of normal elastic fiber assembly or disruption of the crosslinking process can result in structurally fragile vessel walls and possible sites for atherosclerotic lesions to develop.

The cardiovascular pathology associated with high blood levels of homocysteine generally follows an extended time of exposure to this risk factor. The resulting disruption of the architecture of the vessels and the interactions between the components of the vessels make it difficult to determine the primary physical or biochemical lesions associated with this disease. The purpose of the research presented here was to determine the consequence of homocysteinemia on aortic elastic fiber development in an animal model during the period of rapid vascular growth and matrix development.

**MATERIALS AND METHODS**

**Animals and experimental diets**

Broiler chicks from the North Carolina State University poultry facility were obtained on the day of hatching and were fed the experimental diets. The chicks were housed in conventional battery brooders with raised wire floors. The basal corn–soybean meal diet (Table 1) and tap water were available ad libitum. The vitamin mixture supplied approximately twice the requirements for growth (30). The basal diet was calculated to contain 0.39% DL-methionine and 0.37% cysteine compared to requirements of 0.5% DL-methionine and 0.93% DL-methionine + DL-cysteine. In a series of experiments the chicks were fed the basal diet supplemented with either DL-methionine, DL-homocysteine, homocysteine thiolactone or DL-cysteine. In a series of experiments the chicks were fed the basal diet supplemented with either DL-methionine, DL-homocysteine, homocysteine thiolactone or DL-cysteine, or DL-methionine combined with folic acid, vitamin B-12 and B-6 individually or all three combined at the concentrations indicated for 3 wk. The chicks were weighed at weekly intervals and blood was drawn by heart or wing vein puncture at the times indicated. The chicks were killed by cervical dislocation at the times indicated. To determine whether extending the period of DL-methionine supplementation would exacerbate the vessel pathology, other experiments were conducted in which aortas from control and 2% DL-methionine–fed groups were taken at designated times over 9 wk. To maintain elevated plasma homocysteine levels, day-old chicks were fed the control and the 2% DL-methionine–supplemented diet for 2 wk. At that time, the 2% DL-methionine–fed group was divided and half of the chicks were supplemented each week with supplements of

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/100 g</th>
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<tr>
<td>Oil</td>
<td>40.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.0</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>0.4</td>
</tr>
<tr>
<td>Limestone</td>
<td>1.9</td>
</tr>
<tr>
<td>Vitamin mix2</td>
<td>1.9</td>
</tr>
<tr>
<td>Trace mineral mix3</td>
<td>1.0</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>As indicated</td>
</tr>
<tr>
<td>Ground yellow corn</td>
<td>to 100</td>
</tr>
</tbody>
</table>

1 Cottonseed oil. 2 Supplies/kg diet: retinyl acetate, 3000 units; α-tocopherol acetate, 2.0; mg menadione sodium bisulfate, 1 mg; riboflavin, 7.2 mg; calcium pantothenate, 20 mg; niacin, 54 mg; thiamine, 3.6 mg; folic acid, 1.1 mg; pyridoxine HCl, 6 mg; biotin, 0.3 mg; vitamin B-12, 18 µg; cholecalciferol, 400 Icu. 3 Supplies in mg/kg diet: CuSO4·5H2O, 77; Na2SeO3·3H2O, 0.1; ZnCO3, 60.

**FIGURE 1** Histological changes in aortas of chicks fed a 2% methionine diet after 3 wk. Chick aortas stained with Harts elastic stain are shown in panels a, b and f. A representative control aorta is shown in panel a and a representative aorta from chicks fed 2% DL-methionine is shown in panels b and f. The rather diffuse elastic fibers and lamellae separation are observed in the aortas of chicks fed 2% methionine as well as marked smooth muscle proliferation (arrow, b). Representative aortas stained with Gomori’s trichrome stain for collagen (blue) and smooth muscle (red) are shown in panels c, d and e. Smooth muscle proliferation is indicated by the arrow in panel d. Aneurysms typical of those found in aortas of chicks fed 2% methionine after 3–6 wk are illustrated with trichrome stain in panel e and elastin stain in panel f. The arrow in panel f indicates the complete fracturing of the elastic fibers around the aneurysm. Magnification: 20× (c, d, e) and 40× (a, b, f).
DL-methionine calculated to maintain a constant high plasma level of this amino acid and, as a consequence, a constant high plasma level of homocysteine.

We also monitored the effect of changes in food consumption relative to body weight on vessel pathology as the chicks grew older. Methionine intake was calculated from the amount of food ingested per group each week during the 9 wk trial.

All animal research was approved by the University Animal Research Committee. The young chicken animal model was selected for these studies for several reasons. Chick aortas are very large relative to mouse or rat models and during the first 3 wk there is extensive depositions of elastin in this vessel. We were able to start the various feeding regimens on d 1 after hatching and analyze the effects of these dietary components on elastin fiber formation during this period of rapid elastogenesis.

Biochemical analyses

Plasma total homocysteine and cysteine were determined by the method of Araki and Sako (31). To determine free plasma methionine levels, 25 µL of 20% sulfosalicylic acid was added to 100 µL of plasma. The sample was vortexed and microfuged and 5- to 25-µL aliquots of the clear supernatant were analyzed for methionine by amino acid analysis.

For the l lysyl oxidase assay, aortas were removed, cleaned and immediately frozen on dry ice and stored at -70 °C until assayed within 3 d. The llysyl oxidase substrate was made from recombinant tropoelastin expressed and labeled with L-[4,5]H-lysine in a bacterial expression system essentially as described (32). The recombinant tropoelastin expression vector was a generous gift from Dr. Herb Rongish, University Kansas Medical Center, Kansas City, KS. The expression system essentially as described (32). The recombinant tropoelastin expression vector was a generous gift from Dr. Herb Rongish, University Kansas Medical Center, Kansas City, KS. The recombinant tropoelastin expression vector was a generous gift from Dr. Herb Rongish, University Kansas Medical Center, Kansas City, KS.

Supplementation with DL-methionine, DL-homocysteine, DL-homocysteine thiolactone and DL-cystine

Plasma methionine increased 5-fold with the addition of 1% DL-methionine to the diet, whereas the addition of 2% DL-methionine to the diet resulted in a 20-fold increase in serum methionine compared to normal serum levels. Plasma homocysteine levels were increased about 2-fold in both the 1 and 2% DL-methionine groups and as much as 12-fold in the 1% homocysteine thiolactone–supplemented chicks. Aortic llysyl oxidase activity and desmosine and hydroxyproline levels were not affected by any of the dietary supplementations. Body weight was significantly depressed in the 2% DL-methionine–supplemented and 1% DL-homocysteine thiolactone–supplemented groups compared with controls.

The histopathology in the aortas of the 2% DL-methionine group was pronounced, with marked smooth muscle proliferation and separation of the elastic lamellae (Fig. 1). Staining for elastin indicated marked elastin lamellae separation and disrupted elastic fibers (Fig. 1b, f) compared to the aorta from a control chick (Fig. 1a). These were not isolated lesions but encompassed the whole aorta. The outer regions of the media were the most severely disrupted by the smooth muscle proliferation; however, the intima was thickened and elastic lamellae were also interrupted in this region. There was no unambiguous evidence of elastic fiber degeneration. Figure 1c was from the aorta of a control chick and Figure 1d was from a 2% methionine-fed chick's aorta stained with Gomori's trichrome, which more selectively illustrates the smooth muscle hypertrophy throughout the vessel. There were many instances of aneurysms in the aortas of chicks fed the 2% DL-methionine diet as illustrated (Fig. 1e, f). No discernable pathology was observed in the aortas of chicks receiving 1% DL-homocysteine thiolactone, 1% DL-cysteine or 1% DL-methionine.

A neurological pathology occurred in 10–20% of the chicks fed 2% DL-methionine. The chicks at 10–14 d appeared normal when undisturbed, but when they were handled or otherwise startled, they retracted their heads so that their eyes were directed upward in a staring-like manner. This symptom was reminiscent of thiamine deficiency, which results in paralysis of the anterior neck muscle, except that in the 2% DL-methionine-fed chicks the symptoms were less severe and were transient, lasting only 5–10 min. These neurological symptoms were temporary and were not observed after >5 wk in the chicks fed the 2% DL-methionine diet.

Supplementation with biotin, ascorbic acid and pyridoxine

Extra vitamins involved in methionine metabolism, singly or in combination, did not reverse the growth retardation or
the increases in plasma homocysteine and methionine concentrations attributed to the addition of 2% DL-methionine to the diet (Table 3). Histologically, the extent of smooth muscle proliferation in the 2% DL-methionine group was not diminished by the addition of these vitamins. These data indicate that excess DL-methionine did not cause a deficiency of these vitamins.

**Supplementation with DL-methionine for 9 wk**

Growth retardation was evident early in the 2% methionine group and continued throughout the experiment (Fig. 2, *upper panel*), although there was no mortality during this time. In the 2% methionine-fed chicks, the plasma methionine rose sharply and remained at a high level at 1, 2, and 3 wk and then fell precipitously by 5 wk, declined further at 7 wk and rebounded somewhat by 9 wk (Fig. 2, *middle panel*). The plasma homocysteine of this group also increased rapidly, reached a maximum concentration by 2 wk and decreased in a manner similar to that of methionine levels. The concentration of aortic desmosine of both control and DL-methionine-fed chicks increased with time and reached a maximum at 35 d (Fig. 2, *lower panel*). Although the desmosine concentration was consistently higher in the older chicks that received 2% DL-methionine, the differences were not significant (P = 0.187-0.746).

There was an apparent reversal in the histopathology that paralleled the plasma homocysteine and methionine patterns over time. At 3–5 wk, chick aortas from the 2% DL-methionine group showed major areas of smooth muscle proliferation with many occurrences of aneurysms. At 7 wk the histopathology had not worsened and by 9 wk, visible signs of smooth muscle proliferation and lamellae separation had virtually disappeared and the aortas of DL-methionine–treated chicks could not be distinguished from those of the controls. The repair of the elastic fibers was quite remarkable, given that we found no evidence of aneurysms after 7 wk in chicks fed the 2% DL-

**FIGURE 2** Effect of feeding chicks 2% DL-methionine for 9 wk on body wt (*upper panel*), plasma methionine and homocysteine (*middle panel*) and aortic desmosine levels (*lower panel*). Values are means ± SD, n = 5. *Different from control, P < 0.01.

**FIGURE 3** Morphological measurements of the aorta lumen diameter with increasing age in chicks fed control and 2% methionine diets. Values are means ± SD, n = 5. *Different from control, P < 0.01.

**FIGURE 4** Methionine consumed per chick per 100 g per week over a 9-wk period. The chicks were fed either the control diet, the same diet supplemented with 2% DL-methionine (2% met) or the 2% DL-methionine diet with weekly increases in DL-methionine to maintain a high plasma level (2% met+). Total food consumption was measured each week for each group and the amount of ingested methionine was calculated. Values are means, n = 5.
methionine diet. A quantitative measure of the histopathology reversal was illustrated by comparing the aortic wall thickness and diameter of the lumen (Fig. 3). Starting at 3 wk and extending through 5 wk, the diameter of the lumen as a percentage of the diameter of the whole vessel was constricted in the DL-methionine-fed chicks compared to that in controls. This was not just a reflection of a thicker wall as a result of smooth muscle proliferation, but an actual stricture of the lumen. By 7 wk, the vessels of the two groups of chicks were indistinguishable and remained so through 9 wk.

**Effect of food consumption per body weight on maintaining homocysteinemia**

There was a gradual decline in the methionine intake/chick−1,100 g body wt−1·wk−1 over 9 wk by the control chicks as well as those receiving constant 2% DL-methionine supplementation (Fig. 4). In chicks receiving the weekly supplement of additional DL-methionine, the intake of methionine was maintained near peak levels for at least 7 wk. The plasma methionine and homocysteine levels among the chicks receiving the increasing supplemental DL-methionine did not remain at peak levels but fell noticeably by 9 wk in a pattern very similar to that observed in Figure 2 (middle panel). Incremental increases of DL-methionine to 3.75% by 9 wk did not alter the level of aorta desmosine. Hydroxyproline levels were unaffected by the graded increase in dietary DL-methionine and after 9 wk, the values (expressed as nmol/mg protein) were 193.1 ± 26.4, 193.6 ± 43.0 and 193.6 ± 15.8 for the controls; 2 and 3.75% DL-methionine-supplemented chicks, respectively. Histologically, elevated plasma methionine levels had no noticeable effect on aortic pathology. The severity of the lesions was maximal at 3 wk and did not worsen over the 9-wk period.

There was a possibility that differing food consumption would have some effect on nitric oxide metabolism, which could impact the vascular lesions we observed in chicks fed high levels of methionine. In two experiments we fed 50 μg/g sodium nitroprusside as a source of nitric oxide and observed no effects on the histology of the controls or the 2% methionine-fed chicks.

**Electron microscopy of elastic fibers**

The elastic fibers in the aorta from control chicks were long, continuous fibers with few areas of branching or disorder (Fig. 5A). Elastic fibers from the aortas of chicks fed 2% DL-methionine, however, had a more irregular surface and showed a greater degree of branching than fibers in the chicks fed a normal diet. There were also many small patches of darkly staining elastin in regions close to the larger fibers (e.g., arrow in Fig. 5C). Under higher magnification, the normal microfibrillar network that surrounds the elastic fiber (Fig. 5B, E) was greatly reduced in the aortas of the chicks receiving the high level of methionine (contrast Fig. 5D and Fig. 5F).

Although some fibers were visible in the methionine-treated chicks, they were generally smaller and less dense than the microfibrils associated with elastic fibers of control chicks.

**Fibrillin-2 immunohistochemistry**

In aortas of 3-d-old control chicks, fibrillin-2 was detected using an anti-fibrillin-2 antibody at relatively high levels in
the intima, with lesser amounts in the media and adventitia. By 2–3 wk, fibrillin-2 was confined mostly to the intimal region with some reaction in the adventitia, as indicated by the red color over the blue background (Fig. 6A, C). By 9 wk of age, chick aortas showed virtually no immunoreactivity for fibrillin-2. By contrast, the aortas of the chicks fed 2% DL-methionine showed a marked loss of fibrillin-1 immunoreactivity at 2 and 3 wk (Fig. 6B, C), although there was a low level of immunoreactivity in the adventitia of some aortas. Even by 7 d of age there was a noticeable decrease in immunoreactive fibrillin-2 in most, but not all, of the aortas from the DL-methionine–supplemented chicks.

**DISCUSSION**

Vascular elastogenesis in chicks is an early event that occurs just before hatching and then continues at an elevated rate during the next few weeks. Elastin production slows perceptibly as chicks reach adulthood and the organs attain maximum size. Because turnover of elastin in most organs, including the blood vessels, is extremely slow, there is only a maximum size. Because turnover of elastin in most organs, there is only a minimal requirement for new elastin synthesis in the mature animal. By feeding chicks the experimental diets immediately after hatching, tissues undergoing rapid elastin synthesis would be exposed to high circulating levels of homocysteine. During this period any effect of methionine, homocysteine or their metabolites on elastin synthesis, crosslinking or assembly would have their maximal consequences.

Desmosine levels in the aortas, an indicator of total elastin content, were not decreased as a result of homocysteinemia in the chick model. This was evident even during the first 5 wk when smooth muscle proliferation and aneurysm development was maximal. Aortic lysyl oxidase activity was consistently lower in the 2% methionine-fed groups but evidently not enough to affect the desmosine crosslinking process.

The metabolism of homocysteine is influenced by the activity of several enzymes that are dependent on folic acid, vitamin B-12 or pyridoxine for activity. It was possible that under conditions of homocysteinemia, the vitamin levels in the basal diet were insufficient. This was not the case, however, because supplemental vitamins had no effect on the histopathology of the aortas of chicks fed 2% methionine.

We had anticipated a dose response in plasma homocysteine levels as dietary methionine or homocysteine was increased. This did occur (Table 2) and plasma levels exceeding 500 μmol/L were achieved when 1% DL-homocysteine thiolactone was added to the diet, compared to levels of around 140 μmol/L with 2% methionine. However, even with comparable circulating levels in the 1% methionine-fed group, aortic pathology was minimal compared to that in the chicks fed the 2% DL-methionine diet. This raises the question of whether it is a derivative or metabolite of methionine or homocysteine, rather than a direct effect of homocysteine, that causes the lesions.

The progression of the lesions and serum markers of the chick model of homocysteinemia was transient in nature. One possible explanation was a reduction in food consumption per unit body weight (and per unit blood), as the chicks grew older. This in fact was true; however, adding supplemental DL-methionine to the diet to keep the circulating levels of homocysteine elevated did not cause a progressive worsening of the disease.

An interesting observation in the homocysteinemic chicks was the occurrence of tonic contractions and paralysis observed when they were startled. The seizures lasted 5–10 min, after which the chicks were able to move normally. As far as we know, this is the first report of neurological symptoms in methionine-induced homocysteinemia in experimental animals. Homocysteine is associated with neurodegenerative disorders in humans (38). Alzheimer-type dementia, psychosis and ataxia not only have a close association with vitamin B-12, B-6 and folic acid deficiency, but are also strongly associated with elevated homocysteine levels. Epileptic seizures have been associated with severe hyperhomocysteinemia in patients with homocysteine β-synactase deficiency (39). Administration of high doses of homocysteine systemically to rats and mice produces similar epileptic-like seizures (40–42). There have also been several case reports relating severe homocysteinemia to dystonia (43). Homocysteine and some of its metabolites are potent agonists for N-methyl-D-aspartate receptors, which may play a role in the pathogenesis of these neurodegenerative diseases (44,45).

Homocysteinylation of the epsilon amino group of lysine residues results in protein damage and has been shown to inactivate several enzymes (46). This reaction occurs under physiologic conditions with as little as 10 nmol/L homocysteine thiolactone. Homocysteinylation or similar reactions may also occur with other matrix molecules such as the fibrils, which contain 47 cysteine-rich endothelial growth factor–like repeats. Even minor disruption of these disulfide

<table>
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**Effect of DL-methionine, DL-homocysteine thiolactone and DL-cysteine supplementation on body weight, plasma methionine, homocysteine and cysteine and aorta lysyl oxidase, desmosine and hydroxyproline levels in chicks**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body wt</th>
<th>Met</th>
<th>Cys</th>
<th>Hcys</th>
<th>LO</th>
<th>Des</th>
<th>OHP</th>
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<tr>
<td>Control</td>
<td>615 ± 41</td>
<td>174 ± 51</td>
<td>328 ± 22</td>
<td>41.3 ± 2.6</td>
<td>46.3 ± 12.3</td>
<td>5719 ± 260</td>
<td>167 ± 62</td>
</tr>
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<td>2% Met</td>
<td>470 ± 23</td>
<td>4057 ± 332</td>
<td>404 ± 22</td>
<td>137.8 ± 9.8</td>
<td>36.3 ± 7.6</td>
<td>5719 ± 260</td>
<td>167 ± 72</td>
</tr>
<tr>
<td>1% Met</td>
<td>630 ± 63</td>
<td>997 ± 338</td>
<td>254 ± 23</td>
<td>123 ± 41</td>
<td>39.7 ± 5.3</td>
<td>6494 ± 613</td>
<td>172 ± 303</td>
</tr>
<tr>
<td>1% Cys</td>
<td>420 ± 31</td>
<td>167 ± 24</td>
<td>383 ± 37</td>
<td>526 ± 113</td>
<td>ND</td>
<td>6274 ± 454</td>
<td>ND</td>
</tr>
<tr>
<td>7% Cys</td>
<td>641 ± 63</td>
<td>163 ± 18</td>
<td>588 ± 109</td>
<td>37.5 ± 4.7</td>
<td>ND</td>
<td>5346 ± 752</td>
<td>ND</td>
</tr>
</tbody>
</table>

1 ND designates measurements that were not determined in those particular experiments. Abbreviations: Met, methionine; Cys, cysteine; Hcys, homocysteine; LO, lysyl oxidase; Des, desmosine; OHP, hydroxyproline.

2 Values represent means ± SEM, n = 78 chicks from eight experiments.

3 Values represent means ± SD, n = 10.

4 Significantly different from the control, P < 0.01.
TABLE 3

Effect of supplemental vitamin B-12, folic acid and vitamin B-6 on body weight, plasma levels of homocysteine, cysteine and methionine and aorta desmosine in chicks fed a 2% DL-methionine diet.1,2

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body wt</th>
<th>Hcys</th>
<th>Met</th>
<th>Cys</th>
<th>Des</th>
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<tr>
<td>Control</td>
<td>738 ± 113</td>
<td>60 ± 23</td>
<td>341 ± 22</td>
<td>209 ± 60</td>
<td>5122 ± 314</td>
</tr>
<tr>
<td>2% Met</td>
<td>476 ± 110*</td>
<td>117 ± 34</td>
<td>4509 ± 743*</td>
<td>386 ± 55</td>
<td>5059 ± 915</td>
</tr>
<tr>
<td>+ Folate, 5.5 mg/kg</td>
<td>455 ± 134*</td>
<td>134 ± 31</td>
<td>4509 ± 244*</td>
<td>388 ± 31</td>
<td>5574 ± 291</td>
</tr>
<tr>
<td>+ B-12, 0.9 mg/kg</td>
<td>472 ± 92*</td>
<td>120 ± 26</td>
<td>3940 ± 853*</td>
<td>452 ± 65</td>
<td>4448 ± 720</td>
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<tr>
<td>+ B-6, 30 mg/kg</td>
<td>492 ± 110*</td>
<td>116 ± 30</td>
<td>4088 ± 390*</td>
<td>376 ± 57</td>
<td>5193 ± 531</td>
</tr>
<tr>
<td>+ Folate + B-12 + B-6</td>
<td>513 ± 77*</td>
<td>129 ± 40</td>
<td>3711 ± 459*</td>
<td>422 ± 103</td>
<td>4766 ± 536</td>
</tr>
</tbody>
</table>

1 Abbreviations: see Table 2.
2 Values represent means ± se, n = 10.
3 Different from control, P < 0.01.

bonds can disrupt fibrillin aggregation and block microfibril formation (47).

Microfibrils are assumed to act as a scaffold for normal elastin deposition and fiber formation. In Marfan syndrome in humans or in the right skin defect in mice, abnormal elastin formation results from errors in fibrillin-1 assembly (48,49). Many of the pathological features of these disorders are also seen in homocysteinemia.

Electron micrographs of vascular tissue from chicks with homocysteinemia suggest that the microfibrillar component was greatly affected by this condition. In normal chicks, microfibrils were evident on the surface of large elastic fibers and were particularly prominent in small patches of newly synthesized elastin close to the surface of vascular cells. In chicks fed methionine, however, large elastic fibers had fewer microfibrils on their periphery and there were fewer filaments connecting the elastic fiber with the cell surface. In the smaller patches of elastin close to the cell surface, some microfibrils were evident but in most cases only fine, wispy filaments could be seen. These filaments were smaller in diameter than those of normal microfibrils. It is not surprising that some normal microfibrils were evident in the methionine-treated chicks, given that elastin deposition began in the late gestational phase of development before they were fed methionine.

The reduction in elastin-associated microfibrils in the methionine-fed chicks resembles what was observed in the aorta of fibrillin-1–deficient mice (50). In addition to a deficiency of microfibrils evident by electron microscopy, smooth muscle cells in the vessels of these mice acquired an altered phenotype characterized by abnormal matrix synthesis and upregulation of matrix-degrading enzymes. This phenotypic change was thought to result from the reduced number of fibrillin-containing connecting filaments that are postulated to provide contextual signals to smooth muscle cells. Whether vascular cells in the methionine-fed chicks exhibit a similar phenotype to that observed in the fibrillin-deficient mice was not directly examined, although the morphological changes in the vessel wall suggest this possibility. In this context it should be noted that the consequences of homocysteinemia may target a wider range of tissues and have more extensive effects than mutations in any of the individual fibrillin genes because homocysteinylated elastin is at the protein level and can alter any of the three fibrillins or other cysteine-rich proteins associated with microfibrils.

A marked decrease in fibrillin-2 immunostaining indicated that the fibrillin moiety of the microfibrils was one of the targets for the adverse action of homocysteine. Fibrillin-2 is expressed early in development and is preferentially localized to elastin-rich tissues (51). It may play a role in directing elastin fiber assembly by binding and aligning tropoelastin molecules (52,53). Our finding that the formation of desmosine and elastin crosslinking was not significantly affected by the loss of fibrillin-containing microfibrils raises the possibility that microfibrils may not be critical for elastin crosslinking. An alternative explanation is that the elastin produced when homocysteine levels are high can self-assemble onto the pre-existing elastic fibers in a process that does not use microfibrils.

In summary, we have shown that high methionine diets fed to young growing chicks elevated serum homocysteine levels. This led to severe vessel wall pathology, characterized by marked vascular smooth muscle proliferation, disordered elastic fibers, aneurysms and neurological damage. Despite prolonged consumption of the high methionine diet, there was no evidence of a significant decrease in lysyl oxidase activity or a reduction in desmosine crosslink formation. Our findings do suggest, however, that high levels of methionine, homocysteine or their metabolites adversely affect microfibril assembly and, possibly, function.

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