Vol. 91, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 14, 1979 Pages 41-49

ATHEROCALCIN, A γ-CARBOXYGLUTAMIC ACID CONTAINING PROTEIN FROM ATHEROSCLEROTIC PLAQUE

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Received September 20, 1979

SUMMARY

The specialized calcium binding amino acid, γ-carboxyglutamic acid (Gla) is quantitated in developing atherosclerotic plaque relative to progression of the disease, and a Gla-containing protein isolated from calcified atherosclerotic plaque is partially characterized. Low levels of Gla are found in fatty streak and fibrous plaque lesions, and a marked increase in Gla content occurs in calcified plaque. A unique Gla-containing protein is purified from 0.5M EDTA (pH 8.0) extracts of calcified plaque, named atherocalcin. The protein containing 19 Gla residues/1000 amino acids is 80,000 molecular weight, with a pI of 4.16-4.3 and is uniquely different from other known Gla-containing proteins. The implications of this work for the further understanding of the pathogenesis and therapy of atherosclerosis are discussed.

INTRODUCTION

Atherosclerosis is thought to progress pathologically from fatty streak lesions to fibrous plaques, and finally to complex calcified atheromata. The mechanisms controlling this pathological process are largely unknown, although many theories exist (1).

Of possible relevance to the pathogenesis of atherosclerosis, our laboratory has demonstrated that proteins containing the vitamin K dependent, calcium binding amino acid, γ-carboxyglutamic acid (Gla) are present in calcified atherosclerotic plaque, as well as other pathological calcifications (2,3). Gla is found normally in the vitamin K dependent clotting factors, and in normal bone in a protein called osteocalcin (4). The finding of different proteins containing Gla in bone and ectopic mineralizations including hard plaque suggest a hypothesis that certain calcium binding proteins containing this amino acid may

0006-291X/79/210041-09$01.00/0

be of importance in the sequelae ultimately leading to the deposition of calcium. This may occur through calcium binding interactions similar to those of other Gla containing proteins such as the vitamin K dependent clotting factors, which undergo a unique calcium-phospholipid interaction necessary for the reactions of the coagulation cascade (5). Therefore, the identification and origin of the Gla protein(s) present in the calcified atherosclerotic plaque could have important clinical significance. These proteins might have their origins in the vitamin K-dependent clotting factors or in other vitamin K-dependent plasma proteins, or by de novo synthesis by cells present in the arterial wall lesions. In the present work, Gla levels have been quantitated in atherosclerotic tissue pools at various levels of clinical severity, and the Gla-containing proteins of atherosclerotic tissue have been extracted, the non-dialyzable components isolated, and partially characterized.

METHODS

Pathological Tissues: Atherosclerotic lesions were dissected at autopsy from twenty-two aortas (12 males and 10 females; age range 33-89). This material was rinsed with copious amounts of saline to remove blood, and then pooled as either fatty streak, fibrous plaque, or calcified plaque. Fatty streaks were defined as nonraised white to yellow intimal streaks. Fibrous plaques were defined as raised, firm, fibrotic lesions, without gross mineralization. Calcified plaques were taken as those lesions with grossly hardened mineral involvement. In addition, several grossly normal aortas obtained at autopsy were stripped of adventia and minced over ice for control tissue. The tissue pools were next lyophilized, and milled to a coarse powder in a Spex liquid nitrogen mill. The calcified plaque powder was extracted for seventy-two hours in 0.5M EDTA (pH 8.2) containing proteolytic inhibitors as previously described (4). The extract was dialyzed exhaustively at 4°C against distilled water using Spectropor 3 dialysis tubing and then freeze-dried.

Amino Acid Analysis and Chemical Determinations: Gla detection and quantitation was performed on 2N KOH hydrolysates using a Beckman Spinco 121M amino acid analyzer according to methodology established in our laboratory (6). The presence of Gla was confirmed by 6N HCl decarboxylation to convert the putative Gla peak to glutamic acid as previously described (6). O-phosphoserine amino acid analysis was performed and calculated as described previously for bone extracts (7).

Protein Isolation and Purification Procedures: EDTA extracts were chromatographed on Sephacryl S200 Superfine columns (2.5 x 100 cm) with 0.1M ammonium acetate pH 7.1 plus 0.7mM dithiothreitol at a flow rate of 8.0 ml/hr. Ion exchange chromatography of gel filtration pools rich in Gla was performed with DEAE Cellulose employing 1.5 x 25 cm columns run at 4°C with a buffer containing 20mM imidazole, 2mM EDTA, 20mM $CaCl_2$ at pH 7.0 with a NaCl gradient (as shown in Figure 2) run at a flow rate of 60 ml/hr. Column fractions were assayed for protein using the Coomassie blue procedure of Bradford (8). Column fraction pools were prepared for further analyses with extensive dialysis against distilled water in Spectropor 3 followed by lyophilization.

Electrophoretic Techniques: SDS disc-gel electrophoresis with 15% acrylamide vertical slab gels was performed at 14°C (9). Isoelectric focusing was carried out on a BioRad 1415 electrophoresis cell in 4% acrylamide gels with 2% pH 4-6 ampholine maintaining constant power at 8.0 watts for three hours. The electrofocused gels were stained with 0.05% Coomassie blue (10).

Immunological Techniques: Purified atherosclerotic protein extracts were assayed for serum protein contamination using immunodiffusion and immunoelectrophoresis techniques (11).

Materials: The materials used and their suppliers were as follows: Spectropor 3 (Spectrum Industries); Sephacryl S-200 (Pharmacia); DEAE Cellulose (Whatman), Ampholine (LKB); rabbit antihuman serum albumin and whole human serum antisera (Calbiochem-Behring). The other chemicals used were either reagent grade or the best quality available.

RESULTS

Atherosclerotic tissue contains protein-bound Gla at all levels of pathological severity; trace Gla levels are also found in so-called "normal" aorta (Table I). As shown, calcium and phosphorus content of aortic lesions was significantly elevated in the fatty streak and fibrous plaque pools, demonstrating calcium/phosphorus molar ratios typical of hydroxyapatite. Calcified plaque, however, had relatively higher calcium suggesting the increased presence of calcium carbonate. Of importance is the very high Gla level found in the calcified plaque. Of further interest is the almost constant ratio of calcium/Gla in tissues of various levels of pathologic severity. Gla recovery calculations (Table II) reveal that the dialyzed EDTA extract contains 5.7% of the total tissue Gla, with the insoluble residue containing 12.5%. The remainder of Gla was lost as dialyzable low molecular weight peptides perhaps as a result of the action of tissue proteases in intact lesions.

TABLE I

AORTIC GLA AND CALCIUM CONTENT

TISSUE	GLA (nmol/mg) PROTEIN	CALCIUM (nmol/mg) PROTEIN	PHOSPHORUS (nmol/mg) PROTEIN	CALCIUM (nmol) / PHOSPHORUS (nmol)	CALCIUM (nmol) / Gla (nmol)
Normal Aorta	0.39	295	665	0.44	756
Fatty Streak	0.37	1,190	747	1.59	3,220
Fibrous Plaque	0.31	1,240	746	1.66	3,990
Calcified Plaque	8.47	30,000	12,400	2.43	3,550

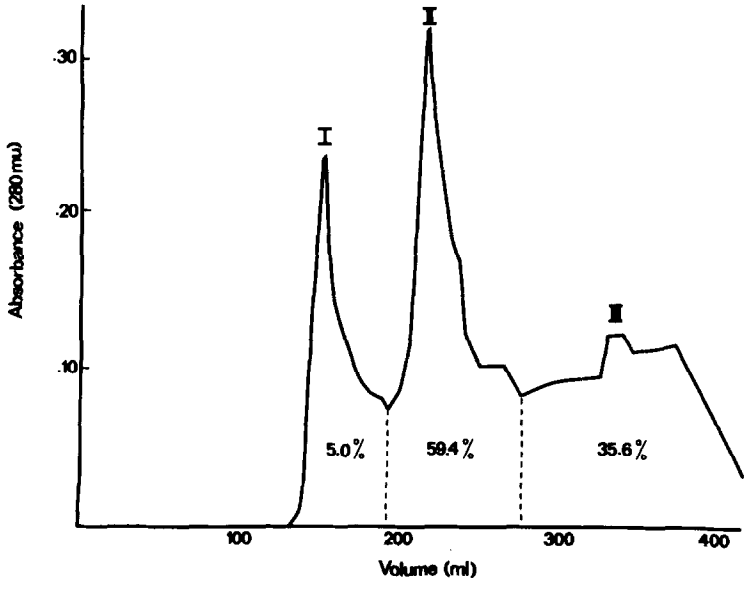

Figure 1. Sephacryl S-200 profile of EDTA plaque extract, indicating percentage distribution of protein-bound Gla.

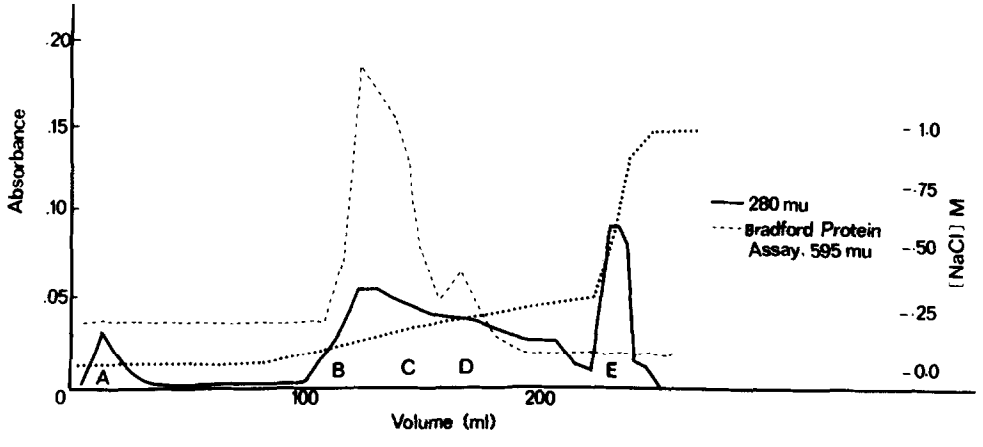

Figure 2. DEAE cellulose chromatography of Sephacryl S-200 peak II utilizing a linear NaCl gradient (0.0 to 0.25M) followed by a 1.0M NaCl wash (E).

Gel filtration chromatography of the EDTA extract with Sephacryl S200 (Fig. 1) yielded two Gla-rich peaks: one occurring at 240-260 ml (Peak II) and a peptide fraction eluting between 280 and 400 ml (Peak III). Chromatography of peak II on DEAE cellulose (Fig. 2) resulted in four protein peaks, only one of which (Peak D) contained Gla (Table II). SDS gel electrophoresis (Fig. 3) of DEAE peaks reveals B and C to have similar mobility with molecular weight of approxi-

TABLE II

GLA-AORTIC PLAQUE PROTEIN PURIFICATION

γ-CARBOXYGLUTAMATE CONTENT

PURIFICATION	RES/1000 GLU	RES/1000 AA	TOTAL nmol	% RECOVERED
Calcified plaque	8.6	0.82	3.5×10^4	100.0%
EDTA extract of calcified plaque	15.1	2.29	2.0×10^3	5.7%
Residue after EDTA extraction	2.8	0.23	4.3×10^3	12.5%
S200 Peak II	35.6	-	-	-
S200 Peak III	86.1	-	-	-
DEAE Peak D (Atherocalcin)	105.2	19.0	217.2	0.63%

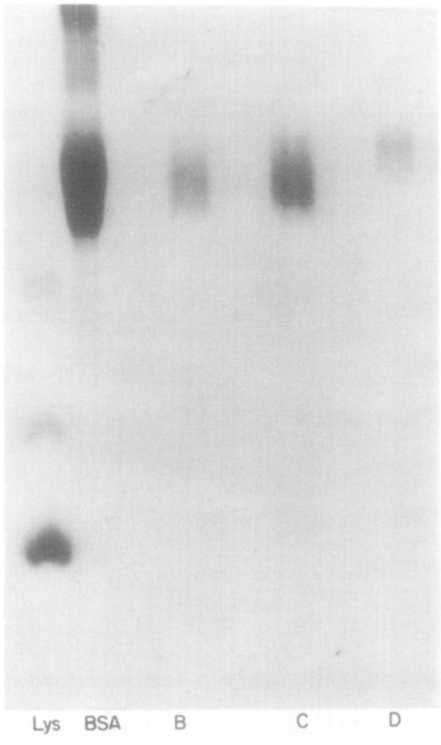

Figure 3. 15% SDS-acrylamide gel electrophoresis. Bands are coded as follows: Lys, lysozyme; BSA, bovine serum albumin; B,C,D, correspond to DEAE peaks as shown in Figure 2.

mately 68,000 while the Gla containing peak D migrated with less anodal mobility and had a molecular weight of approximately 80,000. Isoelectric focusing of the DEAE peaks revealed that peaks B and C contained two protein bands which focused

TABLE III

AMINO ACID COMPOSITION OF ATHEROCALCIN COMPARED TO VITAMIN K DEPENDENT PLASMA PROTEINS

(residues per 1000 amino acids)

	ATHEROCALCIN	PROTHROMBIN	FACTOR IX	FACTOR X	PROTEIN C	PROTEIN S
ASP	153	101	100	85	94	116
THR	62	50	59	66	43	52
SER	69	68	74	64	64	76
GLU	164	106	96	113	133	92
PRO	50	61	36	45	50	47
GLY	59	80	86	90	97	68
ALA	68	57	54	68	53	69
CYS	19	36	47	54	60	37
VAL	63	59	71	57	77	63
MET	10	14	12	17	17	9
ILE	38	33	61	30	38	56
LEU	68	77	51	67	76	90
TYR	17	31	38	21	30	28
PHE	36	35	45	48	33	34
HIS	21	18	19	25	17	20
LYS	50	56	66	50	45	75
ARG	36	72	42	62	59	35
TRP	-	27	17	16	-	-
GLA	19	18	28	22	23	17
MOLECULAR WEIGHT	80,000	71,500	57,100	58,900	56,100	69,000
pI	4.16-4.3	4.7-4.9	4.0-4.5	4.9-5.2	4.4-4.8	5.0-5.5

at pH 4.2-4.36 while peak D focused at pH 4.16-4.3 with a single protein band. The amino acid composition and pI of peak D, designated as atherocalcin, is presented in Table III, in comparison with other selected Gla containing proteins in the same molecular weight range. As shown, there are 19 Gla residues/1000 amino acids, or about 12 per atherocalcin molecule. Furthermore, the amino acid phosphoserine, which was significiantly present in the EDTA extract (5.6 res/1000 amino acids) was not demonstrable in any of DEAE cellulose peak pools.

The possible plasma origin of atherocalcin was examined. Immunoelectrophoresis of the S200 peak II fraction revealed reactivity of this material with rabbit antiserum against human serum, corresponding to albumin when compared with a plasma control. Immunodiffusion studies, shown in Fig. 4, also revealed immunoprecipitation with DEAE peaks B and C when these were reacted with antiwhole human serum, and anti-human albumin antisera. Serial dilutions of peaks B and C revealed the level of serum protein contamination to be about 20% for both peaks. Peak D, atherocalcin, was unreactive.

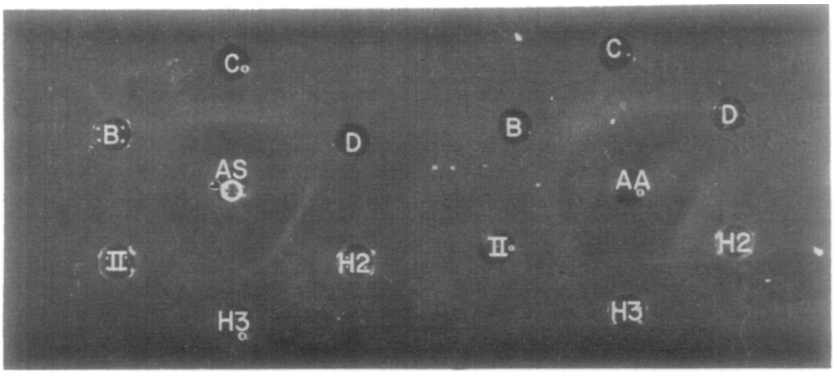

Figure 4. Double-immunodiffusion of anti- whole human serum (AS), and anti-human albumin (AA) versus the following antigens: II, Sephacryl S200 peak II (200 μg/ml); B, DEAE peak B (200 μg/ml); C, peak C (200 μg/ml); DEAE peak D (200 μg/ml); H2, human plasma diluted 1:100; and H3, human plasma diluted 1:1000 (albumin concentration of 40 μg/ml).

DISCUSSION

We have demonstrated for the first time a unique Gla containing protein in calcified atherosclerotic plaque which we have named atherocalcin. To date, of the Gla-proteins, only prothrombin and protein S have similar molecular weights, but differ significantly in charge and composition (Table III). Other known human Gla proteins, the F1 fragment of prothrombin (12), the Gla containing proteins of kidney stones (3) and osteocalcin (4) are of much lower molecular weights than atherocalcin, and bear no obvious resemblance by amino acid composition. However, proteolytic fragments of atherocalcin might more closely resemble these lower molecular weight components.

Atherocalcin and related Gla-containing proteins may be of importance in the development of the atherosclerotic lesion. Work from a number of laboratories suggests that mineral deposition may be a consequence of sequelae involving thrombosis and tissue necrosis (1). Perhaps Gla-containing proteins play an integral part in the process. Our hypothesis is supported by the finding that Gla-content increases with pathologic severity. Since the demineralization of hardened lesions with neutral-EDTA buffer results in extraction of 87% of the Gla-containing proteins, a strong association of these proteins with the mineral component is indicated.

The cellular or tissue origin of atherocalcin is unknown. It may be synthesized de novo by cells in the atherosclerotic lesion as a concomitant event in the sequelae of the atherosclerotic disease process. Perhaps, a similar protein may also be a normal component in bone development, as a high molecular weight precursor of osteocalcin. Indications (13,14) are that osteocalcin is synthesized in bone as a high molecular weight precursor protein. In addition to its location in atheromata, some atherocalcin may circulate or be released into the plasma. Perhaps, circulating levels of the atherocalcin, measurable by radioimmunoassay, might correlate with disease status, and could therefore be a valuable diagnostic and therapeutic parameter. Since all Gla-containing proteins appear to have a requirement for vitamin K for their posttranslational modification, further development of vitamin K antagonists with a more defined target organ specificity than that displayed by the current coumarin or indanedione drugs, might have considerable utility for pharmacological therapy of the ultimately lethal consequences of atherosclerotic and arteriosclerotic disease.

ACKNOWLEDGMENTS

The authors thank Mr. John Zenker for expert technical assistance, and Mr. Mark Knowlton of the Peter Bent Brigham Hospital for obtaining pathologic specimens. Thanks also to Drs. A.S. Nadas, M.J. Glimcher and P.V. Hauschka for valuable discussions and to Dr. F.W. Keeley, University of Toronto, for communicating his work to us on a similar protein. This work was supported by National Institutes of Health Grants: DE 04641, AG00376, HL05606, and HL24463.

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