The borohydride-reducible compounds of human aortic elastin

Demonstration of a new cyclic amino acid in alkali hydrolysate, and changes with age and in patients with annulo-aortic ectasia including one with Marfan syndrome

Tapio HALME,* Mirjami JUTILA,† Timo VIHERSAARI,* Pentti OKSMAN,† Nicholas D. LIGHT‡ and Risto PENTTINEN*§

*Departments of Medical Chemistry and †Department of Chemistry and Biochemistry, University of Turku, SF-20520 Turku, Finland, and ‡A.F.R.C. Meat Research Institute, Langford, Bristol BS18 7DY, U.K.

Human aortic elastin reduced with [³H]borohydride was analysed by ion-exchange chromatography after alkali or acid hydrolysis. Alkali hydrolysates of elastins contained a radioactive peak that was eluted between proline and leucine. This peak was not present in foetal elastin, but its proportion increased steadily during aging. Aortic samples from patients with annulo-aortic ectasia (aneurysm of the ascending aorta), including one with classical Marfan syndrome, contained less elastin (CNBr-insoluble material) than did the age-matched controls. The proportion of radioactivity in the new peak of all these aortas was low when compared with age-matched controls. Gas-chromatographic/mass-spectrometric analysis suggested that it contained a cyclic derivative of a hydrated aldol-condensation product. The concentration of the cross-link precursors, lysine aldehyde and aldol-condensation product (estimated from the acid-hydrolysis product 6-chloronorleucine and the acid-degradation product of reduced aldol-condensation product) was high in very young aortas but remained quite stable after childhood. No differences were observed in cross-link profiles of acid hydrolysates between pathological and control aortas. A low proportion of radioactivity in the new peak may indicate the presence of young or immature elastin in the pathological aortas.

INTRODUCTION

Elastin is the main structural non-collagenous protein of the aortic wall. It is extensively cross-linked and metabolically quite inert. The precursor, tropoelastin, is secreted into the extracellular space and cross-linked into insoluble elastin (Sandberg *et al.*, 1982). The cross-linking begins by an enzymic step catalysed by lysyl oxidase (Pinnell & Martin, 1968; Eyre *et al.*, 1984) to form lysine aldehydes. They react with each other to yield an aldol-condensation product (ACP) (Salcedo *et al.*, 1969), probably via a β -hydroxy-ACP, which is then dehydrated to $\alpha\beta$ -unsaturated ACP (Rojkind *et al.*, 1966; Robins, 1982). Alternatively, lysine aldehydes react with peptidyl lysine residues to yield dehydrolysinonorleucine, which in elastin is mostly converted into lysinonorleucine, an unreducible cross-link (Lent & Franzblau, 1967). The unique cross-links of elastin, desmosine and isodesmosine, are formed via reaction of ACP with dehydrolysinonorleucine (Sandberg, 1976; Gray, 1977) or via reaction of ACP with a peptidyl lysine residue to yield dehydromerodesmosine followed by addition of a third peptidyl lysine aldehyde residue (Francis *et al.*, 1973).

Age-dependent changes in elastin cross-links have been evaluated only in a few studies. Increased (Ledvina & Bartos, 1968) or decreased concentration of desmosines and a concurrent increase in the concentration of lysinonorleucine (John & Thomas, 1972) have been suggested in aging elastin. Elastin fluorescence increases with age (Deyl *et al.*, 1981), but the molecular

background of this phenomenon is not known (Robins, 1982). Hexosyl-amino acids in proteins, e.g. in collagen and haemoglobin, have been used as a marker of the protein age (Robins, 1982; Mayer & Freedman, 1983), but such markers are not available for elastin.

Reduction of elastin with [³H]borohydride and subsequent amino acid analysis is a common technique used in studies of elastin cross-links. Borohydride reduces lysine aldehydes, ACP, dehydrolysinonorleucine, dehydromerodesmosine, merodesmosine and desmosines (Robins, 1982; Paz *et al.*, 1982).

In annulo-aortic ectasia the ascending aorta and aortic annulus are dilated and the aortic valve is insufficient (Ellis *et al.*, 1961). Annulo-aortic ectasia is often one manifestation of the Marfan syndrome (McKusick, 1972), but in most cases there is no generalized connective-tissue weakness. Elastin concentration is decreased in the dilated aortic wall of many of these patients (Halme *et al.*, 1982).

The Marfan syndrome is a dominantly inherited connective-tissue disorder with skeletal, ocular and cardiovascular manifestations (McKusick, 1972). The basic molecular defect is obviously variable. Many studies point to altered structure or metabolism of collagen (Laitinen *et al.*, 1968; Krieg & Müller, 1977; Boucek *et al.*, 1981; Byers *et al.*, 1981) or elastin (Gunja-Smith & Boucek, 1981; Derouette *et al.*, 1981; Halme *et al.*, 1982). Decreased concentration of desmosines in purified (hot-alkali-extracted) elastins from three Marfan-syndrome aortas has been shown by Abraham *et al.* (1982).

Abbreviation used: ACP, aldol-condensation product of two lysine aldehyde residues.

§ To whom reprint requests should be addressed.

Table 1. Proportion of elastin in neutral-salt-insoluble aortic residues

The aortic samples were extracted with phosphate-buffered saline and 4 M-guanidine, reduced with borohydride and digested with CNBr. The CNBr-insoluble material is considered to represent elastin.

Age of donor of the aorta	Sex of donor	CNBr-resistant material (%)
Controls		
Foetal (gestational age 32 weeks)	Male	67
3 months	Male	75
7 years	Female	50
18 years	Male	67
29 years	Male	60
35 years	Male	61
60 years	Male	51
Patients		
10 years (Marfan syndrome)	Female	22
19 years	Male	36
32 years	Male	35
54 years	Male	38
60 years	Male	19

The purpose of the present investigation was to study qualitative changes in aortic elastin during aging and in patients with annulo-aortic ectasia. This was performed by analysing the NaB³H₄-reducible components of elastin. We found evidence of a previously unknown cyclic amino acid, 2-amino-6-(6-carboxy-3-piperidyl)-6-hydroxyhexanoic acid, in alkali hydrolysates of reduced elastin of aged aorta.

MATERIALS AND METHODS

Aortas (Table 1)

Control aortas were from autopsies made for healthy people who died in accidents. The 'foetal' sample was from a prematurely born boy who died of respiratory difficulties (gestational age 32 weeks). The pathological samples were from surgical corrections of annulo-aortic ectasia. All samples were from the ascending aorta.

Preparation of the aortic samples

Intima media of the aortae (200–300 mg wet wt.) was pressed under 1600 kg/cm² and then homogenized in 10 ml of phosphate-buffered saline (0.15 M-NaCl/0.02 M-sodium phosphate buffer, pH 7.3) with a Teflon/glass Potter–Elvehjem homogenizer. The white homogeneous suspension was then extracted with phosphate-buffered saline at 4 °C overnight with continuous stirring and centrifuged at 30000 g for 30 min at 4 °C. The pellet was extracted twice for 24 h at 4 °C with 20 ml of 4 M-guanidine/0.05 M-Tris/HCl buffer, pH 7.5, with centrifugation as described above and washed with phosphate-buffered saline twice for 24 h to remove guanidine.

Reduction with NaB³H₄

The insoluble material was suspended with a glass rod in phosphate-buffered saline (1 ml/100 mg wet wt.). NaB³H₄ (specific radioactivity 209 mCi/mmol; The

Radiochemical Centre, Amersham, Bucks., U.K.) dissolved just before in ice-cold phosphate-buffered saline (3 mg/ml) was added (1 mg/100 mg wet wt. of the pellet). After 2 h at room temperature the reduction was stopped by adjusting the pH to 3.0 with acetic acid, and the free radioactivity was washed away with water.

Isolation of elastin by CNBr digestion

The insoluble residue was denatured in 70% (v/v) formic acid at 60 °C for 5 min, cooled to 30 °C and digested with CNBr (20 mg/ml) overnight at 30 °C with shaking. A 2-fold volume of water was added, and the solubilized peptides were separated by centrifugation at 2000 g for 10 min. The residue was washed with 10% formic acid, and, after dilution, the pellet and the supernatant were freeze-dried. The pellet was regarded as elastin.

Amino acid analysis

Amino acids of the CNBr-resistant NaB³H₄-reduced residue were analysed after hydrolysis in 6 M-HCl at 105 °C for 48 h as 4'-dimethylaminoazobenzene-4-sulphonyl-amino acids by reversed-phase h.p.l.c. (Chang *et al.*, 1981, 1982) on a Lichrosorb RP 18 250 mm × 4 mm column (particle size 5 µm) (LKB 2134–215) with an LKB 2150 pump, LKB 2151 detector and LKB 2152 controller (LKB, Bromma, Sweden).

Analysis of the NaB³H₄-reduced amino acids

Alkali hydrolysis of elastin was performed in 2 M-KOH at 110 °C for 22 h in sealed test tubes under N₂ atmosphere, and acid hydrolysis in 6 M-HCl at 105 °C for 48 h in air-cooled open test tubes. After hydrolysis, HCl was removed by evaporation, and KOH by precipitation as KClO₄.

The amino acids were separated by ion-exchange chromatography in volatile pyridine/formate buffers. The elution positions of standard amino acids were used to identify radioactive components (Light & Bailey, 1982).

The alkali hydrolysates were analysed on an 80 cm × 0.72 cm column of Amberlite IR-120/AS (particle size 28–35 µm) resin (Serva, Heidelberg, West Germany) at 60 °C by using a gradient buffer system from 0.1 M-pyridine/formate, pH 2.9, to 0.8 M-pyridine/formate, pH 5.0 (175 ml + 175 ml) (Light & Bailey, 1982). Fractions at 4 min intervals (2.5 ml) were collected, 1.0 ml of each of which was used for determination of radioactivity as described previously (Järveläinen *et al.*, 1982).

The acid hydrolysates were analysed on a 250 mm × 4 mm h.p.l.c. cation-exchange column of Aminex A-9 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) at 60 °C, with the starting buffer (0.1 M-pyridine/formate, pH 2.9) during 0–15 min, and then a linear gradient to 30% (v/v) starting buffer/70% (v/v) 0.8 M-pyridine/formate, pH 5.0, during 15–185 min; the flow rate was 0.35 ml/min, and fractions were collected at 1.6 min intervals. Before each run the column was regenerated with 2 M-pyridine for 30 min and equilibrated with starting buffer for 90 min. A 0.3 ml sample of each fraction was taken for counting of radioactivity.

Gas chromatography-mass spectrometry (g.c.-m.s.)

The radioactive peak was collected from chromatography of a pooled alkali hydrolysate of ten elastin

Fig. 1. Chromatogram of alkali hydrolysate (a) or acid hydrolysate (b) of reduced foetal aortic elastin

For full experimental details see the text. The elution positions of standard amino acids are shown as black triangles. Abbreviations: HNL, 6-hydroxynorleucine; CNL, 6-chloronorleucine; ALDOL, acid-degradation product of reduced ACP.

preparations (from aortas of 7–60-year-old humans). The peak was evaporated to dryness under N₂ flow, and its contents were converted into *N*-trifluoroacetyl *n*-butyl esters (Roach & Gehrke, 1969) and analysed by using a 3800 HR gas chromatograph (DANI, Monza, Italy) and a V.G. Analytical 7070 E Organic Mass Spectrometer (V.G., Manchester, U.K.). The capillary column (25 m long × 0.32 mm internal diam., coated with OV-1 liquid stationary phase; Orion Analytica, Espoo, Finland) was coupled directly to the mass spectrometer. The injector temperature was 250 °C; the column was oven-temperature-programmed from 90 °C to 300 °C at 5 °C/min, and the helium carrier gas flow rate was 1 ml/min. With the capillary column a splitless injection with a delay of 30 s was used. The electron-impact mass spectra were obtained at 70 eV.

RESULTS

Identification of the radioactive peaks

Chromatograms of the alkali and acid hydrolysates of foetal aortic elastin are shown in Fig. 1. For identification, the putative hydroxynorleucine (the reduced form of lysine aldehyde) and reduced ACP peaks of alkali hydrolysates were collected and evaporated to dryness. After HCl hydrolysis the products were fractionated again. The hydroxynorleucine fraction yielded two peaks, 6-hydroxynorleucine and 6-chloronorleucine. Roughly 70% of the initial 6-hydroxynorleucine was converted into 6-chloronorleucine, which was eluted between leucine and phenylalanine. Acid degradation of reduced ACP yielded one major peak, which was eluted just before leucine. These findings, as well as the elution positions of

6-hydroxynorleucine and reduced ACP, are consistent with previous observations (Lent *et al.*, 1969; Volpin *et al.*, 1973).

The main radioactive peak in the chromatograms of acid hydrolysates was detected between hydroxylysine and lysine (Fig. 1b). Most probably it contains tetrahydrodesmosines (reduced desmosine and isodesmosine and their dihydro forms; Paz *et al.*, 1982), and it might also contain traces of reduced dehydromerodesmosine and reduced merodesmosine (Starcher *et al.*, 1967; Paz *et al.*, 1971). The 'reduced desmosine' peak yielded two smaller peaks when isolated and degraded by 2 M-KOH at 110 °C for 22 h (results not shown), as previously reported (Lent *et al.*, 1969).

Lysine aldehyde and ACP concentrations were determined as the acid-hydrolysis products of their reduced forms. 6-Chloronorleucine was a marker for lysine aldehyde because no other component was eluted in the same region in our chromatography system. However, the 6-hydroxynorleucine/6-chloronorleucine ratios varied, being greater than expected (Lent *et al.*, 1969), indicating contamination of the hydroxynorleucine peak by some other radioactive material.

CNBr-resistant material

The proportion of CNBr-resistant material in the insoluble aortic residue was clearly smaller (about one-half) in all pathological samples than in the controls (Table 1). The CNBr-resistant material of aortas from 3-month-old, 18-year-old and 60-year-old humans contained no methionine (indicating that the digestion was complete), no hydroxylysine and very low amounts (< 1/1000 residues) of histidine or cysteine (results not shown), indicating that the residue was practically pure elastin at all ages.

Fig. 2. Chromatograms of alkali hydrolysates of reduced elastins from aortas of humans of different ages

Elastins were prepared from aortas of foetal (a), 7-year-old (b) and 60-year-old (c) human controls. For full experimental details see the text. The elution positions of standard amino acids are shown as black triangles. For discussion of peak X see the text. Abbreviation: HNL, 6-hydroxynorleucine.

Proportion of radioactivity in reducible components of elastin

Chromatograms of alkali hydrolysates of aortic elastins (from foetal, 7-year-old and 60-year-old humans; Fig. 2) showed 6-hydroxynorleucine and reduced ACP and a well-separated peak (called 'X' in this text) between 6-hydroxynorleucine and reduced ACP. The ratio peak X/reduced ACP increased steadily with age in control aortic elastins (practically no peak was detected in foetal elastin) (Fig. 3). In elastins from all pathological aortas this ratio was lower than in age-matched controls ($P = 0.025$).

Another unidentified peak was eluted just before proline (Fig. 2). The radioactivity of this peak tended to increase with age, but some of it was present in foetal elastin and its proportion was lowest at the age of 7 years. Similar behaviour was observed when 'hydroxynorleucine' was quantified, but by means of subsequent acid hydrolysis and chromatography this peak appeared to

Fig. 3. Ratio peak X/reduced ACP in alkali hydrolysates of elastins from different aortas

The ratios were calculated from radioactivities in the peaks. $P = 0.025$ for difference between the groups of pathological (●) and control (○) aortas (Wilcoxon matched-pairs signed ranks test). M indicates the case of Marfan syndrome. For discussion of peak X see the text.

contain several components (results not shown). The significance of these findings therefore remains in doubt.

Chromatograms of acid hydrolysates of reduced elastins from control aortas showed that the ratio 6-chloronorleucine/acid-degradation product of reduced ACP was highest in elastin of foetal aorta, and that of acid-degradation product of reduced ACP/reduced desmosines at 3 months age. These values decreased to almost constant levels after cessation of growth. The values were similar in pathological and age-matched control samples.

Gas-chromatographic/mass-spectrometric data

Gas chromatography of the component of peak X showed only one well-separated peak, which was not present in an equivalent amount of foetal aortic elastin or in the buffer blank (results not shown). This component emerged from the g.c. column at 145 °C.

The electron-impact mass spectrum of this peak is shown in Fig. 4. The proposed fragmentation scheme of *N*-trifluoroacetyl di-*n*-butyl ester of the component is shown in Scheme 1, and the proposed pathway of formation and structure of this cyclic amino acid in Scheme 2.

DISCUSSION

In this study we have analysed the borohydride-reducible compounds of human aortic elastin. The elastin preparations were isolated from healthy aortic walls of different ages and from patients with annulo-aortic ectasia, one of whom had the classical Marfan syndrome.

Elastins were prepared by CNBr digestion of borohydride-reduced insoluble aortic intima-medial material. CNBr cleaves proteins at methionine residues (Scott & Veis, 1976) and thus leaves elastin insoluble (Gotte, 1980).

Chromatograms of alkali hydrolysates showed a radioactive peak that was eluted between proline and leucine. This peak was absent from the preparation from foetal elastin and its proportion (in relation to reduced ACP) increased during aging. It was most probably not due to protein contamination, as histidine, cysteine and hydroxylysine concentrations of elastin preparations from aortas of different ages were below the level 1/1000

Fig. 4. Mass spectrum of the *N*-trifluoroacetyl di-*n*-butyl ester derivative of the component included in peak X

The peak X was collected from ion-exchange chromatography with the use of radioactivity as a marker. For full experimental details see the text.

Scheme 1. Suggested fragmentation scheme of the *N*-trifluoroacetyl di-*n*-butyl ester derivative of the new cyclic amino acid in electron-impact mass-spectral analysis

The m/z 481 (Fig. 4) ion is suggested to correspond to $M-1$ ion. The main ion at m/z 97 (Fig. 4) supports the view that a stable cyclic structure forms a part of the component, and the proposed form of this cyclic ion is indicated.

amino acids. Its elution position (between proline and leucine, close to valine) suggested hexitol-valine (Bailey *et al.*, 1976), but since it was not found in acid hydrolysates this possibility is unlikely.

G.c.-m.s. analysis of this new peak showed a reduced cyclic derivative of the aldol condensation product (Scheme 2). The question of the means of formation of this component is open, but it could be explained by a

Schiff reaction between the free aldehyde of hydrated ACP and the adjacent primary amino group of the same ACP residue (Scheme 2). The cyclization reaction presumes that the ACP is the *N*-terminal amino acid. The cross-linking sites of elastin of young tissues are surrounded by alanine, tyrosine and glycine residues (Sandberg *et al.*, 1982; Rucker & Murray, 1978; Foster *et al.*, 1973). Determination of the *N*-terminal amino acid

Scheme 2. Proposed pathway of formation, alternative structures and sites of borohydride reduction of the suggested component of peak X

The possible Amadori-like rearrangement is indicated in the Scheme.

of elastin has given variable results depending on the purification procedure used (Gotte, 1980). As far as we know, lysine has not been reported to be the *N*-terminal amino acid of tropoelastin. It could be postulated, however, that during aging non-specific proteinases might cleave the elastin peptides outside the cross-linked stable network. As a result the cross-links would be situated at the ends of polypeptides and the reaction described above would be possible with time.

Many questions arise concerning the compound in 'X'. One is the probability of an Amadori-like rearrangement, where the double bond would move from the Schiff base to the hydroxy group. The primary form of ACP, at least in collagen, is likely to be β -hydroxy-ACP (Rojkind *et al.*, 1966). A cyclic derivative of $\alpha\beta$ -unsaturated ACP might be very unstable because of its inability to undergo an Amadori-like rearrangement. The 'disappearance' of peak X in chromatograms of acid hydrolysates can also be related to this rearrangement reaction. In the rearranged product the borohydride reduction site would be the keto group, and acid-catalysed dehydration of the labelled β -hydroxy group (Rojkind *et al.*, 1966) would make this component non-radioactive and explain its 'disappearance'.

The possibility that this new cyclic amino acid is formed as a result of alkali hydrolysis from a more complex structure must also be kept in mind. Further studies are evidently needed to establish the origin of the compound.

Faris *et al.* (1976) found a radioactive component that was eluted near valine in alkali-hydrolysed elastin from [^{14}C]lysine-labelled aortic smooth-muscle cell culture. This and our component of peak X may be identical.

The lysine aldehyde/ACP and ACP/desmosine ratios were highest in elastin preparations from the aortas of foetal and the 3-month-old humans, but no major changes occurred after the age of 10 years. Most probably the high proportion of cross-link precursors reflects rapid elastin synthesis, but these ratios seem to be quite insensitive markers for the rate of elastin synthesis.

Elastins from the pathological aortas had values comparable with controls.

The ratio peak X/reduced ACP was lower in alkali hydrolysates of elastins from all pathological aortas as compared with age-matched controls (Fig. 3, $P = 0.025$). If the occurrence of the peak X component is a marker for elastin maturation, this may reflect the presence of recently synthesized elastin in pathological aortas, possibly indicating a repair process as a response to tissue damage.

In the present study the results of the Marfan-syndrome aorta did not differ from those of other annulo-aortic ectasia patients. This suggests that annulo-aortic ectasia in several non-Marfan patients may be caused by a mutation(s) in structural proteins. Familial clustering has been shown in many cases of annulo-aortic ectasia (e.g. the 32-year-old patient; Table 1) (Halme *et al.*, 1985).

Aging of elastin seems to be a complicated process, probably as a consequence of its long metabolic turnover. Unknown radioactive peaks (in addition to peak X) appeared in chromatograms of hydrolysates of elastins of aging aortas. Our results also suggest that 6-hydroxy-norleucine cannot be determined after alkali hydrolysis of elastin by the single chromatographic procedure used. On acid hydrolysis this 'hydroxynorleucine' peak of elastins from aortas of older humans (54–60 years old) behaved quite differently from that expected (Lent *et al.*, 1969). However, the peak X was well separated from other radioactive components. The proposed structure of the cyclic derivative of ACP fits well with the elution position of its reduced form in cation-exchange chromatography (one amino group is converted into an imino group, which moves the elution position towards that of proline). These facts suggest that the cyclic amino acid described by us is a real moiety or alkali-hydrolysis product of a real moiety of aged elastin. It might be used as an indicator for aging of elastin. Further studies are needed, however, to confirm its chemical structure and biological significance *in vivo*.

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