INTERACTION OF ENZYMATICALLY MODIFIED LOW-DENSITY LIPOPROTEINS AND FIBRONECTIN

T. M. Chulkova

UDC 616.13-004.6-092:616.13-008.939.15-07

KEY WORDS: atherosclerosis; low-density lipoproteins; fibronectin.

Interaction between serum lipoproteins and connective-tissue components of the extracellular matrix of the arterial wall is regarded as one of the mechanisms of lipid accumulation in atherogenesis [5]. Disturbance of metabolism and enzymic modification of lipoproteins can intensify such interaction. Data in the literature on this question are extremely limited. It has been shown that the quantity of insoluble complexes with glycosaminoglycans from the human aorta is increased after treatment of the low-density lipoproteins (LDL) with trypsin [3].

Assuming that enzymic modification of lipoproteins can enhance their atherogenicity, it was decided to study interaction between enzymatically modified LDL and fironectin. Fibronectin possesses adhesive and ligand-binding activity and it is one component of the extracellular matrix. In blood vessel walls it is located mainly in the glycocalyx, the subendothelium, and in the basal membrane of endothelial cells. It is these structures that are involved in the initial stage of atherosclerosis [2, 11]. Fibronectin is also found in atherosclerotic plaques [10].

EXPERIMENTAL METHOD

LDL (density 1.019-1.063 g/cm^3) were isolated from serum of healthy blood donors by preparative ultracentrifugation [6] and kept in medium containing 0.05M NaCl, 0.50M KBr, 0.05% EDTA, at 4°C for not more than two weeks. After preliminary dialysis against 0.02 M Tris-HCl, pH 7.4, containing 0.05% EDTA, the LDL were hydrolyzed with pepsin at pH 4.0 for 60 min at 37°C, with enzyme and substrate in the ratio of 1:300. At the end of hydrolysis the incubation medium was alkalified with 0.2 M Tris to pH 7.5. LDL treated in the same way but without addition of the enzyme served as the control. To determine the degree of hydrolysis, protein from the incubation mixture was precipitated with 10% TCA. The residue was separated by filtration and the concentration of liberated peptides determined in the filtrate [8]. The protein concentration in the original preparations of LDL was determined by Lowry's method.

Interaction of LDL with fibronectin was determined by measuring removal of fibronectin from a column containing fibronectin-collagen-sepharose, on passage of solutions of native and modified LDL through it. Collagen-sepharose was obtained by immobilizing denatured collagen on CNBr-sepharose 4B by the method described previously [12]; 1 ml of sepharose contained 1 mg of immobilized collagen. All procedures connected with chromatography on collagen-sepharose were carried out in the presence of 10^{-4} M phenylmethylsulfonyl fluoride. Fresh human serum (5 ml), clarified by centrifugation, was applied to 1 ml of collagen-sepharose, equilibrated with 0.05 M Tris-HCl, pH 7.5, at the rate of 10 ml/h. The column was washed with 0.5 M NaCl and the corresponding buffer until there was no difference between the optical densities of the displacing solution and the original buffer. Next, 1 ml of 0.5% solution of native LDL, or of pepsin-treated LDL, was passed through the fibronectin-collagen-sepharose at the rate of 1 ml/h. The column was then washed with buffer and with 0.5 M NaCl. The fibronectin was eluted with 4 M urea and estimated quantitatively by measuring its absorbance at 280 nm. From 5 ml of serum, 0.7 mg of fibronectin bound with 1 ml of collagen-sepharose.

Protein was identified in eluates from the column by electrophoresis on 5% polyacrylamide gel (PAG) plates in the presence of sodium dodecylsulfate (SDS). The samples were heated

Institute of Biological and Medical Chemistry, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR V. N. Orekhovich.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 100, No. 9, pp. 301-303, September, 1985. Original article submitted October 16, 1984.

 0007-4888/85/0009-1202$09.50

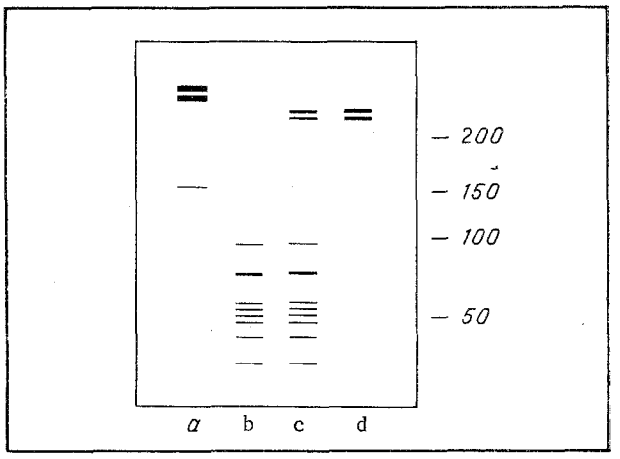

Fig. 1. Electrophoresis of LDL preparations: a) LDL after keeping at pH 4.0 for 60 min at 37°C; b) LDL after hydrolysis with pepsin at pH 4.0 for 60 min at 37°C with enzyme and substrate in the ratio of 1:300; c) eluate of pepsin-modified LDL from column with fibronectin-collagen-sepharose; d) fibronectin. Scale of molecular weights of proteins (in kilodaltons) on right.

beforehand to 100°C for 2 min in buffer containing 0.06 M Tris-HCl, pH 6.7, 2% solution of SDS, 3% solution of mercaptoethanol, 0.1% solution of bromphenol blue, and 6% solution of glycerol. The gels were stained with a 0.25% solution of Coomassie g-250 [8].

EXPERIMENTAL RESULTS

Pepsin, an analog of the tissue proteinase, catepsin D, was used for enzymatic modification of the LDL. Proteolysis with pepsin was carried out at pH 4.0 for 60 min at 37°C until, to judge from the results of determination of the quantity of TCA-soluble material, 10% of protein of LDL had been removed. The LDL modified in this way sedimented during analytical ultracentrifugation in one symmetrical peak. Only the speed of sedimentation was slowed, evidently on account of separation of proteolysis products from LDL. Keeping native LDL at pH 4.0 for 60 min at 37°C without pepsin did not lead to any change in the sedimentation properties of LDL.

During electrophoresis of 50 μg LDL, hydrolyzed by pepsin, in 5% PAG in the presence of SDS, only protein components with mol. wt. below 100 kilodaltons (kD) were determined. No low-molecular-weight components were present in the preparations of native LDL (Fig. 1: a, b).

To study interaction between LDL and fibronectin, solutions of native LDL and of pepsin-modified LDL were passed through a column containing fibronectin-collagen-sepharose. In cases when pepsin-modified LDL were passed through the fibronectin-collagen-sepharose, partial removal of the fibronectin adsorbed on collagen took place, and during subsequent elution with 4 M urea its concentration was reduced by 50% or more. Electrophoresis revealed fibronectin in the solution of modified LDL flowing from the column (Fig. 1c). Next day, a precipitate was thrown down in this eluate at 4°C. The precipitate was harvested by centrifugation and dissolved in 1% SDS. Electrophoresis revealed fibronectin and LDL in this precipitate.

In experiments with native LDL not treated with pepsin, no fibronectin was removed and no such precipitates were formed.

On the addition of increasing doses of fibronectin (from 10 to 200 μg) to the solution of modified LDL in 0.05 M Tris-HCl, pH 7.5, containing 0.025 M $CaCl_2$, turbidity began to appear, and it increased with an increase in the quantity of fibronectin. This turbidity was due, as electrophoresis showed, to the formation of insoluble LDL—fibronectin complexes. On the addition of fibronectin to a solution of native LDL, only traces of precipitate were formed, and the amounts of precipitate did not increase with an increase in the fibronectin concentration.

The results of this investigation show that enzymatically modified LDL interact with fibronectin immobilized on collagen, and also with fibronectin present in solution, to form a precipitate. The results accord with the view that chemical and enzymic modification of LDL lead to an increase in their atherogenicity [1, 3-4]. It can be postulated that interaction of enzymically modified LDL with fibronectin, as a component of the extracellular matrix, will

lead to deposition of these LDL in the blood vessel walls. In addition, the phagocytosis-stimulating activity of fibronectin may lead to more active ingestion of its complexes with modified LDL by macrophages.

LITERATURE CITED

1. I. M. Karmanskii, A. L. Pichugin, and V. O. Shpikiter, Vopr. Med. Khim., No. 6, 738 (1979).
2. R. I. Litvinov, Kazan. Med. Zh., No. 3, 203 (1984).
3. M. Bihari-Varga, S. Goldstein, D. Lagrange, and E. Gruber, Int. J. Biol. Macromol., 4, 438 (1982).
4. M. S. Brown and J. L. Goldstein, Annu. Rev. Biochem., 52, 223 (1983).
5. G. Camejo, Adv. Lipid Res., 19, 1 (1982).
6. R. J. Havel, H. A. Eder, and J. H. Bragdon, J. Clin. Invest., 34, 1345 (1955).
7. U. K. Laemmli, Nature, 227, 680 (1970).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr, et al., J. Biol. Chem., 193, 256 (1951).
9. S. Stenman and A. Vaheri, J. Exp. Med., 147, 1054 (1978).
10. S. Stenman, R. Smitten, and A. Vaheri, Acta. Med. Scand., 642, Suppl. 165 (1980).
11. T. Vartion and A. Vaheri, J. Biol. Chem., 256, 13085 (1981).

GENERATION OF SUPEROXIDE RADICALS BY MITOCHONDRIA OF THE ISCHEMIC HEART

A. N. Ledenev and E. K. Ruuge

UDC 616.12-005.4-07:616.12-018.1:576.311.347

KEY WORDS: superoxide radicals; mitochondria; ischemia.

Evidence of an increase in the concentration of free radicals in myocardial tissues during ischemia and subsequent reperfusion has recently been published [5, 10, 13, 14]. Various workers have suggested that the formation of these radicals may lead to death of the heart cell, the mechanisms of this phenomenon have not been explained. As long ago as in 1973, Chance found [2] that small quantities of superoxide radicals and hydrogen peroxide may be formed in mitochondria under normal physiological conditions. A detailed investigation of the process of superoxide radical formation by the mitochondrial respiratory chain of heart and the participation of free-radical forms of coenzyme Q in this process has been undertaken in [1, 11, 12]. It has been shown that in the ischemic heart mitochondria undergo pathological changes, directly involving the respiratory chain [8, 15].

The ability of mitochondria of the ischemic myocardium to generate superoxide radicals was studied in the present investigation.

EXPERIMENTAL METHOD

Mitochondria were isolated by the method in [7]. Ischemia was produced by incubating the isolated heart of a male Wistar rat in a water bath at 37°C for 60 min [9]. Mitochondria isolated immediately before removal of the heart from the animal served as the control. Protein was determined by Lowry's method [6]. The rate of oxygen consumption was measured by means of a Clark's electrode in medium containing 0.25 M sucrose, 20 mM Tris-buffer (pH 7.4), 0.2 mM EDTA, 4 mM KH_2PO_4, 3 mM $MgCl_2$, 5 mM succinate (sodium salt), and 5 μM rotenone at 25°C. Superoxide radicals were detected by means of the spin trap tiron (sodium 1,2-dihydrobenzene-3,5-disulfonate) by the method in [1]. Quantitative graduation of the method was carried out by the writers jointly with E. Yu. Popova and A. A. Konstantinov, on the basis of the rate of reduction of cytochrome c in medium in which the source of superoxide radicals was the reaction of oxidation of xanthine, catalyzed by xanthine oxidase (from Calbiochem-Behring, West

Research Institute of Experimental Cardiology, All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow.

(Presented by Academician of the Academy of Medical Sciences of the USSR V. N. Smirnov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 100, No. 9, pp. 303-305, September, 1985. Original article submitted November 20, 1984.

0007-4888/85/0009-1204$09.50