Induction of Increased Collagen and Elastin Biosynthesis in Copper-Deficient Pig Aorta

Kenneth E. Hill and Jeffrey M. Davidson

Young pigs raised on a copper-deficient diet develop severe abnormalities of connective tissue due to defective cross-linking of collagen and elastin. They eventually succumb to anemia and cardiovascular damage, the latter apparently due to the defective connective tissue metabolism. We evaluated the effects of nutritional copper deficiency upon collagen and elastin synthesis using short-term explant cultures of the medial portion of four successive segments of the descending aorta from 110-day-old pigs raised on a copper-deficient diet. Collagen synthesis was evaluated by collagenase susceptibility, and elastin synthesis was quantified by immunoprecipitation with an antiporcine-elastin antiserum. In the normal developing aorta, elastin synthesis was maximal in the upper thoracic aorta, while levels of collagen synthesis were highest in the lower abdominal aorta. Both activities subsided by 110 days postpartum. Compared with controls, the copper-deficient group showed: 1) histopathologic changes confined to the luminal half of the thoracic aorta; 2) a 1.3- to 1.6-fold increase in cellularity along the entire length of the organ; 3) a 1.3- to 2.4-fold increase in relative collagen synthesis, the greatest change occurring in the thoracic portion; 4) a 3- to 4-fold increase of relative elastin synthesis in the thoracic aorta, the abdominal aorta remaining unchanged; 5) 4- to 10-fold increases in collagen production; and 6) a greater than 15-fold increase in elastin production by the tissue of the thoracic aorta. Although copper deficiency produced a complex set of alterations, the combination of defective cross-linking, hypertension, and increased cardiac output appeared to be responsible for the hyperplasia of vascular smooth muscle and a striking induction of connective tissue production. These effects may represent an authentic compensatory mechanism.

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Collagen and elastin are the connective tissue proteins responsible for the structural integrity of the aorta.1,2 They impart the resiliency and tensile strength required to withstand the hydrostatic pressures exerted by the heart. Dietary copper directly influences connective tissue biosynthesis due to its requirement in lysyl oxidase activity.3 This enzyme plays a major role in the cross-linking of collagen α-chains, collagen molecules, and tropoelastin molecules by catalyzing oxidative deamination of lysyl residues which then condense to form characteristic, covalent cross-links.4 Previous studies have shown that removal of copper from the diet of various juvenile animals can produce a diseased state in part resembling lathyrism but accompanied by anemia and cardiac hypertrophy.5 The structural abnormalities associated with copper deficiency are pronounced in many connective tissues including the aorta and coronary arteries. These organs become fragile and prone to dissecting aneurysms. There is gross anatomical and histological damage to the tunica media, and the biochemical composition of the tissue is altered.6

Nutritional copper deficiency has been extensively studied with respect to collagen and elastin accumulation.7-10 Altered cross-linking affects collagen types,11 amino acid composition,12 mechanical properties,5 glycosaminoglycan content,13,14 and collagen degradation in the vascular wall.15 Little is known, however, about the way in which connective tissue cells respond to alterations in the mechanical...
properties of the extracellular matrix. The combined effects of copper deficiency on hematopoiesis and connective tissue cross-linking provide a complex, but intriguing, form of vascular disease.

We have recently shown that in the newborn pig, collagen synthesis and concentration progressively increase in the aorta distal from the heart, while elastin synthesis and accumulation follow the opposite trend. The presence of these biosynthetic gradients determines the regional physical properties of the vascular wall. Recent studies have further indicated that the shape of these biosynthetic gradients is a function of developmental age and that synthetic activity subsides to basal levels by 110 days (Davidson JM, Hill KE, and Alford J, unpublished results). However, it is not known how altered cross-linking or anemia-induced hypertension affects these patterns of synthetic activity.

In this study, we describe the altered patterns of connective tissue biosynthesis in the copper-deficient aorta and the relationship of these changes to position along and within the vessel. We demonstrate that the tissues of the arterial wall respond dramatically to the effects of copper deficiency, by what appears to be compensatory hypertrophy and increased connective tissue biosynthesis by the vascular wall.

**Materials**

**Animals**

We obtained 3-day-old pigs from the Department of Animal Science, Brigham Young University, and studied three normal and four experimental animals. The experimental group was placed on a copper-deficient diet, while the control animals were fed a similar but copper-supplemented diet. The animals were maintained for 15 weeks on their respective regimens and then sacrificed by an intravenous injection of 50 mg of sodium barbital per kilogram of animal weight.

**Isolation of Aortic Segments**

The experimental protocol was similar to previously published procedures. The aortas were removed from the conus arteriosus to the iliac bifurcation by sterile dissection, and the tissues were further dissected free of fat, lymph nodes, and adventitia. Four segments were analyzed for biosynthesis: A, conus arteriosus to the upper midpoint of the thoracic aorta; B, upper midpoint to the diaphragm; C, diaphragm to the lower midpoint of the abdominal aorta; D, lower midpoint to the iliac bifurcation.

**Histological Procedures**

Tissue strips were fixed in 10% buffered formalin and processed by standard methodologies. Hematoxylin and eosin, Masson’s trichrome, Verhoeff-Van Gieson, and alcian blue stains were used to estimate cellularity and discriminate among collagen, elastic tissue, and glycosaminoglycans, respectively. Nuclei were counted by using a microscope equipped with a 1 cm² grid in a ×10 ocular to estimate cell density.

**DNA Content**

Strips of each segment were weighed, lyophilized, minced in 0.05 N NaOH, and extracted overnight at 4°C. The DNA content of the tissue extract was determined by a fluorometric assay. Data were normalized to the wet weight of the tissue. Parallel determinations were done by extraction in 8 M guanidinium-HCl, precipitation with perchloric acid (1.0 M), and a diphenylamine assay, as previously described.

**Biosynthetic Labeling of Collagen and Elastin**

Tissues were minced and incubated in Dulbecco’s modified Eagle’s medium containing 20 µCi/ml 3H-proline (New England Nuclear, > 100 Ci/mmol) for 3 hours at 37°C on a gyratory shaker bath. Labeling was terminated by aspirating the media and rinsing with ice-cold phosphate-buffered saline. Tissue was resuspended in 3 to 5 ml of 0.5 M acetic acid containing 1 µg/ml pepstatin and 10 mM iodoacetic acid. Samples were then homogenized and extracted overnight at 4°C. Homogenates were dialyzed first against 10 volumes and then extensively against 0.5 M acetic acid. The initial dialysate was saved for estimation of praline pool size. The samples were clarified by centrifugation and the supernatants were used for biosynthetic quantification.

**Quantification of Protein Synthesis**

Collagen synthesis was determined by the amount of radioactivity released into trichloroacetic (TCA)-soluble material after limited digestion with highly purified bacterial collagenase. Elastin synthesis was estimated by immunoprecipitation of the radiolabeled material with an antiporcine-α-elastin antiserum. Absolute rates of protein synthesis were estimated from the specific activity of dialyzable free proline as determined by amino acid analysis and liquid scintillation counting.

**Results**

**Anatomic and Histological Differences**

The gross appearance of the copper-deficient aortas was markedly different from that of aortas from normal animals. The entire organ was paler, waxen in appearance, thicker, and more friable. The differences were more apparent in the thoracic area than in the abdominal area and were consistent with previously published histological results.

The most striking morphologic feature was the increase in the thickness and the diameter of the thoracic aorta, almost twice the normal size. The abdominal aorta did not show any noticeable distortion.
when compared with normal tissue (data not shown). The copper-deficient aorta showed a significant increase in the number of medial smooth muscle cells per unit area throughout the length of the organ (Table 1). Subjectively, the ratio of connective tissue matrix to cells appeared to be lower in the thoracic aorta and higher in the abdominal portion.

Histochemical staining of the normal aorta showed a regular staining pattern throughout the length and width of the organ; that is, collagen, elastin, and glycosaminoglycans were evenly distributed within the media. As shown in previous studies, copper-deficient thoracic tissue showed that the elastic lamellae of the internal, luminal portion were split or fragmented in appearance, and glycosaminoglycans were increased and appeared to be pooled in the interfibrillar spaces. The outer portion of the media was less affected than the inner, and the abdominal segments did not show any extensive histological damage (data not shown).

**Cellularity**

Comparison of the guanidine-diphenylamine and fluorometric DNA assays showed the former method to give slightly lower and more variable results than accepted literature values in connective tissue. Consequently, the fluorometric data were utilized as the basis for determining cellularity.

Based on the DNA content per wet weight, the normal aorta showed similar cell densities in Segments A through D, averaging about 3 x 10⁶ cells/g wet weight or 0.2% (Table 1). In contrast, the average number of cells per unit weight was at least 50% greater in the copper-deficient aorta. Microscopic enumeration of cell nuclei corresponded to the values derived from biochemical analysis (Table 1).

**Collagen and Elastin Biosynthesis**

Explanted aortic segments were incubated under standardized conditions which gave linear incorporation of ³H-proline with time, and the tissues were extracted with 0.5 M acetic acid, which solubilized over 90% of incorporated radioactivity. The TCA-insoluble proteins were then analyzed for relative collagen and elastin synthesis.

Figure 1 presents the biochemical data from normal and copper-deficient tissues. The normal group (white bars) showed uniform relative collagen synthesis and absolute production (synthesis corrected for precursor pool specific activity) along the longitudinal axis of the organ. In contrast, the copper-deficient group (dark bars) showed a prominent decreasing gradient of collagen synthesis from Segments A to D (12.20% ± 1.03% to 5.57% ± 0.48%). The greatest variation from normal was the elevated synthesis in the upper thoracic aorta. Net production of collagen per cell increased dramatically, being over 10-fold higher in the copper-deficient thoracic segments.

Relative elastin synthesis in normal 110-day pigs exhibited a shallow, decreasing gradient which was paralleled in the absolute production of elastin. The copper-deficient aortas had a much steeper gradient of synthesis (17.0% ± 1.56% to 2.5% ± 0.1%). Relative synthetic activity increased fourfold in Segment A, and Segment B had a threefold increase, while the abdominal aorta (Segments C and D) remained similar to the normal. The net production of elastin per cell was markedly increased in the thoracic aorta and elevated in the entire organ.

Figure 1 also illustrates the total protein production of the four segments. Production progressively increased in the more distal aortic segments in control animals, but a similar protein production profile in the experimental animals was considerably higher.

To determine the actual biosynthetic output of the aortic tissue, the production per cell was multiplied by the cell density of the tissue. Table 2 shows the average total protein, collagen, and elastin production of copper-deficient aortas compared to normal aortas. These data combine the effects of increased cellular production and cellularity. Thus, the net production of collagen increased markedly (> 15-fold) in

### Table 1. Cellularity of Aortic Segments

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Thoracic segments</th>
<th>Abdominal segments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Biochemical (cells per gram tissue wet weight (x 10⁶))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copper-deficient</td>
<td>493 ± 16</td>
<td>521 ± 64</td>
</tr>
<tr>
<td>Normal</td>
<td>376 ± 46</td>
<td>332 ± 15</td>
</tr>
<tr>
<td>Change*</td>
<td>+1.3</td>
<td>+1.6</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Histologic (nuclei per mm²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copper-deficient</td>
<td>16.3 ± 2.1</td>
<td>11.5 ± 0.3</td>
</tr>
<tr>
<td>Normal</td>
<td>10.8 ± 0.8</td>
<td>8.4 ± 0.2</td>
</tr>
<tr>
<td>Change*</td>
<td>+1.5</td>
<td>+1.4</td>
</tr>
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Values are means ± SEM. Probability factor (p) was determined by one-tailed t-test. Segment A = conus arteriosus to the upper midpoint of the thoracic aorta; Segment B = upper midpoint to the diaphragm; Segment C = diaphragm to the lower midpoint of the abdominal aorta; Segment D = lower midpoint to the iliac bifurcation.

*Copper-deficient divided by normal values.
Figure 1. Connective tissue protein biosynthesis in copper-deficient and normal aortas. Collagen synthesis and production, elastin synthesis and production, and total protein production are compared between the copper-deficient (●) and normal (○) pig aortas. The diagram of the aorta corresponds to the segmental dissection of the intact organ. Error bars represent SEM, and statistical significance (t test) is shown by asterisks (*** = \( p < 0.001 \); ** = \( p < 0.05 \); * = \( p < 0.1 \)).
the thoracic aorta, while elastin production was elevated to an even greater extent (20- to 30-fold). The abdominal aorta showed a three- to sixfold increase of collagen and elastin output. Net protein production was increased fourfold along the length of the copper-deficient aorta.

**Discussion**

Collagen and elastin biosynthesis comprise a significant fraction of total synthetic activity during aortic development, but the steep, inverse gradients noted at birth were no longer apparent at 110 days postpartum. Peak biosynthesis was observed between 1 and 14 days postpartum; as expected, connective tissue protein synthesis subsided to maintenance or residual levels as the organs matured. The introduction of nutritional copper deficiency at the time of maximal growth and connective tissue synthesis produced a dramatic variation in the formation of the extracellular matrix in the developing vascular wall. By producing a severe anemia, copper deficiency also induced high demands on the cardiovascular system; moreover, the tissue appeared to counteract the defective nature of the principal structural proteins and increased cardiovascular output by a compensatory development of the aortas.

These experiments confirmed the gross anatomic and histological changes previously reported in copper-deficient animals. They reiterate the fact that dietary restrictions of copper will eventually produce severe aortic damage in growing animals. As in previous studies, the principal vascular connective tissue histopathology was confined to the thoracic aorta and in particular the inner luminal portion of the vessel. The elastic lamellae were irregular and split in appearance, and there were prominent pools of glycosaminoglycans as has also been seen in lathyritic tissue. These patterns were markedly different from the uniform histochemical staining of normal thoracic tissue. The abdominal aorta did not show obvious morphologic derangement.

Both microscopic and biochemical determination of cellularity in the experimental tissue showed similar relative increases in cell number along the entire length of the organ. Therefore, the thickening of the vessel walls in the copper-deficient animals was not only a result of altered matrix metabolism but also of cellular hyperplasia. Some of the increases in tissue mass and DNA content could have also been due to the cellular hypertrophy and polyploidy that has been reported in a rat model of hypertension. The increased cellularity may have been a response to both the hypertension and the weakening of the artery wall that is associated with copper deficiency. Although increased numbers of smooth muscle cells would not be expected to impart the strength required to overcome the defective connective tissue formation that is associated with copper deficiency, they could increase the contractility of the tissue as well as its biosynthetic capacity.

Nutritional copper deficiency can affect a wide variety of enzyme systems, resulting in neurologic, hematopoietic, and connective tissue defects which can vary, depending on the species, its age, and the severity and timing of copper depletion. Genetic defects in copper transport are manifested in connective tissue abnormalities in the aneurysm-prone, mouse mottled mutant series. In Ehlers-Danlos Type IX syndrome and in the hamster, copper deficiency produces a form of emphysema. The dominant effects of copper deficiency in the juvenile pig were 1) defective crosslinking of collagen and elastin, and 2) severe anemia, which leads to weakened connective tissue matrix and increased cardiac output, respectively. It is likely that this combination of factors provides a stimulus to cells of the vascular wall to proliferate and produce greater quantities of matrix, albeit imperfect. Thus, the overall tissue production of elastin and collagen in the thoracic aorta of the copper-deficient animal was 15 to 30 times greater than that which is found in normal...
animals. Increased production appears to be a compensatory response by the tissues to the altered properties of the extracellular matrix.

A limited examination of the relative increases in both Type I collagen mRNA and elastin mRNA has supported the conclusions of this study (Alford J, Hill KE, and Davidson JM, unpublished results), but more precise quantitation is required to determine whether the absolute, as well as relative, contents of these transcripts have been amplified by vascular cells from the copper-deficient pig.

Both relative and absolute production of collagen and elastin were markedly stimulated in the copper-deficient tissue, and the greatest effects were in the upper thoracic segment, nearest the heart. This increase was great enough to reverse the proximodistal gradient of collagen synthesis and steepen the gradient of elastin gene expression to levels approaching those of the newborn animal.

Nevertheless, the extent to which collagen and elastin production were stimulated was obviously greater than the increases in contents of these proteins. Extensive degradation of newly synthesized soluble precursors could have accounted for this discrepancy. Previous studies in the copper-deficient chick have shown that soluble elastin is inefficiently converted to an insoluble form, and more recent studies indicate a very high rate of turnover for tropoelastin which fails to be cross-linked. Further studies should be able to establish the extent of rapid proteolysis in this experimental system.

These results show that the responses of the copper-deficient porcine aorta were localized within certain regions of the vessel. There was an apparent induction of collagen and elastin gene expression, since the synthesis and production were markedly different from a normal developing vascular tissue. A greatly increased cell density and an elevated net production of connective tissue matrix were reflective of the tissues’ compensatory response to vascular damage. Despite the pleiotropic consequences of copper deficiency, this model may be instructive in understanding the response of vascular tissue to injury.

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Index Terms: aorta • copper deficiency • collagen • elastin • crosslinking • protein synthesis • pig

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