A Possible Role for Elastin Ligands in the Proteolytic Degradation of Arterial Elastic Lamellae in the Rabbit

HERBERT M. KAGAN, PAUL E. MILBURY, JR., AND DIETER M. KRAMSCH

SUMMARY Thoracic aortae of normal rabbits were perfused with pancreatic elastase in vitro at 37°C and 70 mm Hg pressure in the presence or absence of elastin ligands previously shown to stimulate or inhibit the enzymatic degradation of elastin. Perfusion with elastase resulted in an average of 3.8 lamellae degraded, whereas addition of sodium linoleate before and during the perfusion with elastase increases this value to 7.9 \((P < 0.001)\). Conversely, perfusion with the cationic detergent, dodecyltrimethylammonium chloride, completely prevents the degradation of elastic lamellae by elastase. These effects do not reflect alterations of the intrinsic catalytic activity of elastase, but apparently indicate the formation of complexes between the elastin ligands and arterial elastic lamellae, as is consistent with prior studies indicating such interactions between fatty acids or detergents and purified elastin.

These studies suggest that agents such as fatty acids may significantly alter the metabolic susceptibility of elastin in vivo and possibly contribute to the degradation of elastic lamellae seen in arteries with advanced atherosclerosis. Circ Res 44: 95–103, 1979

THE PROTEOLYTIC degradation of elastin by elastolytic enzymes has been the subject of intensive investigation for many years (Baló and Banga, 1950; Feinstein and Janoff, 1975; Werb and Gordon, 1975; Robert et al., 1970; Hornebeck and Robert, 1977). Much of the interest in this process stems from its possible involvement in the pathology of human disease including atherosclerosis (Loeven, 1977). Much of the interest in this process stems from its possible involvement in the pathology of human disease including atherosclerosis (Loeven, 1977). Much of the interest in this process stems from its possible involvement in the pathology of human disease including atherosclerosis (Loeven, 1977). Much of the interest in this process stems from its possible involvement in the pathology of human disease including atherosclerosis (Loeven, 1977). Much of the interest in this process stems from its possible involvement in the pathology of human disease including atherosclerosis (Loeven, 1977). Much of the interest in this process stems from its possible involvement in the pathology of human disease including atherosclerosis (Loeven, 1977). Much of the interest in this process stems from its possible involvement in the pathology of human disease including atherosclerosis (Loeven, 1977). Much of the interest in this process stems from its possible involvement in the pathology of human disease including atherosclerosis (Loeven, 1977).
elastin protein (Loeven, 1969; Spina and Garbin, 1976; Spencer and Stahmann, 1977). It is of interest, therefore, that lipid-related molecules can influence the susceptibility of elastin to elastolysis. The rate of hydrolysis by pancreatic elastase of purified elastin is increased as much as 30-fold by elastin-bound, hydrophobic, anionic molecules, including saturated and unsaturated fatty acids and sodium dodecyl sulfate (Kagan et al., 1972; Jordan et al., 1974). In contrast, cationic elastin ligands such as dodecyltrimethylammonium chloride (DTAC) also bind to elastin but prevent elastolysis by pancreatic elastase (Kagan et al., 1972; Jordan et al., 1974; Hall and Czerkowski, 1961). These results are consistent with the models of Hall and Czerkowski (1961) and Gertler (1971) indicating that the interaction between elastase and elastin relies heavily on the distribution of a complementary electrostatic charge between the normally cationic elastase and anionic sites on elastin. The elastolytic activity of macrophages is also strongly stimulated by sodium dodecyl sulfate (Werb and Gordon, 1975), indicating that similar charge relationships probably exist between elastin and the macrophage-derived enzyme.

Although the elastase of human neutrophils is less affected by charge-altering agents, it also is a cationic protein (Feinstein and Janoff, 1975).

The presence of neutral lipid in the atherosclerotic artery is well established. It has been reported recently (Claire et al., 1976) that a variety of free fatty acids exist in apparent association with the insoluble elastin and collagen fraction of human arteries. Furthermore, the amounts of free fatty acid as well as of neutral lipid increase in this fraction as the severity of atherosclerosis advances. We now have tested further the hypothesis that free fatty acids and other elastin ligands may influence the susceptibility of arterial elastin to digestion by elastolytic enzymes. As noted, our previous studies have shown that elastolysis of purified, powdered ligament elastin is markedly enhanced or prevented by the appropriate elastin ligands. However, it seems particularly important to test the applicability of this control mechanism to intact elastic fibers situated in the complex environment of the whole arterial wall before hypotheses about the possible relevance of this mechanism to elastolysis in vivo can be supported further. Such studies would also serve to test further the validity of extrapolating information obtained about elastin in vitro to the properties of this protein in vivo. Therefore, in the present communication, we describe the effects of elastin ligands on the digestion by pancreatic elastase of arterial elastic lamellae in rabbit aortae tested in an in vitro perfusion system.

Methods

Pancreatic elastase was purified to homogeneity by the method of Shotton (1974) and was kindly supplied by Dr. Philip Stone of the Department of Biochemistry of Boston University School of Medicine. The sodium salts of fatty acids used in this study were obtained from Applied Science and used without further purification. DTAC was obtained from Eastman Organic Chemicals. New Zealand male rabbits obtained from the Gloucester Rabbitry and weighing 2.5–3.0 kg were housed in single cages and fed Purina rabbit chow ad libitum for 3 days to 1 week prior to use. Fatty acid-free bovine serum albumin was obtained from Sigma Chemical Co.

Rabbits were decapitated 45–60 minutes after sedation by intramuscular injection of diazepam, 3.3 mg/kg. A quick midline incision was made through the abdomen and thorax, the thoracic aorta was exposed, and the branch arteries of the aortic arch were ligated. The aorta then was perfused by cannulation through the left ventricle and filled with a solution of 6% bovine serum albumin (BSA) in Krebs-Ringer bicarbonate buffer (KRB), pH 7.4, during the operative procedures used to prepare for the resection of the thoracic aorta. The sedation and perfusion with BSA-KRB solution were techniques adapted from the studies of Morrison et al. (1976), who described such procedures for maintaining the endothelium of the aorta intact and for prolonging the viability of aortic tissue in vitro. As quickly as possible, the associated aortic fat was removed and all remaining branch arteries of the thoracic aorta were ligated. The length of the thoracic aorta to be used in the experiment was measured in situ so that linear stress equivalent to that which is physiologically normal could be applied to the aorta in vitro in the perfusion apparatus. The thoracic aorta then was removed from the aortic root to the diaphragm after all ligated branches were cut. Both ends of the aorta were attached to the perfusion chamber. The perfusion chamber used in the studies was designed and constructed to permit control of aortic length, perfusion flow rate, temperature, and hydrostatic pressure on the perfusion solution, and to permit intermittent sampling of the aliquots of the recirculating perfusate solution and fixation under physiological conditions of pressure and linear stress. The direction of flow of perfusate through the aorta was the same as that of the circulating blood. The aorta was flushed with KRB buffer at 70 mm Hg pressure for 2 minutes prior to the start of the experiment to rinse out remaining blood and to check for leaks in the aorta. The rinse solution then was replaced with perfusion solution consisting of KRB, or KRB plus elastin ligand, and perfusion was continued for 30 minutes at 37°C and 70 mm Hg pressure, which was the average systemic blood pressure in the rabbits used in this study. In those experiments in which the effect of elastase was studied, the enzyme was introduced into the circulating perfusate after this 30-minute period, and perfusion continued for an additional 30-minute period. Controls were treated identically except for the appropriate omission of ligand or elastase. In each case, total perfusion time was 1 hour. The perfusion chamber in which the
aorta was suspended was filled with KRB buffer and maintained at 37°C and atmospheric pressure during the experiment. The total volume of perfusate circulating through the aorta was 25 ml, although as little as 15 ml can be used in this system. This perfusate was continuously recirculated through the aorta at a rate of 3 ml/min by a Gilson Minipuls pump placed in the perfusion system between the perfusate reservoir and the aorta (Fig. 1).

The aorta was perfused briefly with fresh KRB after the perfusion experiment was complete to flush out the enzyme and/or elastin ligand solution. The aorta then was perfusion-fixed with 2% glutaraldehyde in 0.09 m sodium cacodylate buffer, pH 7.4, at 70 mm Hg pressure for 18 hours at 37°C. After fixation, the aorta was removed from the perfusion chamber, dehydrated in 1,4-dioxane, embedded in paraffin, and sectioned at 5-μm thickness. The sections were stained with the Verhoeff's-Van Gieson elastica stain and examined by light microscopy.

The extent of damage by perfused elastase was determined by morphological examination of the elastic lamellae in each of several sections of treated aortae. The number of damaged lamellae per field was established by counting a lamella completely missing from the field as one damaged unit and adding to this the estimates of fractional damage to other lamellae in the same field. Serial sections were taken from the thoracic aorta through the area from just below the arch to just above the first pair of intercostal arteries. The damage per section, n, was determined as follows: Degraded or damaged lamellae were counted at the four poles of two perpendicular diameters for each aortic section. The sum of the damaged lamellae so measured was divided by four to give the average damage per section. This procedure was repeated with several serial sections so that approximately a 1-cm length was sampled and counted from each perfused aorta in this fashion. The average lamellar damage per aorta was taken as the mean of the n values for each of the sections examined. In each case, the enumeration of damaged lamellae established by one of the authors was checked with unidentified stained sections by the other two authors. This procedure yielded independent values for the mean damage per aorta which were within 10% of each other. It was noted in this sampling procedure that the n values of serial sections taken from any one fixed tissue block were in excellent agreement with each other, suggesting that there had been relatively even penetration of ligands and elastase within the block of tissue examined.

Assays for elastase activity employed the synthetic elastase substrate p-nitrophenyl-N-tert-butyloxycarbonyl-L-alanine (Visser and Blout, 1972) at 25°C, in 0.1 m potassium phosphate buffer at pH 6.5, following the change in absorbance at 410 nm.

Results

Perfusion Studies

A section from the upper thoracic aorta that was perfused with KRB buffer without elastase for 60 minutes at 70 mm Hg pressure and fixed at this pressure, as described, is shown in Figure 2. Note that the elastic lamellae are intact in this preparation, and that they are distended as would be expected under physiological conditions due to the method of pressure fixation employed (Wolinsky and Glagov, 1967). The medial elastic lamellae approximate 22 in number, as previously described (Wolinsky and Glagov, 1967). Densely stained nuclei of endothelial cells are apparent at the intimal surface of the aorta, indicating the presence of this cell layer in the organ after the perfusion and fixation treatment. Although the endothelium is present, examination of the intimal surface at higher magnification revealed that some fragmentation and disruption of the endothelium had occurred at some luminal areas. It was therefore assumed that a variable but possibly significant degree of endothelial damage may have resulted from the perfusion treatment beyond probable spontaneous defects of the endothelium present in vivo.

Perfusion with Elastase

A representative section of an aorta perfused with elastase (50 μg/ml) is shown in Figure 3. The inter-
nal elastic lamina is mostly degraded, as is the first medial lamella, whereas lesser damage is noted in the next one to two medial lamellae. This result is consistent with the degree of damage noted in other sections of the same aorta and in other aortae of different rabbits treated identically. The endothelium appears present but is damaged in this section.

Effect of Fatty Acid in the Perfusate

Perfusion with sodium linoleate \(2.5 \times 10^{-3} \text{ M}\) in the absence of elastase apparently does not alter the elastic lamellae of the intima or media, as shown in Figure 4. Some loss of endothelium is noted in this section. In contrast, perfusion of an aorta with elastase in the presence of \(2.5 \times 10^{-3} \text{ M}\) linoleate (Fig. 5) causes considerably more damage to the elastic lamellae than was observed with elastase alone. The endothelium of the section shown in Figure 5A is partially intact, and eight to nine lamellae are affected by elastase in this case. The endothelium of the section shown in Figure 5B apparently is missing, and there is somewhat greater damage, amounting to 9 to 10 lamellae degraded. Examination of several sections did not reveal a consistent correlation between the degree of endothelial damage and lamellae degraded.

Effect of DTAC in the Perfusate

Elastolysis of purified elastin is prevented or strongly inhibited by the binding to elastin of the cationic detergent DTAC, as previously shown (Jordan et al., 1974; Hall and Czerkowski, 1961). Perfusion of the aortae for 60 minutes with \(5 \times 10^{-3} \text{ M}\) DTAC alone does not disrupt the elastic lamellae but does cause some damage to the cellular structures of the aortic media. However, this agent completely prevents the enzymatic degradation of elastic lamellae when present in the perfusate together with elastase (Fig. 6).

Elastase Assays of Perfusate

The design of the perfusion apparatus used in these studies allows the removal of samples of perfusate for analysis during the perfusion experiment. The enzyme activity in the perfusate was determined by assays of samples of perfusate, as described in the Methods section. The activity of elastase in the perfusate of an aorta treated with the enzyme in the absence of added elastin ligand was determined at varying times during each of the experiments cited. The soluble enzyme activity remained essentially constant during the course of
each experiment. The activities obtained at 60 minutes are compared in Table 1. These results indicate that the perfused aorta contains little if any soluble inhibitor of elastase, consistent with the treatment of the aorta prior to the perfusion experiment. It is also important to note that the elastin ligands which either stimulate or inhibit elastolysis in the aortae do not significantly affect the level of enzyme activity initially added to the perfusate. This observation supports the hypothesis that the effects observed reflect interactions of these molecules with aortic wall components, presumably elastin, rather than modulation of the intrinsic catalytic properties of the enzyme by these agents. This is consistent with previous studies showing that fatty acids or DTAC stimulate or inhibit elastolysis, respectively, through interactions of these agents with purified elastin (Kagan et al., 1972; Jordan et al., 1974).

Summary of Perfusion Studies
A summary and a statistical analysis of the results are presented in Figure 7. Enumeration of the number of lamellae partially or totally damaged was established in each of several fields examined in multiple sections taken from each treated aorta, as described in the Methods section. As shown, the data indicate that the fatty acid enhances the susceptibility of the arterial elastic lamellae to elastolysis more than 2-fold, whereas the cationic detergent completely prevents elastolysis in all sections examined. Analysis of the data in Figure 7 by a nonpaired t-test yielded a \( P \) value of <0.001 for the comparison of the enzyme-treated aortae and aortae treated with enzyme plus fatty acid.

Discussion
Many of the static and mechanical features of the aortic media have been related to the close association of its elastin, collagen, microfibrillar, and smooth muscle components in concentric layers, termed lamellar units (Wolinsky and Glagov, 1967; Bergel, 1961a and b; Ross and Bornstein, 1970). The average tension per lamellar unit is remarkably constant in a number of mammalian species, suggesting that the elastic lamella and the contents of its adjacent interlamellar zone represent the unit of structure and function of the mammalian aortic wall (Wolinsky and Glagov, 1967). It seems clear, therefore, that the elastic lamellae play essential roles in the structure and function of the normal arterial wall. Therefore, elastolysis, and factors
which affect this process, could have far-reaching effects upon the status of the arterial wall.

Although elastin is generally regarded as a very stable protein whose half-life approximates the life of the animal, there has been chemical evidence that degradation of elastin occurs with aging in the human aorta (Robert, 1977). This evidence is consistent with observations that elastic lamellae appear fragmented with age and severity of atherosclerosis (Spina and Garbin, 1976; Adams and Tuqan, 1961; Schlattmann and Becker, 1977). Loeven (1969) postulated that such damage reflects proteolytic events occurring in these aging or diseased tissues. An elastolytic enzyme has been partially purified from pig aorta extracts, and it has also been noted that elastolytic activity in human aorta increases with increasing extent of atheroma (Hornebeck and Robert, 1977).

The present study focuses on the possibility that factors in addition to elastolytic enzymes may play roles in the degradation of elastin in aortic tissue. The present studies show that molecules such as fatty acids, previously shown to bind to purified elastin and to promote elastolysis by enhancing the normally minimal negative charge of the substrate, have the potential to promote elastolysis in whole aortic tissue as well. Conversely, the cationic detergent, DTAC, previously shown to inhibit elastolysis by coating the substrate with sufficient positive charge to repel cationic elastase enzymes, also inhibits elastolysis in the perfused aorta. Therefore, this control mechanism apparently can operate within the complex environment of the arterial wall, as well as with pure elastin.

It is likely that pancreatic elastase is not of primary importance to damage seen in aortic tissue because of its compartmentalization in the gastrointestinal tract and, more importantly, because of the presence of circulating elastase inhibitors. It should be noted, however, that immunological evidence has been presented that pancreatic elastase is present in plasma as an inhibited complex (Geokas et al., 1977). However, other elastolytic enzymes of neutrophil and macrophage origin clearly can influence aortic elastin. There is evidence for infiltration of such cells into the arterial media with accompanying damage to elastic lamellae after mechanical dilation of the thoracic aorta of rabbits with a balloon catheter (Helin et al., 1971). It is also of interest that apolipoprotein B, the major protein in human plasma low-density and very low-density lipoprotein, has been identified apparently bound to intima-medial elastina and collagen fibers in atherosclerotic human arteries (Hoff and Gaubatz,
FIGURE 5  Rabbit aorta perfused with sodium linoleate for 30 minutes and then with linoleate and elastase for an additional 30 minutes.
Thus, a potential source of esterified fatty acids, at least, can penetrate to elastic fibers in the arterial wall. Although elastolysis may not be the initiating event in atherosclerosis, it is likely that it plays an important role in the total pathology of the atherosclerotic process. The disruption of elastic lamellae, possibly facilitated by elastin-bound fatty acids and the presence of elastolytic enzymes, would be expected to weaken severely the strength of the arterial wall and potentially contribute to the development of aneurysms. Further, partial digestion of elastin would induce marked changes in the conformation and polarity of this normally apolar protein. Such events could clearly influence the interaction of the fragmented elastin fibers with lipids, calcium, glycosaminoglycans, collagen, cell membranes, and the closely associated microfibrillar protein component, thus altering the intimal-medial environment significantly with possible consequences to the development of the atherosclerotic lesion. In this regard, it has been shown recently that elastin peptides are immunogenic (Daynes et al., 1977), and thus that elastolysis could initiate an immune response. These possibilities are consistent with the observation that treatment of the abdominal aorta in vivo with elastase markedly increases the formation of subintimal fibrous plaques and lipid deposition upon subsequent feeding of the experimental animals with high cholesterol diets (Nabseth et al., 1963).

Although linoleate was selected for the present studies because of its relative solubility, saturated fatty acids, including sodium oleate, have been shown to enhance elastolysis quite as effectively

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**Table 1** Esterase Activity of Elastase in Perfusates

<table>
<thead>
<tr>
<th>Sample</th>
<th>Esterase activity</th>
<th>Percent control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elastase stock (50 µg/ml)</td>
<td>0.032</td>
<td>(100)</td>
</tr>
<tr>
<td>Perfusates at 60 minutes:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elastase alone</td>
<td>0.034</td>
<td>106</td>
</tr>
<tr>
<td>Elastase + linoleate</td>
<td>0.036</td>
<td>109</td>
</tr>
<tr>
<td>Elastase + DTAC</td>
<td>0.029</td>
<td>91</td>
</tr>
<tr>
<td>Krebs-Ringer bicarbonate without elastase</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Esterase activity in 0.1-ml aliquots of each sample was assayed using p-nitrophenyl-N-tert-butylxycarbonyl-alanine as substrate and following its hydrolysis spectrophotometrically, as described. $\Delta A_{410}$ = change in absorbance at 410 nm.

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**Figure 6** Rabbit aorta perfused with DTAC for 30 minutes and then with DTAC and elastase for an additional 30 minutes.
Figure 7. Summary of perfusion studies. Each bar represents one perfused aorta. The heights of the bars represent the average number of lamellae damaged per aorta. The bracketed vertical lines indicate two standard deviations. The numbers in parentheses reflect the number of sections examined per aorta. Damaged lamellae per section were enumerated by examination of four fields per section, as detailed in the Methods section.

(Jordan et al., 1974). The formation of such elastin-fatty acid complexes also markedly decreases the mechanical strength of elastic fibers, likely through disruption of hydrophobic bonding within the elastin protein (Mukherjee et al., 1976). In summary, it seems reasonable to consider that agents such as fatty acids may induce significant physical and metabolic changes affecting the integrity of the elastic lamellar units of the arterial wall.

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H M Kagan, P E Milbury, Jr and D M Kramschi

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