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INDUCTION OF LIPID PEROXIDATION IN ERYTHROCYTES DURING CHOLESTEROL OXIDATION CATALYZED BY CHOLESTEROL OXIDASE

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UDC 612.111.1: 612.397.2]. 015.11: 577.152.1

KEY WORDS: lipid peroxidation; cholesterol; cholesterol oxidase; erythrocytes.

Lipid peroxidation (LPO) is a universal method of modification of biomembranes under normal conditions and when injured by the development of pathological processes [6, 10]. That is why it is particularly important to study mechanisms of initiation of the LPO reaction in vivo. LPO reactions can be initiated in vivo both enzymatically and nonenzymatically [2, 6]. The enzyme systems that participate in the formation of LPO products in biomembranes include various NADPH- and NADH-dependent generators of active forms of oxygen [2, 8, 12] and, in particular, the system of mixed-function microsomal oxygenases. Much less is known about the participation of oxidase systems generating H_2O_2 in the induction of LPO. It has recently been shown that deamination of biogenic amines, catalyzed by monoamine oxidase, may under certain conditions be accompanied by accumulation of LPO products [9]. Hence the importance of studying to what extent the participation of oxidases could be a source of lipid peroxides in vivo.

The aim of this investigation was to study the ability of cholesterol oxidase (ChO), which catalyzes oxidation of cholesterol (Ch) to cholest-4-en-3-one and, at the same time, reduction of O_2 to H_2O_2, to induce the LPO in erythrocyte membranes, for we know that Ch is a natural inhibitor of LPO in plasma membranes [3].

EXPERIMENTAL METHOD

Erythrocyte ghosts were obtained from guinea pig blood by the method in [13]. The reaction of oxidation of Ch in the erythrocyte ghosts or in micelles with Triton X-100 was carried out in the following medium: Tris-HCl 0.2 M, pH 7.0 (at 37°C), Triton X-100 0.25%, and ChO 0.05 U/ml. The activity of ChO was determined by the chemiluminescence method which detected H_2O_2 with the presence of luminol (10^{-4} M) and horseradish peroxidase (1.0 U/ml) [4, 11]. To separate Ch from cholest-4-en-3-one the method of thin-layer chromatography was used in a solvent system of chloroform – methanol (98 : 2) as described in [7]. For densitometry of the plates in ER165M densitometer was used. The level of LPO products interacting with 2-thiobarbituric acid (TBA) was determined as described previously [5].

EXPERIMENTAL RESULTS

ChO catalyzes the reaction of Ch oxidation with simultaneous formation of H_2O_2:

$$\text{Ch} + O_2 \xrightarrow{\text{ChO}} \text{cholest-4-en-3-one} + H_2O_2.$$

Activity of the enzyme can be estimated by determing accumulation of either reaction product. It was shown (Fig. 1) that during incubation of Ch dispersed in Triton X-100 with ChO the concentration of Ch (the spot with $R_f = 0.36$) falls and that of cholest-4-en-3-one (the spot with $R_f = 0.50$) rises. H_2O_2 formation can be recorded highly sensitively by a chemiluminescence method, using a luminol – peroxidase system. Curves showing the kinetics of chemiluminescence arising during incubation of Ch, dispersed if Triton X-100, in the presence of ChO, luminol, and peroxidase, are given in Fig. 2. In the absence of ChO or of Ch, and also of

Institute of Physiology, Bulgarian Academy of Sciences, Sofia. Higher Medical Institute, Pleven, Bulgaria. (Presented by Academician of the Academy of Medical Sciences of the USSR. G. N. Kryzhanovskii.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 100, No. 8, pp. 179-181, August, 1985. Original article submitted January 18, 1985.

 0007-4888/85/0008-1052 $09.50

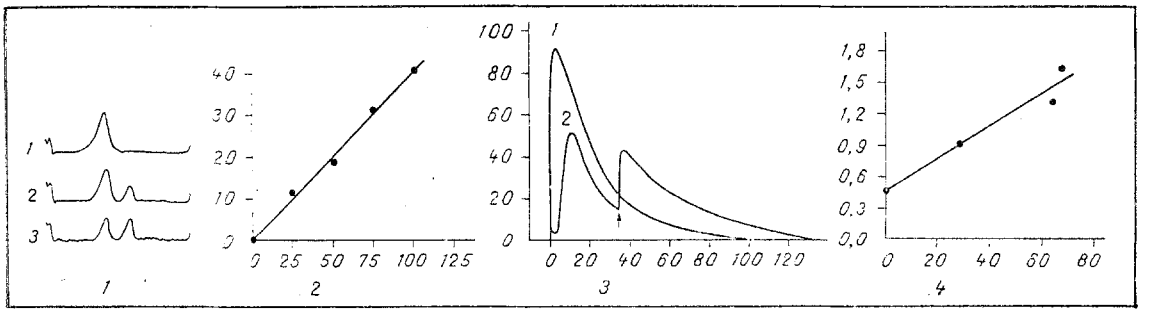

Fig. 1. Densitograms of thin-layer chromatograms of reaction products formed by the action of ChO on Ch (30.0 μg/ml), dispersed in Triton X-100. Reaction products extracted from incubation mixture after end of incubation by addition of chloroform (1 : 1 by volume). 1, 2, 3) Incubation for 0, 15, and 30 min respectively.

Fig. 2. Dependence of light sum of chemiluminescence developing during oxidation, catalyzed by ChO, of Ch dispersed in Triton X-100 in the presence of peroxidase and luminol, on Ch concentration. Abscissa, concentration (in $M \cdot 10^6$); ordinate, light sum of chemiluminescence (in relative units).

Fig. 3. Kinetic curves of chemiluminescence developing during oxidation, catalyzed by ChO, of endogenous Ch in guinea pig erythrocyte ghosts (0.15 mg protein/ml). Abscissa, time (in min); ordinate, intensity of chemiluminescence (in relative units). 1) In presence of Triton X-100; 2) in absence of Triton X-100, followed by its addition later (indicated by arrow).

Fig. 4. Dependence of MDA accumulation during cholesterol-oxidase reaction in erythrocyte ghosts (0.15 mg protein/ml) on light sum of developing chemiluminescence. Abscissa, light sum of chemiluminescence (in relative units); ordinate, MDA concentration (in nanomoles/mg protein).

luminol or peroxidase, no chemiluminescence response was recorded. If all components were present in the incubation medium the amplitude and light sum of the chemiluminescence were proportional to the concentration of Ch in the system.

On addition of ChO to a suspension of erythrocyte ghosts chemiluminescence also developed, and its intensity depended on the presence of Triton X-100 in the system. In the absence of detergent the intensity of chemiluminescence was significantly lower than in its presence; addition of detergent to the suspension after a fall in the intensity of chemiluminescence to the initial level caused a new flash of chemiluminescence (Fig. 3). Under these circumstances the light sum of chemiluminescence recorded when the detergent was present initially in the suspension was equal to its value obtained by addition of the light sums before and after addition of Triton X-100 to the erythrocyte ghosts. This means that in the presence of Triton X-100, more Ch was exposed to enzyme attack than in its absence. In the absence of detergent, the Ch pool located in the outer monolayer of the erythrocyte membrane was perhaps accessible for ChO, whereas after addition of the detergent Ch located in both monolayers of the membrane is so accessible.

During the reaction of Ch oxidation catalyzed by ChO, LPO was induced in the erythrocyte membranes and could be recorded as the formation of products interacting with TBA, principally malonyl dialdehyde (MDA). The intensity of LPO (concentration of MDA) for instance (Fig. 4), increased with an increase in ChO activity, or with an increase in the light sum of chemiluminescence. Incubation of the erythrocyte suspension in the absence of ChO was accompanied by negligible accumulation of LPO products (Table 1), without chemiluminescence. Exclusion of peroxidase and luminol from the incubation system had little or no effect on the development of LPO in erythrocytes ghosts. 4-Methyl-2,6-di-tert-butylphenol (ionol), an inhibitor of free-radical oxidation, completely blocked LPO.

As regards the possible mechanisms of LPO induction in the system under consideration, the first suggestion to be made is that hydroxyl radicals, formed by the reaction of ferrous ions with H_2O_2:

$$H_2O_2 + Fe^{++} \rightarrow OH^- + Fe^{+++}$$

TABLE 1. Formation of LPO Products in Erythrocytes during Cholesterol-Oxidase Reaction under the Influence of Various Agents

Experimental conditions	MDA concn., nanomoles/mg protein	
	0,05 mM Fe^{2+}	10,0 mM Fe^{2+}
IM, incubation for 50 min	9,1	1,8
IM, incubation for 0 min	1,2	1,2
IM without ChO	1,6	1,1
IM without peroxidase	10,0	—
IM without luminol	10,2	—
IM without exogenous Fe^{++}	1,4	1,4
IM + catalase (0,7 unit)	5,3	—
IM + catalase (1,4 units)	3,8	—
IM + catalase (14,0 units)	1,2	—
IM + ionol ($5 \cdot 10^{-5}$ M)	1,2	—
IM + EDTA (10^{-4} M)	2,0	—

Legend. IM) Incubation medium: Tris-HCl 0.2 M, pH 7.0 (37°C), Triton X-100 0.25%, ChO 0.5 unit/ml, luminol 10^{-4} M, peroxidase 1.0 unit/ml, erythrocyte ghosts 0.15 mg protein/ml.

In fact, addition of increasing quantities of catalase to the incubation medium caused inhibition of MDA formation (Table 1). The transition metal chelating agent EDTA has a similar inhibitory action. Finally, addition of exogenous Fe^{++} in a concentration optimal for LPO induction (50 μM) stimulated MDA formation, whereas high concentrations of Fe^{++} (10 mM) had an inhibitory action. Another cause of LPO activation in the course of the reaction catalyzed by ChO, incidentally, could be a reduction in the quantity of free Ch in the membrane, where it exerts an antioxidant action [3].

At the present time ChO is often used to study the asymmetry of distribution of Ch in biomembranes and the velocity of its trans-bilayer migration [7]. It is suggested that changes in membrane permeability do not take place during the reaction catalyzed by the enzyme, and no products capable of affecting flip-flop in biomembranes are formed. Accumulation of LPO products in erythrocyte membranes discovered in the present investigation under the influence of ChO compels critical re-examination of these results, for we know that accumulation of LPO products causes a sharp increase in permeability of biomembranes for ions and nonelectrolytes [10] and also an increase in the rate of trans-bilayer migration of phospholipids in the membrane [1].

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