PROTECTIVE EFFECT OF α-TOCOPHEROL ON THE MEMBRANE Ca^{++}-TRANSPORTING SYSTEM OF THE SARCOPLASMIC RETICULUM IN HYPERCHOLESTEROLEMIA

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The development of hypercholesterolemia (HCh), like that of other diseases, is accompanied by activation of lipid peroxidation (LPO) as well as by a high cholesterol (Ch) concentration in cell membranes [11, 12]. The functioning of Ca-ATPase in the sarcoplasmic reticulum (SR) of rabbit skeletal muscles is disturbed under these conditions: the rate of Ca^{++} accumulation and of hydrolysis of ATP, and the efficiency of operation of the Ca pump are reduced [10, 11]. Disturbances of the structure and functions of the membrane systems due to uncompensated activation of LPO can be prevented by administration of natural or synthetic antioxidants [4]. The writers showed previously that addition of α-tocopherol (TP) along with Ch to the diet of rabbits normalizes the level of LPO products and restricts the fall in the rate of Ca^{++} accumulation and ATP hydrolysis [10].

The aim of this investigation was to study whether structural and functional disturbances in SR membranes can be prevented by administration of the natural antioxidant TP.

EXPERIMENTAL METHOD

Experiments were carried out on 24 male chinchilla rabbits weighing 2.5-3 kg. The animals were divided into four groups with six rabbits in each group. Animals of control group 1 were kept on the standard animal house diet. The diet of the rabbits of group 2 contained added Ch (1 g/kg daily). Besides Ch, the animals of group 3 received TP acetate in the form of a 10% solution in oil (30 mg/kg). The diet of the rabbits of group 4 contained TP in the same dose, but not Ch. All the animals were kept on the diet for 30 days. Preparations of SR were obtained as described previously [6]. The reaction of interaction between 4-chloro-7-nitrobenzo-2-oxo-1,3-diazole (NBD-Cl) and free SH-groups of Ca-ATPase, and measurement and calculation of the parameters were carried out as described in [8]. Treatment of SR Ca-ATPase with maleimide spin label, recording of the EPR spectra, and calculation of the parameters were undertaken by the method in [9]. Ca-ATPase activity was determined pH-metrically [7]. Lipids were extracted from a suspension of SR membranes by the method in [5]. The lipid composition of the SR membranes was determined by the micromodification of thin-layer chromatography on glass plates with silica-gel in a system of chloroform–methanol–water (65:25:4) [5]. Phospholipids and Ch were separated in a system of diethyl ether–petroleum benzin (1:1). The plates were developed with concentrated H_2SO_4, and Dragendorff's and Vaskovsky's reagents [5]. Densitometry of the chromatograms was carried out on an EP-65m densitometer (East Germany) and the fatty acid composition of the SR membrane lipids was determined on the "Chrom-4" gas-liquid chromatograph. The stationary phase consisted of 10% PEGA, the length of the column was 2500 mm, and its internal diameter 3 mm. The carrier gas was nitrogen and a frame ionization detector was used. The sensitivity was 1:200. The temperature of the vaporizer was 300°C. Methyl esters of fatty acids were fractionated under two different temperature programs: 4 min at 60°C, followed by elevation of the temperature at the rate of 5°C/min up to a final temperature of 200°C, followed by isothermic conditions at 200°C. Peaks were identified by the internal standards method. The volume of the sample was 1-5 μl in hexane. The protein concentration was determined by Lowry's method.

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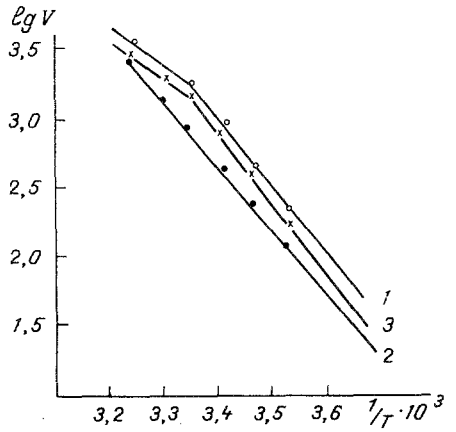

Fig. 1. Temperature dependence of Ca-ATPase activity. 1) Control, 2) cholesterol diet, 3) cholesterol diet + TP.

TABLE 1. Kinetic Parameters of SH-Groups of Ca-ATPase under Normal Conditions and in HCh (M ± m)

Experimental conditions	n_{tot}	n_1	k_1	n_2	k_2
Control	12,20±0,78	6,50±0,63	0,11±0,004	5,70±0,68	0,016±0,002
HCh	8,85±0,51*	4,40±0,39*	0,09±0,017*	4,40±0,78	0,022±0,002

Legend. n_{tot}) Total number of SH-groups titrated with NBD-Cl. n_1 and n_2) Number of fast and slow SH-groups, respectively (in moles/10^5 g protein), k_1 and k_2) constants of modification of fast and slow SH-groups, respectively (in min^{-1}). *P < 0.001 compared with control.

TABLE 2. Binding of Maleimide Spin Label with SH-Groups of SR Ca-ATPase (M ± m)

Experimental conditions	Time, min			
	5	10	20	30
Control	0,036±0,003	0,071±0,0045	0,145±0,006	0,213±0,02
HCh	0,023±0,003*	0,045±0,005*	0,09±0,008*	0,145±0,023*
HCh + TP	0,033±0,002	0,060±0,004	0,13±0,007	0,183±0,031

Legend. *P < 0.01 compared with control. Data expressed in negative logarithms (to base 10) of ratio $1/1_o$, where 1 denotes the amplitude of the EPR signal of the label not bound with protein, 1_o the same at the initial moment of time.

EXPERIMENTAL RESULTS

To assess the properties of Ca-ATPase under normal conditions and in HCh, the dependence of activity of this enzyme on temperature was studied. The curve obtained for Ca-ATPase isolated from muscles of normal animals, plotted between Arrhenius coordinates, had a kink in the 20°C region (Fig. 1), reflecting intramolecular rearrangements of the enzymes [7]. During the development of HCh the kink disappeared and the dependence became linear. Addition of TP to the cholesterol diet restored the normal character of the temperature dependence of Ca-ATPase activity (Fig. 1). The rate of binding of the thiol reagents NBD-Cl and spin-labeled maleimide by "fast" SH-groups of Ca-ATPase in HCh fell below normal (Tables 1 and 2). The reason for this could be structural rearrangement affecting the protein-lipid complex of Ca-

TABLE 3. Content (in %) of Principal Lipid Components in SR under Normal Conditions and in HCh (M ± m)

Components	Control	HCh
Phosphatidylcholine	59,5±5,13	59,94±5,75
Phosphatidylethanolamine	30,32±2,11	27,74±2,94
Phosphatidylserine	11,75±1,45	7,48±1,52*

Legend. Here and in Table 4: *P < 0.05 compared with control.

TABLE 4. Fatty Acid Composition (in %) of Phospholipids of SR under Normal Conditions and in HCh (M ± m)

Fatty acid	Control	HCh	HCh + TP
$C_{16:0}$	23,9±1,40	30,6±1,75*	33,00±2,05
$C_{18:0}$	12,70±2,76	9,32±0,57	8,95±1,06
$C_{18:1}$	18,40±2,80	13,00±0,13*	16,40±0,10
$C_{18:2}$	24,90±1,02	19,30±0,72*	25,00±1,34
$C_{18:3}$	0,69±0,08	3,28±1,11	1,31±0,23
$C_{20:4}$	9,47±1,00	6,47±0,51*	9,50±1,87

ATPase on account of modification of SH-groups by LPO products [1-4]. This hypothesis is confirmed by the fact that addition of TP to the animals' diet simultaneously with Ch increased the rate of binding of thiol reagents by "fast" SH-groups and brought it close to normal.

Also in this investigation changes in the lipid components of Ca-ATPase, as the probable cause of disturbances of function of the Ca pump in HCh, and the possibility of preventing these changes by means of TP, were studied. The results showed that concentrations of phosphatidylcholine and phosphatidylethanolamine remained virtually unchanged in HCh, whereas the phosphatidylserine concentration fell somewhat (Table 3). The fatty acid composition of the SR membranes showed more significant changes in HCh: the fractions of arachidonic, linoleic, and oleic acids decreased whereas the relative percentage of palmitic acid increased (Table 4). Administration of TP to animals with HCh restored the concentrations of unsaturated acids (the most probable peroxidation substrate) to normal, but did not affect the concentration of saturated palmitic acid. The writers showed previously that administration of TP does not lower the Ch level in SR membranes in HCh [10].

It can thus be concluded that an essential role in damage to the Ca pump in HCh is attributable to activation of LPO: addition of TP to the diet of animals with HCh considerably restored the structural parameters and transport function of membrane SR.

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INHIBITION OF RADIATION-INDUCED PYCNOSIS OF CELL NUCLEI BY CADMIUM IONS

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Ions of many heavy metals possess marked toxicity. For instance, if cadmium salts enter the body of man and other mammals they induce the development of pathological changes in the kidneys, lungs, and other organs and systems [10]. The problem of the concrete mechanisms of these biological effects has not been finally solved. It can be tentatively suggested that Cd^{++} ions, competing with ions of other trace elements, inhibit activity of certain metabolic processes [12]. The corresponding shifts at the cellular level can be studied by complex anlysis, for it is possible to modify nuclear structures and to produce changes in cell membranes. The lymphoid cells of the thymus are very convenient for such investigations, for they are highly sensitive *in vivo* to the action of Cd^{++} ions [13].

This paper describes a study of the effect of cadmium chloride ($CdCl_2$) on the frequency of nuclear pycnosis in a thymocyte population *in vitro* and the results are compared with data on the supercoiled structure of DNA and integrity of the cell membranes.

EXPERIMENTAL METHOD

Noninbred female albino rats weighing 130-150 g were used. Thymocytes were isolated and counted and suspensions ($5 \cdot 10^6$ cells in 1 ml) prepared in medium 199 with 10% homologous serum by the method described previously [4]. A 4 mM solution of $CdCl_2$ and its dilutions were made up in 0.15 M NaCl solution and kept at −10°C. To each sample of 2 ml, 0.05 ml of $CdCl_2$ solution was added immediately before irradiation. In the control, 0.15 M NaCl was added.

Gamma-irradiation of the cells (^{60}Co) was carried out at 20°C in an atmosphere with 5% CO_2 on a "Luch-1" apparatus with a dose rate of 0.9 Gy/min. The cells were subsequently incubated for 5 h at 37°C.

Pycnotic forms of thymocytes were counted by staining with acridine orange and the fractions of cells carrying receptors for autologous erythrocytes (ARFC) were determined as described previously [14]. Cells in contact with two or more erythrocytes were taken to be ARFC. The thymocytes were washed once for supravital staining. The cells were stained with alcian blue (from Gee Lawson, England) or erythrosin [5], with a final concentration of the dye of 0.02%. Conformational changes in the supercoiled structure of DNA (scDNA) of the thymocytes were assessed as changes in relative viscosity of the cell lysate (nucleoids), by means of an Ostwald viscometer [2].

The results were subjected to statistical analysis by Student's t test [1].

EXPERIMENTAL RESULTS

After incubation of unirradiated thymocytes for 5 h moderate pycnotization of the cell nuclei, characteristic of the model used [14], was observed (Table 1). The presence of Cd^{++} ions (10-100 μM) did not reduce the fraction of ARFC or change the number of cells which

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