Changes in Aortic Lysyl Oxidase Activity in Diet-Induced Atherosclerosis in the Rabbit

Herbert M. Kagan, Jayashree Raghavan, and William Hollander

This study assessed the response of lysyl oxidase, the enzyme that initiates covalent crosslinking in elastin and collagen, by studying the aortic tissue of rabbits after arteriosclerosis had been induced by diet. Rabbits in the experimental group were fed an atherogenic diet of rabbit chow supplemented with 8% peanut oil and 2% cholesterol for varying periods of time, while the control group was fed only rabbit chow. Lysyl oxidase activity was found to be distributed throughout the length of the thoracic and abdominal aortas of the normal rabbits. However, rabbits fed the atherogenic diet showed marked increases in enzyme activity in the aortic arch, a change that was initially evident after 30 days and became greatest (2.5 times that of the controls) after 90 days. Enzyme activity in the study rabbits increased only minimally in the abdominal aortic wall. Aortic prolyl hydroxylase activity measured after 60 days of feeding changed in degree and manner similar to lysyl oxidase activity. These region-specific changes in enzyme activities correlated with the distribution and severity of aortic lesions in this model of the disease. Lysyl oxidase activity increased dramatically in this model of atherosclerosis, suggesting that this extracellular enzyme activity may prove to be a vulnerable and accessible point of control of the fibrotic response in atherosclerosis. (Arteriosclerosis 1981; 1:287-291)

Early lesions in the atherosclerotic arterial wall are characterized by their contents of accumulated lipid and the presence of fibrogenic cells that probably migrate from the media and proliferate in the intima. Such lesions are seen to eventually contain newly formed fibers of collagen and elastin. As the lesion develops and becomes raised, it may become covered by a collagenous fibrous cap. It has been suggested that the presence of the fibrous cap tends to retard or prevent regression of the lesion if the atherogenic diet is withdrawn from experimental animals, possibly because of the retention of increased aortic cholesterol by the excess connective tissue protein content. The accumulation of connective tissue proteins appears to be influenced by the specific nature of the atherogenic diet, since a diet containing 2% cholesterol further supplemented with 8% peanut oil induces lesions that are more fibrotic than a diet containing only high levels of cholesterol. Such alterations in the content and metabolism of collagen and elastin may be critical to the development and irreversibility of atherosclerotic lesions since these proteins can serve as lipid-binding sites. Further, lipid complexes of elastin have increased susceptibility to elastolytic enzymes and exhibit markedly decreased elasticity. Such effects clearly can have considerable impact on the structure and function of the arterial wall.
The posttranslational events involved in the biosynthesis of both elastin and collagen include the essential role of lysyl oxidase in the initiation of covalent crosslinkage formation between and within the polypeptide chains of elastin and collagen. This enzyme oxidatively deaminates lysyl side chains of these proteins to peptidyl α-aminoadipic-5-semialdehyde. This peptidyl aldehyde residue can spontaneously condense with other aldehyde residues or with unreacted epsilon amino functions to yield a variety of lysine-derived covalent crosslinkages identified in these proteins. Thus, the reaction catalyzed by lysyl oxidase is essential to the accumulation of insoluble fibers and may represent an important site for the biological and, possibly, the chemotherapeutic control of fibrotic processes.

This study examines the response of aortic lysyl oxidase in rabbits fed an atherogenic diet containing 2% cholesterol and 8% peanut oil. The experimental diet consisted of 90 g of rabbit chow, while the control animals were fed the same diet supplemented with cholester and peanut oil. The experimental animals were fed the same diet regimen described by Kritchevsky et al. Animals were sacrificed in groups of five after 30, 60, 90, and 105 days by intravenous injections of sodium pentobarbital (Nembutal, 80 mg/kg). Aortas were quickly removed, cleaned of gross adventitial tissue, and the abdominal aorta, from the coeliac axis to the iliac bifurcation, were separated after planimetric analyses had been performed.

**Methods**

**Animals**

Male white New Zealand rabbits weighing between 2.5 and 3.5 kg were housed two to a cage and fed Purina rabbit chow and water ad libitum for 1 week before the start of the studies. Rabbits matched for body weight were assigned to control and experimental groups. The control animals were maintained on rabbit chow, while the experimental animals were fed the same diet supplemented with cholester and peanut oil. The experimental diet consisted of 90 g of rabbit chow, 8 g of peanut oil, and 2 g of cholester per rabbit per day, similar to the diet regimen described by Kritchevsky et al. Animals were sacrificed in groups of five after 30, 60, 90, and 105 days by intravenous injections of sodium pentobarbital (Nembutal, 80 mg/kg). Aortas were quickly removed, cleaned of gross adventitial tissue, and the abdominal aorta, from the coeliac axis to the iliac bifurcation, were separated after planimetric analyses had been performed.

**Experimental Procedure**

In preparation for biochemical assays, the intima and media were stripped from the adventitia by the method of Wolinsky and Daly. Intima-media tissue samples to be assayed for lysyl oxidase activity were individually homogenized at 0° to 4°C in 2.5 volumes (ml/g wet weight) of 4 M urea, 0.016 M potassium phosphate, pH 7.7, with a Brinkmann Polytron homogenizer at high speed for 90 seconds. The homogenates were centrifuged at 10,000 × g for 20 minutes at 4°C, and the isolated supernatants were dialyzed vs 0.016 M potassium phosphate, pH 7.7. The dialyzed supernatants were directly assayed for lysyl oxidase activity. In cases in which both prolyl hydroxylase and lysyl oxidase activity were to be assayed from the same tissue sample, the intima-media tissues were first homogenized in 0.15 M NaCl, 0.016 M potassium phosphate, pH 7.7, and the supernatant obtained after centrifugation was directly assayed for prolyl hydroxylase activity. Subsequent extraction of the saline-extracted pellets with the 4 M urea-extracting buffer solubilized 90% to 95% of the total lysyl oxidase activity, consistent with the known solubility characteristics of this enzyme in aortic tissue. The phosphate-buffered saline extracts and the dialyzed urea extracts were separately assayed for prolyl hydroxylase and lysyl oxidase activity, respectively.

Assays for lysyl oxidase and prolyl hydroxylase were carried out by the tritium release methods described for each enzyme. Substrate for lysyl oxidase was prepared by pulsing 16-day-old chick embryo aortas with L-4,5-3H-lysine in the presence of β-aminopropionitrile. Extraction of the pulsed aortas with saline, 1 M HCl, and 0.01 M sodium borate, pH 8.0, yielded the saline-insoluble tritiated aortic substrate pellet. Aliquots of the dialyzed urea extracts (50 μl) were added to reaction mixtures containing 125,000 cpm of the substrate pellet in 0.1 M sodium borate, 0.15 M NaCl, pH 8.0. The reaction mixtures (0.75 ml total volume) were incubated at 37° for 2 hours. Tritiated water formed by the action of the added enzyme was isolated by distillation in vacuo, and radioactivity was measured by liquid scintillation spectrometry. All assays for enzyme activity were corrected for enzyme-free controls, and all activities were fully inhibited by the addition of β-aminopropionitrile fumarate to a concentration of 3.5 × 10^-4 M in the assay mixtures.

Prolyl hydroxylase substrate was prepared from the calvaria of 16-day-old chick embryos pulsed in organ culture with L-4,5-3H-proline in the presence of α,α'-dipyridyl. Assays were conducted by the procedure of Hutton et al., modified to use tritiated collagen substrate prepared from the radioactive chick calvaria. All prolyl hydroxylase activities were corrected for appropriate controls and were fully inhibitable by 1 mM α,α'-dipyridyl.

Total soluble protein was determined by the Lowry et al. procedure, and DNA was quantified by the method of Burton. Total serum cholesterol was measured by the method of Schoenheimer and Sperry.
Results

Serum cholesterol levels were markedly elevated in rabbits fed the experimental diet for 30 days or longer. Total cholesterol values ranged from 2300 to 3500 mg/100 ml, with significant variations (table 1). The extent of atherosclerotic involvement was significantly greater in the aortic arch than in the abdominal segment in each of the cholesterol-fed groups. Grossly visible lesions were not observed in the abdominal aorta after 30 days of experimental diet, although they were noted in the arch at this time (table 1).

Distribution of Lysyl Oxidase in the Rabbit Aorta

It has been noted that the thoracic aorta has a greater concentration of elastin than collagen, while this relationship reverses as measurements of the relative contents of these proteins progress from the arch to the abdominal region of the rabbit aorta. In addition, the peanut oil-cholesterol diet induces a greater number and more severe lesions in the arch than in the abdominal aorta in this species. Therefore, we wanted to assess the distribution of lysyl oxidase activity in the normal rabbit aortic wall. As shown in figure 1, lysyl oxidase activity was found throughout the aortic wall from the root to the iliac bifurcation, consistent with the presence of collagen and elastin in all aortic regions. Although there were no marked differences in enzyme activity between the various aortic segments, the activity tends to decrease as measurements proceed toward the bifurcation in the abdominal aorta.

Response of Aortic Lysyl Oxidase to the Atherogenic Diet

The lysyl oxidase activity of the arch and abdominal segments were each assayed from dialyzed urea extracts of tissues of rabbits fed the control or atherogenic diet for specific periods of time (figure 2). This profile of the effects on lysyl oxidase activity was essentially the same regardless of whether enzyme activity was expressed per gram (g) of wet weight as in figure 2, or per milligram (mg) of total protein, per microgram (μg) of DNA, or per square centimeter (cm²) of aortic surface. Lysyl oxidase activity in the aortic arch of rabbits fed the cholesterol-peanut oil diet became elevated approximately 2.5 to 3 times that of control rabbits between 30 and 90 days. The activity remained elevated for up to 105 days, the maximum feeding time used in these

<table>
<thead>
<tr>
<th>Days on Diet</th>
<th>Total serum cholesterol (mg/100 ml)</th>
<th>Gross anatomic disease (% area involved)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aortic arch</td>
</tr>
<tr>
<td>0</td>
<td>56 ± 5</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>2862 ± 121</td>
<td>9.9 ± 1.8</td>
</tr>
<tr>
<td>60</td>
<td>2295 ± 382</td>
<td>22 ± 6.3</td>
</tr>
<tr>
<td>90</td>
<td>2885 ± 397</td>
<td>35.7 ± 9.5</td>
</tr>
<tr>
<td>105</td>
<td>3450 ± 435</td>
<td>57.1 ± 10.1</td>
</tr>
</tbody>
</table>
experiments. In contrast to the changes seen in the arch, enzyme activity did not change appreciably in the abdominal region of the aorta during the first 60 days of feeding. Lysyl oxidase activity did increase slightly in this region relative to the activity in the abdominal aortas of animals maintained on the control diet, as feeding of the atherogenic diet was continued for 90 and 105 days (figure 2).

**Effects on Prolyl Hydroxylase**

Levels of lysyl oxidase and prolyl hydroxylase activities were compared in both anatomical regions at 60 days to assess whether the effects might be selective for lysyl oxidase or reflect a more generalized response of the activities of enzymes involved in the biosynthesis of connective tissue proteins. The activity of both enzymes was increased to similar extents in the arch, with much less of an increase occurring subsequently in the abdominal aortic wall. These effects are consistent with the development of lesions primarily in the arch in this model of atherosclerosis. These changes in enzyme activity did not seem to be an immediate response to high serum cholesterol levels since the lysyl oxidase activity was only slightly elevated in animals fed cholesterol for 30 days, although serum cholesterol was markedly elevated at this time period. Further, there were minimal changes in enzyme activity in the abdominal segment even at 60 days. It is likely that the elevated enzyme activity was caused by increased metabolic activity of fibrogenic cells contained in the atherosclerotic lesion. There is considerable evidence that the rate of collagen synthesis, as well as the total amount of collagen, increases in this model of atherosclerosis. Thus, the change noted here in lysyl oxidase activity may reflect coordinated activation of the biosynthesis of collagen and elastin as well as of enzymes that are involved in their biosynthesis in response to the induction of atherosclerosis.

Results of this study affirm previous reports describing aspects of the response of connective tissue protein metabolism to diet-induced atherosclerosis. Ehrhart and Holderbaum noted a significant increase in collagen synthesis in vitro in aortic tissue taken from rabbits fed 2% cholesterol for 20 or more weeks. These investigators also noted that collagen synthesis was preferentially increased in comparison to noncollagen synthesis by feeding rabbits a 2% cholesterol-8% peanut oil diet for 90 days. Such evidence is consistent with other reports that the addition of peanut oil to the cholesterol-rich diet generates lesions that are more fibrotic.

It is of interest that previous studies indicated that prolyl hydroxylase activity was not elevated in 60 days after feeding a 2% cholesterol diet, but does become elevated in the aorta of rabbits fed this diet for 60 days followed by 20 or 30 days of feeding a cholesterol-free, regression diet. In our present study, the activity of this intracellular enzyme of collagen biosynthesis was significantly elevated in diseased aortic tissue of animals fed the more fibrogenic peanut oil-cholesterol diet for 60 days without subsequent introduction of a regression diet. This variation may reflect the generally stimulatory, more fibrogenic influence of the peanut oil component of the diet, and may be indirectly attributable to specific components of the peanut oil diet previously suggested to be the bases for its fibrogenic affect.

As noted, the lysyl oxidase activity was not markedly increased at 30 days, although serum cholesterol levels were highest at this time. However, increased aortic prolyl hydroxylase activity correlated well with cholesterol accumulated in aortic tissue. A similar correlation may well exist with this parameter, a possibility which is under continued investigation.

The increase in connective tissue protein synthesis previously shown to occur in this and similar animal models of atherosclerosis probably reflects the increased proliferation and biosynthetic capacity of fibrogenic cells involved in atherosclerotic lesions. Our present studies show that this response included a marked increase in the activity of lysyl oxidase, an essential enzymatic component of fibrogenesis, since this enzyme initiates crosslinkage formation in

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**Table 2. Effect of the Atherogenic Diet on Aortic Prolyl Hydroxylase and Lysyl Oxidase Activities**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Days on diet</th>
<th>Arch Enzyme activity* (cpm ³H/assay/mg protein)</th>
<th>Abdominal Enzyme Activity (cpm ³H/assay/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolyl hydroxylase</td>
<td>0</td>
<td>187 ± 26 (100)</td>
<td>174 ± 12 (100)</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>402 ± 44 (215)</td>
<td>199 ± 18 (114)</td>
</tr>
<tr>
<td>Lysyl oxidase</td>
<td>0</td>
<td>53 ± 16 (100)</td>
<td>35 ± 16 (100)</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>128 ± 22 (241)</td>
<td>36 ± 8 (103)</td>
</tr>
</tbody>
</table>

*Mean ± so of triplicate assays of tissue of each of five animals per group.
elastin and collagen. These lysine-derived crosslinkages are essential to the function, insoluble character, and proteolytic resistance of collagen and elastin fibers. In view of the unique role played by this enzyme and in consideration of its apparent function in the extracellular space, this enzymatic site may prove to be an advantageous target for chemotherapy, possibly as a means of controlling the development of fibrotic lesions in atherosclerotic tissue.

References

13. Pinnell SR, Martin GR. The crosslinking of collagen and elastin: Enzymatic conversion of lysine in peptide linkage to α-amino adipic-5-semialdehyde (allysine) by an extract from bone. Proc Natl Acad Sci USA 1968;61:708-716

Index Terms: lysyl oxidase in atherosclerosis · connective tissue proteins · elastin and collagen crosslinking

Erratum

To The Editor:

Please be advised that we inadvertently defined a ratio upside down in our recent paper published in Arteriosclerosis (1981;1:144-155), entitled "Dietary Ethanol-Induced Modifications in Hyperlipoproteinemia and Atherosclerosis in Nonhuman Primates (Macaca nemestrina)." The cholesteryl ester fatty acid (CEFA) ratio is stated in the paper to be Δ2+/Δ0+Δ1, when in fact we actually used the inverse ratio Δ0+Δ1/Δ2+ for the statistical comparisons. In most cases, the implications are the same, but one reader telephoned me to point out the problem. I apologize for this mistake.

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