Vol. 129, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1985 Pages 179-186

CHANGES IN ELASTIN COMPOSITION IN AORTA OF SPONTANEOUSLY HYPERTENSIVE RATS (SHR)

Z. Deyl, M. Horáková and K. Macek

Institute of Physiology, Czechoslovak Academy of Sciences, Prague , Czechoslovakia

Received March 27, 1985

SUMMARY: Tropoelastin and elastin preparations obtained from aortae of spontaneously hypertensive rats (SHR) show an increased proportion of polar amino acids (aspartic acid, glutamic acid, arginine and tyrosine). The content of these amino acids is 1.43-3.04 times higher in SHR rats than in similar elastin or tropoelastin preparations obtained from normotensive animals. On the other hand elastin and tropoelastin preparations obtained from SHR rats show a lower frequency of the Val-Pro sequence; this was found to be 35.93 per 1000 amino acid residues in SHR rats as compared to 51.04 per 1000 amino acids in the preparations obtained from control animals. Since similar differences were found not only in elastin preparations but also in tropoelastin, contamination of these preparations with an acidic protein seems unlikely. In general the results obtained are similar to those seen in animals kept on a long term high fat diet. It appears feasible to suggest that these differences are caused by a changed proportion of two different elastin type.

INTRODUCTION: Abnormalities in the arterial wall are considered one of the major contributions to the altered vascular function during hypertension. A number of hypotheses envolved concerning the contribution of changed vessel wall geometry and its electrolyte and water metabolism that may be involved in increased peripheral resistence and properties of the arterial wall as such (1). The changes in vessel wall geometry may be due either to the expansion of the extracellular matrix or smooth muscle swelling. Such changes are likely to result in a decrease of the arterial internal radius and affect the contractile and elastic properties of the tissues. It is obvious that in such changes the extracellular matrix will play a crucial role.

Though there is a lot of work being done with regard to the altered proteosynthesis in aorta in animals with hypertension very little is known about the relations of individual proteins produced. In this respect elastin as the major constituent of the arterial wall is certainly of prime importance. There are some indications that elastin, similary to collagen, exhibits a certain degree of tissue dependent compositional variability (for review see 2). There are two problems which complicate investigations in this respect. The first is the problem how to obtain

0006-291X/85 $1.50

a pure sample of a highly insoluble protein as elastin, the second is the possibility that random peptide cleavage and loss of some peptide fragments may occur during the preparation procedure. Nevertheless Field et al. (3) have obtained highly purified elastin preparations from ligamentum nuchae and from rabbit ear cartilage showing that in the latter preparation there was a considerable increase in polar amino acids. Other investigators (4) reported that this increase in polar amino acid composition is at least in part compensated by a lowered occurence of the sequence Val-Pro. In our previous report (5) we were able to show that 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 subjected to long term high fat diet feeding. Concomitantly the number of Val-Pro sequences dropped down in hypertensive animals from 48.7 to 26.8. These 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 of two elastin types the proportion of which is changed by high fat diet feeding. The data obtained in our previous investigation favor the working hypothesis that there are two different genetic types of elastin.

In the present report we have investigated aortae of spontaneously hypertensive rats (SHR) to see whether in this animal model there are changes in aortal elastin that may be interpreted in the terms of the existence of different elastin types.

MATERIALS AND METHODS:

Chemicals

Collagenase was a product of Serva (Heidelberg, FRG) purified before use by bioaffinity chromatography according to Bond and van Wart (6) to remove non-specific proteolytic activity. Trypsin was obtained from Calbiochem (Luzerne). Ostion LG KS 0803 ion exchanger was obtained from Chemical Works (Ústí n L., Czechoslovakia). Dansyl chloride was obtained from Calbiochem (Luzerne) and Val-Pro standard from SERVA (Heidelberg, FRG). All other chemicals were of analytical grade.

Tissue investigated

Aortae were obtained from SHR rats, aged 350 days and kept on standard peletted diet which was available ad libitum. Male Wistar rats of the same age served as controls.

Aortae used for the preparation of tropoelastin were obtained from 90-100 days old rats (both SHR and controls) that were fed sulphide-treated milk diet. Both diets were available ad libitum. Blood plasma copper levels at death were generally less than 10 µg/100 ml as determined by atomic absorption spectrometry; 500 aortae of each type were collected in four batches over a period of two years.

Elastin preparation

The procedure of Field et al. (3) was followed with minor modifications. Aortae were frozen in liquid nitrogen, crushed in a mortar and extracted with 1 percent NaCl at 4°C for 24 hours. The supernatant was brierly spun off and discarded. The pellet was suspended in 2 mol/l $CaCl_2$ for two subsequent 24 hours periods and extracted with 5 mol/l guanidine hydrochloride in 0.1 mol/l Tris-HCl (pH 7.6) at 4°C for two 24 hours period. At the end of each period the residue was collected by centrifugation and supernatant was discarded. The microfibrilar protein was removed by treating the residue with 5 mol/l guanidine. HCl dissolved in 0.1 mol/l Tris-HCl (pH 7.6) containing 0.05 mol/l dithiothreitol and 0.4% EDTA Na_2 for two 48 hours periods at room temperature under nitrogen. The residue was washed by 2 mol/l NaCl and destilled water. Finally the material was suspended in 0.01 mol/l $CaCl_2$ at 37°C and digested with purified collagenase to remove contaminating collagen. Substrate/enzyme ratio was 1000:1 calculated per dry weight. The residue was then collected by centrifugation (10000 x g, 20 min.) and washed with 5 mol/l guanidine. HCl dissolved in 0.1 mol/l Tris.HCl (pH 7.6), followed by 2 mol/l NaCl and water prior lyophilisation.

Elastase digestion of elastin

A sample of the elastin preparation (50 mg) was suspended in 20 ml distd. water and the pH was adjusted to 3.45. Then, 100 µg of elastase was added and digestion was carried out at 37°C for 24 hour during which period all material became soluble.

Tropoelastin preparation

This procedure followed generally that described by Sandberg et al. (7). Aortae obtained from copper deficient rats were frozen in liquid nitrogen, homogenized in 0.02 mol/l formic acid (10 ml per g of tissue) and three extractions were carried out over 24 hours period at 4°C. At the end of the extracti on the supernatant was spun off (10000 x g, 20 min) and processed further, the residue was discarded. Next the extracts were precipitated with 40% ammonium sulphate (the protein extract was stirred at this ammonium sulphate concentration overnight) and the precipitate removed by centrifugation at the same conditions as above. The pellet was redissolved in 0.1 mol/l ammonium formate, pH 5.5 (2 ml/100 mg) and dialysed overnight against the same buffer. In the next step tropoelastin was purified by n-propanol-n-butanol extraction according to Ryhänen et al. (8) and recovered from the alcoholic layer by flash evaporation. Material obtained in this way was extracted twice with 1 ml chloroform to remove any contaminating lipids, the pellet was dissolved in 0.02 mol/l formic acid and lyophilised. For final purification the sample was chromatographed on a Sephadex G 100 column (50 x 1 cm) using 10% acetic acid as mobile phase. The first tailing peak was collected.

Valine-proline assay

Elastin and tropoelastin preparations (5-10 mg) were hydrolyzed in alkali-resistant tubes with 1 ml 2 mol/l NaOH in evacuated vials at 100°C for 22 hours. Hydrolysates were cooled and neutralized with perchloric acid. After further cooling on ice, $NaClO_4$ was removed by centrifugation and the clean supernatant was subjected to ion-exchange chromatography (Technicon Amino Acid Analyzer, MR 205 resin, citrate gradient pH 2.8/0.2 mol/l Na^+ to pH 7.0/0.75 mol/l 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 Phe was used on the presumption that the color yield represents 62% of the color factor of Phe (Keith et al. (9)).

Amino acid analysis

Amino acid analyses were done on an automated Amino acid analyzer (Mikrotechna, Prague, Czechoslovakia) using an Ostion LG KS 0803 ion exchanger packed in a 50 x 1 cm column. Elution was done with a stepwise gradient as follows: 0.2 mol/l citrate buffer pH 3.23 for 120 min., 0.2 mol/l citrate buffer pH 4.1 for 65 min. and 0.2 mol/l citrate buffer made 1 mol/l with respect to KCl pH 5.0 for additional 110 min. The column was operated at 40°C for the first 40 min., then the temperature was raised to 55°C for the rest of the run. The flow rate was kept at 70 ml/h. Ninhydrin reaction was used for detection.

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

Lyophilized samples (5-10 mg) were dissolved in 0.2 mol/l ammonium bicarbonate (1 ml) and trypsin was added (0.05 mol/l Tris pH 8.0, enzyme-substrate ratio 1:100, 2 h, 37°C). After two hours a new portion of trypsin was added and the reaction was let to proceed for another two hours. Finally, the hydrolysate was lyophilized and used for the N-terminal amino acid determination. This was done by the established dansyl method (10).

RESULTS:

As indicated in Tables 1, 2 and 3 both elastin and tropoelastin prepared from aortae of the SHR rats were considerably enriched in polar amino acids, namely aspartic acid, glutamic acid, arginine and tyrosine. The increase varied between 143-304% of controls being different with different amino acids. On the other hand this increase was in part compensated by the dicreased content of valine. The situation observed was quite similar to that found previously in rats kept on atherogenic diet. We have analyzed the present data in a similar way as in the previous report (5). In general there are two situations that may lead to such results. Either all our preparations are contaminated by a protein component that is quite rich in polar amino acids or we are dealing with two different elastin types. As long as we have observed the increased proportion of polar amino acids not only in the insoluble elastin preparations but also in the preparation of tropoelastin and in the latter case the differences were even more pronounced, we are strongly in favour of the hypothesis that there are two elastin types present in our aorta preparation. To support futher this idea we have estimated the number of Val-Pro sequences in different aortal elastin preparations. As indicated in Table 4 the expected result was obtained, namely the number of Val-Pro sequences dropped down from 51.04 per 1000 amino acid residues to 35.93 per 1000 amino acid residues in the preparation obtained from the SHR rats (corrected for recovery). When our preparations were digested by tryptic and the arising N-terminal amino acids were quantitated (Table 5) the same amount of all N-ter-

TABLE I

Amino acid composition of aortal elastin preparations from SHR and control rats (residues per 1000 residues)[a]

Amino acid	Rat aorta		Ligamentum nuchae (bovine)	Auricular cartilage (bovine)
	SHR rats	Controls		
Hydroxyproline	10.3	10.7	7.9	10.1
Aspartic acid	13.0	6.0	5.0	21.4
Threonine	9.8	10.0	9.0	10.0
Serine	9.8	9.8	8.5	13.6
Glutamic acid	30.2	15.8	14.3	37.4
Proline	114.0	112.0	115.0	113.0
Glycine	319.6	337.3	334.9	319.0
Alanine	219.0	214.0	225.0	180.0
Valine	110.0	128.8	131.4	106.0
Half cysteine	0.0	0.0	0.0	0.0
Methionine	0.0	0.0	0.0	0.0
Isoleucine	21.8	23.6	24.1	20.9
Leucine	65.1	39.8	60.0	69.9
Tyrosine	11.0	7.0	6.5	16.8
Phenylalanine	30.0	29.7	29.0	34.1
Hydroxylysine	0.0	0.0	0.0	0.0
Lysine	4.0	6.8	3.2	7.4
Histidine	0.5	0.9	0.5	0.4
Arginine	10.1	6.8	5.0	13.1
Aldol condensation product	4.0	4.0	3.9	5.8
Dehydroxylysinonorleucine	0.1	traces	traces	traces
Lysinonorleucine	2.2	2.2	1.8	2.5
Dehydromerodesmosine	0.4	0.2	0.3	0.6
Merodesmosine	0.4	0.3	0.7	0.1
Isodesmosine	5.7	4.9	3.9	5.2
Desmosine	9.0	10.2	10.1	10.7

[a] The composition of ligamentum nuchae and auricular cartilage elastin is presented for comparison.

TABLE II

Amino acid composition of aortal tropoelastin from controls and SHR rats (residues per 1000 residues)

Amino acid	SHR rats	Controls
Hydroxyproline	10.4	9.0
Aspartic acid	12.8	4.2
Threonine	11.6	11.2
Serine	8.7	9.9
Glutamic acid	26.2	14.8
Proline	112.8	111.3
Glycine	326.5	330.7
Alanine	220.2	225.0
Valine	109.7	131.2
Half cysteine	0.0	0.0
Methionine	0.0	0.0
Isoleucine	20.2	19.8
Leucine	47.8	48.3
Tyrosine	11.8	8.2
Phenylalanine	28.7	28.7
Hydroxylysine	0.0	0.0
Lysine	43.4	42.1
Histidine	0.5	0.7
Arginine	8.7	4.9

TABLE III

Relative changes in the occurrence of polar amino acids and valine in aortal elastin obtained from SHR and control rats

Amino acid	Tropoelastin. SHR versus controls	Insoluble elastin, elastase digest. SHR versus controls	Insoluble elastin, elastase digest. Auricular cartilage versus ligamentum nuchae
Aspartic acid	+ 3.04	+ 2.16	+ 4.28
Glutamic acid	+ 1.77	+ 1.91	+ 2.61
Arginine	+ 1.77	+ 1.48	+ 2.62
Tyrosine	+ 1.43	+ 1.57	+ 2.58
Valine	- 1.19	- 1.17	- 1.23

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

minals was observed in controls and SHR rats, expect valine, the proportion of which was clearly lowered in the tropoelastin preparations obtained from the SHR animals.

DISCUSSION:

As already noted the present results are quite similar to those we have obtained in our previous work in animals kept in a high fat (atherogenic) diet. There are basically two problems that have to be paid attention:

1. Whether the present data are indicative of the presence of two elastin types or whether they merely reflect a tight attachment of a polar protein to the elastin preparations.

TABLE IV

The occurrence of Val-Pro sequences in hydrolysates of various tropoelastin preparations

Source of tropoelastin	Val-Pro sequence (No of sequences per 1000 amino acid residues)	Corrected for recovery[+]
Control aortae	19.6	51.04
SHR aortae	13.8	35.93
Ligamentum nuchae	21.2	55.20

[+] Calculated on the basis of average recovery 38.4% (according to Keith et al.[4]).

TABLE V

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

Sample		PTH-Ala	PTH-Tyr	PTH-Gly	PTH-Val	PTH-Leu	PTH-Phe
Aortae, control rats	nmol	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, SHR rats	nmol	72.1	32.2	14.4	3.7	6.0	5.5
	%	53.8	24.0	10.7	2.7	4.4	4.1

2. How is it possible to explain that in two physiologically fundamentally different models there are the same biochemical results.

In answering the first question one can argue in the way that we have published before in connection with the experiments carried out in rats kept on atherogenic diet. The existing differences in the proportion of polar amino acids in tropoelastin, the decreased frequency of the Val-Pro sequence and the decreased proportion of N-terminal valine in tryptic peptides in the preparations from SHR rats are all in favour of the idea of having two different proteins rather than handling a differently contaminating single protein. If the latter were true than one would expect differencies, e.g., in the free end terminals after trypsinization. Also additional treatment with dithiothreitol and sodium dodecyl sulphate which is known to remove polar acidic proteins from elastin preparation should exert at least some effect upon the N-terminals, Val-Pro sequence proportion or the ratio of polar amino acids. However, no such effects were observed with our preparations (data not shown). Therefore we suggest that what we are dealing with is a changed proportion of two elastin types. If this assumption is true than one can speculate a bit futher and assume that the ligamentum nuchae and auricular cartilage preparations can represent pure elastin types I and II. In such a case it is possible (based on the frequency of the Val-Pro sequence in both molecular species assumed) to calculate the proportion of each type in the aortal tissue: Then tropoelastin obtained from control aortae should contain 11.74% of type II while in the analogous preparation from SHR aortae the proportion of type II was increased to 48.77%.

The other question outlined above can be answered by finding a common physiological condition that in both SHR rats and rats kept on atherogenic diet could lead to identical biochemical alternations. Here one is tempted to conclude that the alternations observed result from the increased pressure in aortae. The verification of this assumption is, however, only to be proved in the future.

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