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VARIATIONS IN AORTAL ELASTIN COMPOSITION AFTER ATHEROGENIC DIET FEEDING IN RATS. POSSIBLE INVOLVEMENT OF TWO DIFFERENT ELASTIN TYPES

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Summary: An increased proportion (about twofold) of polar amino acids (aspartic acid, glutamic acid, arginine and tyrosine) was found both in insoluble elastin and tropoelastin preparations from aortae of rats fed high fat diet. Concomitantly the number of val-pro sequences drops from 48.7 to 26.8 in elastin from control and atheromatous aortae. The observed changes can be explained either by assuming a tight attachment of a non-elastin protein rich in polar amino acids or by assuming the existence two elastin types the proportion of which is changed in aortae by high fat diet feeding. The data obtained are in favor of two different genetic types of elastin.

Introduction

There are some indications that elastin exhibits, like collagen, tissue dependent compositional variability (for review, see 1). Two main problems hamper investigations of this type: first, the difficulty of obtaining a pure sample of the highly insoluble protein and second, the possibility of random peptide bond cleavage and loss of some peptide fragments during the preparation procedure. However, Field et al. (2) were able to prove differences in the amino acid composition of highly purified elastin preparations from ligamentum nuchae and ear cartilage; elastin preparation from the latter source was considerably enriched in polar amino acids. Keith et al. (3) confirmed these results and have further demonstrated that the increase of polar residues in the preparation from auricular cartilage is in part compensated by the lower occurrence of the sequence val-pro. These data give weight to a previous report of Keith et al. (4) about the possibility of sequence differences in elastin of ligamentum nuchae and elastic cartilage. They also proposed the name elastin type II for the cartilage protein.

The compositional variability mentioned above refers not only to different tissues, but can be per analogiam expected to occur with spe-

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cific pathological situations, of which atherosclerosis is the first to be considered. There are limited data available about the composition of aortal elastin: this resembles ligamentum nuchae preparations though a slight but consistent increase in polar amino acids can be seen. These facts prompted our search for possible differences in arterial wall elastin caused by atherogenic circumstances, like high fat diet feeding.

Materials and methods

Tissue investigated

Aortae were obtained from male Wistar rats aged 350 days kept on atherogenic diet composed of standard pellets (2000 g), 5 g NaCl and 150 g fish fat to which 1080 g suet were added. Controls were fed standard pelleted diet. Both diets were available ad libitum.

Aortae used for the preparation of tropoelastin were obtained from 90-100 days old rats that were fed sulfide treated milk diet; tropoelastin from atherogenic animals was obtained from rats that were kept on the milk-sulfide diet supplemented with fish fat and suet (in identical ratios as specified above). Both diets were available ad libitum. Blood plasma copper levels at death were generally less that 10 µg/100 ml as determined by atomic absorption spectrometry. 500 aortae of each type were collected in four batches over a period of one year.

Elastin preparation

The procedure of Field et al. (2) was followed with minor modifications. Aortae were frozen in liquid nitrogen, crushed in a mortar and extracted with 1% NaCl at 4°C for 24 hours. The supernatant was briefly spun off and discarded. Remining tissue was suspended in 2M $CaCl_2$ for two 24 hours periods and subsequently extracted with 5M guanidinium hydrochloride/0.1 M Tris-HCl at 4°C for two 24 hours periods. At the end of each period the residue was collected by centrifugation and the supernatant discarded. The microfibrillar protein was removed by treating the residue with 5M guanidinium hydrochloride/0.1 M Tris-HCl (pH 7.6) containing 0.05 mol/l dithiothreitol and 0.4% EDTA for two 48 hour periods at room temperature under nitrogen. The residue was washed by 2M NaCl and distilled water. Finally the material was suspended in 0.01 M $CaCl_2$ at 37°C and digested with affinity chromatography purified collagenase (substrate enzyme ratio 1000:1) to remove collagen. The residue was then collected by centrifugation and washed with 5M guanidinium hydrochloride/0.1 M Tris-HCl (pH 7.6), followed by 2M NaCl and water prior lyophilization.

Elastase digestion of elastin

50 mg of the above prepared elastin were suspended in 20 ml of water (pH was adjusted to 3.45 with 0.02 M NaOH) and 100 µg elastase were added. Cleavage was done at 37°C for 24 hours during which period practically all material became soluble.

Tropoelastin preparation

Aortae obtained from copper-deficient rats served as source material. The tissue was frozen in liquid nitrogen, homogenized in 0.02 M formic acid (10 ml/g tissue); three extractions were carried out over 24 hours period at 4°C. At the end of the extraction the supernatant was spun off and processed further, the residue was discarded. Further procedure followed that described by Sandberg et al. (5). Briefly the extracts were precipitated with 40% ammonium sulphate (the protein extract was stirred at this ammonium sulphate concentration overnight) and precipitate removed by centrifugation at 10000 g. The pellet was redissolved in 0.1 M ammonium formate pH 5.5 (20 ml per g pellet) and the reaction

mixture together with some undissolved material was dialyzed overnight against the same buffer. Non tropoelastin proteins were removed by n-propanol-n-butanol extraction (6). Tropoelastin was recovered from the butanol layer by flash evaporation. Material obtained in this way was extracted twice with chloroform to remove any contaminating lipids. In the final step the pellet was dissolved in 0.02 M formic acid and lyophilized. Final purification of tropoelastin was done by passing the material through a Sephadex G 100 column (50 x 3 cm) in 10% acetic acid. The first tailing peak was collected.

Valine-proline assay

Elastin and tropoelastin preparations (10 mg) were hydrolysed in alkali resistant tubes with 1 ml 2 M NaOH under vacuum at 100° for 22 hours. Hydrolysates were cooled and neutralized with perchloric acid. After further cooling on ice, $KClO_4$ was removed by centrifugation and the clear supernatant was subjected to ion exchange chromatography (MR 205 resin, citrate gradient pH 2.8/0.2 M Na^+ to pH 7.0/0.75 M Na^+, 55°C). Val-pro elutes in the neighbourhood of phenylalanine; valyl-proline anhydride elutes in the front of the chromatogram. For quantitation the val-pro peak near phenylalanine was used on the presumption that the color yield represents 62% of the color factor of phenylalanine (3).

Amino acid analysis

Amino acid analyses were done on an automated amino acid analyzer (Mikrotechna) using a 50 x 1 cm column. Ostion LG KS 0803 ion exchanger (Chemical Works, Ústí n. Labem, Czechoslovakia) was used as column packing. Elution was done with a stepwise gradient as follows: 0.2 citrate buffer pH 3.23 for 120 min, 0.2 M citrate buffer pH 4.1 for 65 min and 0.2 M citrate buffer 1 M with respect to KCl pH 5.0 for additional 110 min. The column was operated at 40°C for the first 40 min, then temperature raised to 55°C. The flow rate was kept at 70 ml/hour.

Tryptic digestion and N-terminal amino acid determination of the cleaved peptides

Lyophilized samples (10 mg) were dissolved in 0.2 M ammonium bicarbonate (1 ml) and trypsin was added (in 0.05 M Tris pH 8.0, enzyme-substrate ratio 1:100). Reaction was let to proceed 2 hours at 37°C. After this period another portion of enzyme was added. Finally the hydrolysate was lyophilized and used for the N-terminal amino acid determination. This was done by established methods using dansylation of the free N-terminal.

Results

Representative amino acid analyses of aortal elastin as well as elastin from atherogenic diet fed animals and animals kept on an atherogenic diet and refed standard diet thenafter are summarized in table I. In both types of preparations that were obtained from animals that were administered atherogenic diet a distinct increase in polar amino acids is seen: this increase is the highest in aspartic and glutamic acids (almost two times as much as in controls); slightly lower values were observed in arginine and tyrosine (1.6 times the control value). On the other hand this increase is compensated by a decrease in the valine concentration. The increase in polar amino acids is somewhat lower than that observed when comparing auricular cartilage elastin and elastin preparation of ligamentum nuchae: in coincidence with literary data the increase

Table I. Amino acid composition of aortal elastin preparations from high fat diet fed rats (residues per 1000 residues)*

	Rat aorta	Rat aorta fed ather. diet	Ligamen-nuchae (bovine)	Auricular cartilage (bovine)
Hydroxyproline	10.7	10.3	8.0	10.2
Aspartic acid	6.5	12.3	5.4	21.0
Threonine	10.0	10.0	9.0	10.2
Serine	9.5	9.3	8.5	13.6
Glutamic acid	16.0	28.2	14.9	35.2
Proline	112.0	113.2	114.0	115.0
Glycine	339.3	327.0	336.5	317.5
Alanine	215.0	217.0	223.0	185.0
Valine	128.6	114.0	130.1	108.0
Half cysteine	0.0	0.0	0.0	0.0
Methionine	0.0	0.0	0.0	0.0
Isoleucine	24.2	22.2	23.6	21.3
Leucine	58.1	59.8	60.1	69.7
Tyrosine	6.9	10.7	6.5	16.8
Phenylalanine	29.0	28.7	29.0	32.0
Hydroxylysine	0.0	0.0	0.0	0.0
Lysine	4.7	6.2	3.4	7.2
Histidine	0.8	0.5	0.5	0.5
Arginine	6.2	9.7	5.5	12.7
Aldol condensation product	4.0	4.0	4.2	5.7
Dehydrolysinonor-leucine	0.1	tr	0.0	0.0
Lysinonorleucine	2.0	1.8	1.8	2.3
Dehydromerodesmosine	0.7	0.6	0.2	0.3
Merodesmosine	0.5	0.4	0.5	0.1
Isodesmosine	5.4	5.2	5.2	5.2
Desmosine	9.8	8.9	10.1	10.5

*The composition of ligamentum nuchae and auricular cartilage elastin is presented for comparison.

in the amount of aspartic acid is almost fourfold in auricular cartilage while the other polar amino acids, namely glutamic acid, arginine and tyrosine are increased 2.5 times in auricular cartilage preparations in comparison to ligamentum nuchae elastin. As expected, a 20% decrease in the valine concentration was revealed in elastin from auricular cartilage in comparison to ligamentum nuchae preparation. It should be noted that data of aortal elastin from standard diet and atherogenic diet fed animals occur within the limits given by the analyses of ligamentum nuchae and auricular elastin; the composition of aortal elastin from controls approaches that of ligamentum nuchae preparation, while that obtained from aorta of animals fed atherogenic diet is more similar to the auricular cartilage preparation, though the increase of polar amino acids is not so pronounced (there is an additional nearly twofold increase in aspartic acid in the auricular cartilage preparation).

In order to avoid the objection that the increase in polar amino acids may result from the contamination by acidic non elastin proteins,

Table II. Amino acid composition of aortal tropoelastin from controls and high fat diet fed rats (residues per 1000 residues)

Amino acid	Controls	High fat diet fed animals
Hydroxyproline	9.2	10.1
Aspartic acid	4.8	12.4
Threonine	11.3	11.3
Serine	9.8	8.9
Glutamic acid	15.2	24.2
Proline	110.3	112.3
Glycine	337.2	337.2
Alanine	218.2	214.1
Valine	128.4	110.0
Half cysteine	0.0	0.0
Methionine	0.0	0.0
Isoelucine	20.6	20.3
Leucine	48.7	47.2
Tyrosine	8.3	11.4
Phenylalanine	29.4	28.5
Hydroxylysine	0.0	0.0
Lysine	42.1	40.1
Histidine	0.7	0.6
Arginine	5.8	11.4

known to be codistributed with elastin in tissues, tropoelastin samples were prepared from aortae of controls and atherogenic diet fed animals. As visualized in Table II the results about the increase of polar amino acids are confirmed here: the increase of aspartic acid in the high fat diet fed group is 2.6 of the control value, the other amino acids, glutamic acid, arginine and tyrosine increase as well, though the increase is only twofold or less. Relative changes in the occurence of polar amino acids in different elastin preparations are summarized in Tab. III.

Table III. Relative changes in the occurrence of polar amino acids and valine in different elastin preparations.

	Tropoelastin, aorta. Atherogenic diet versus controls	Insoluble elastin, elastase digest. Atherogenic diet versus controls	Insoluble elastin, elastase digest, auricular cartilage versus ligamentum nuchae
Aspartic acid	+ 2.6	+ 1.9	+ 3.9
Glutamic acid	+ 1.6	+ 1.8	+ 2.4
Arginine	+ 2.0	+ 1.6	+ 2.3
Tyrosine	+ 1.4	+ 1.6	+ 2.6
Valine	- 1.16	- 1.12	- 1.20

Plus sign refers to an increased occurrence of the particular amino acid, minus sign refers to a decrease.

Table IV. Val-Pro occurence in different tropoelastin preparations

Source of tropoelastin	Val-Pro sequence (no of sequences per 1000 amino acid residues)	Corrected for recovery[+]
Control aortae	18.1 ± 1.5	48.7
Atheromatous aorta	10.3 ± 2.3	26.8
Ligamentum nuchae	20.0 ± 1.6	52.1

[+]Calculated on the basis of average recovery 38.4% (according to Keith et al. (3)).

If the increase of polar amino acids in atherogenic diet fed animals is paralelled by a decrease of valine, then it seems feasible to anticipate, that it will be reflected in a decreased frequency of the val-pro sequence. Indeed direct estimation of val-pro sequence indicated a drop from 48.7 to 26.5 val-pro sequences per 1000 residues (corrected for recovery) in tropoelastin preparations from controls and atherogenic diet fed animals (Table IV).

When tropoelastin preparations were subjected to tryptic cleavage and the resulting mixture of peptides subjected to N-terminal analysis, then the only difference seen between the atherogenic diet fed animals and controls was a drop in the N-terminal valine: while this constitutes 4.8 per cent of all liberated N-terminals in control preparation, in the tropoelastin preparation obtained from atherogenic diet fed animals it represents only 2.9 per cent (Table V).

Discussion

The differences in the amino acid composition of elastins prepared from aortae of animals fed standard and atherogenic diet can be interpreted in two ways. It may be either assumed that there is some non-elastin protein material rich in polar amino acids closely attached to the elastin fragments or that there are indeed two different types of elastin the proportion of which is changed in the tissue. With mature elastin preparations the difficulties of purification are well known and there is no way how to decide between the above two possibilities. If

Table V. Comparison of N-terminal analyses of tryptic digests of tropoelastin from different sources

		PTH-Ala	PTH-Tyr	PTH-Gly	PTH-Val	PTH-Leu	PTH-Phe
Aortae, control rats	nmoles	87.8	37.8	17.6	8.1	7.6	7.3
	%	52.8	22.7	10.5	4.8	4.5	4.3
Aortae, high fat diet fed animals	nmoles	67.3	31.2	13.9	3.7	5.3	5.2
	%	53.1	24.6	10.9	2.9	4.1	4.1

the alterations seen were caused by a contaminating protein, then the most likely candidate appears to be some acidic protein (7-10). On the other hand the combined action of chaotropic agents and partial proteolysis has been shown to remove this material from elastin preparations (8). Additional treatment of our preparations with dithiothreitol and sodium dodecyl sulfate remained without effect upon the amino acid composition (data not shown). The changes in the occurence of polar amino acids were, however, seen also in neutral salt soluble elastin (tropoelastin), which minimizes the possibility that the changes were caused by some attached acidic proteinous material.

On the other hand the decrease of val-pro sequence was estimated by a direct assay. It may be argued of course that the changed proportion of the val-pro sequence in the contaminating protein could distort the overall image of the analysis. This, however, seems unlikely, as the N-terminal analysis of the partial hydrolysate revealed the drop of valine only, while the proportion of other N-terminals was preserved and no other N-terminals were found indicating that there was no substantial contamination of the sample. Of the two explanations offered it appears therefore more likely that it is indeed dealt with two different elastin species in the tissue.

The presence of two tropoelastin polypeptides in cell free translation of chick aortic mRNA and in organ cultures of chick aortae as reported by Foster et al. (11) and Barrineau et al. (12) is strongly in favor of this view.

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