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

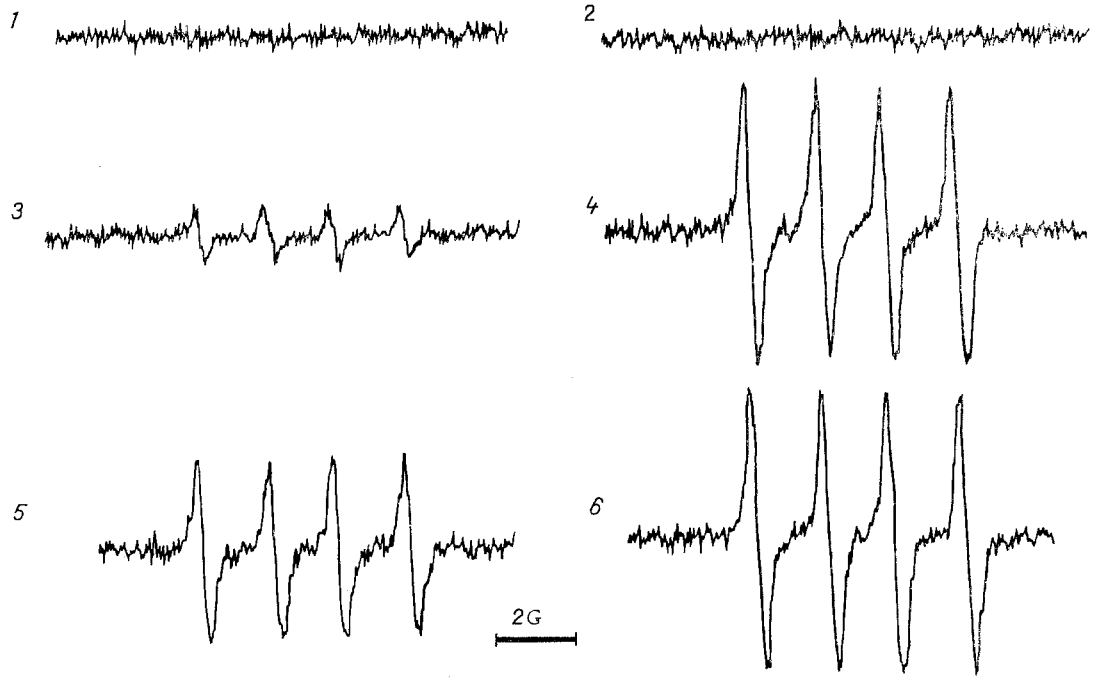

Fig. 1. Generation of superoxide radicals by isolated mitochondria of normal and ischemic rat heat. 1, 3, 5) EPR spectra of tiron radical in control sample; 2, 4, 6) the same in ischemic sample. Concentration of mitochondrial protein 4 mg/ml (control) and 2 mg/ml (ischemia). 1, 2) Basic medium without additives; 3, 4) with addition of antimycin (2 μM); 5, 6) with addition of antimycin and MONCPH (2 μM).

TABLE 1. Effect of Ischemia on Rate of Oxygen Consumption in States 3 and 4 and on Rate of Generation of Superoxide Radicals (in ng-moles/mg protein/min) by Isolated Rat Heart Mitochondria (n = 5; M ± m)

Rate of O_2 consumption			Rate of O_2^- generation	
experimental conditions	state 3	state 4	−MONCPH	+MONCPH
Control	96,0±4,2	21,5±2,5	0,39±0,10	1,20±0,20
Ischemia	37,0±2,8	23,7±2,5	2,74±0,59	2,90±0,48

Legend. +MONCPH, −MONCPH: presence or absence of the uncoupler mesoxalonitrile(3-chlorophenyl)-hydrazone in incubation medium.

Germany), and by comparing this rate with the steady-state concentration of tiron radicals under the same conditions. The main incubation medium to determine the rate of generation of superoxide radicals O_2^- by mitochonrdia contained 0.25 M sucrose, 10 mM Tris-buffer (pH 7.4), 0.2 mM EDTA, 10 mM tiron, and 10 mM succinate. Antimycin A and carbonyl cyanide-m-chlorphenylhydrazone (CCCP), in a concentration of 2 μM, were used as additives. Electron paramagnetic resonance (EPR) spectra of tiron radicals were recorded under the following conditions: power of SHF radiation 20 mW, frequency of modulation 100 kHz, amplitude of modulation 0.5 G, scanning speed of field 10 G/min, time constant 0.064 sec, temperature 25°C. Measurements were made on an E-109E spectrometer (Varian, USA). The reagents used were obtained from Sigma (USA) and Serva (West Germany).

EXPERIMENTAL RESULTS

To determine the effect of experimental ischemia on function of mitochondria, rates of oxygen utilization were measured in states 3 and 4. The results indicate that the model of ischemia which we used depressed the respiratory activity, mainly through its effect on the

rate of oxygen consumption in state 3 (Table 1). It has been shown [8, 15] that a reduced respiration rate may be associated with damage to enzymes of the electron transport chain.

Tiron is oxidized by the superoxide radical into a relatively stable semiquinone, which gives the characteristic EPR spectrum. Addition of succinate and antimycin to the mitochondria was accompanied by the formation of free tiron radicals, whose concentration reached a stable level within a few minutes and, as has been shown [1], serves as an indicator of the rate of O_2^- generation. It has been found that superoxide radicals are formed in the respiratory chain as a result of interaction of the unstable ubisemiquinone with oxygen; under these circumstances stimulation of superoxide-forming activity by antimycin was due to an increase in the steady-state concentration of the ubisemiquinone. The results of one typical experiment with control and ischemic mitochondria are shown in Fig. 1. It was found (Table 1) that the superoxide-forming activity of mitochondria of the ischemic myocardium is significantly increased compared with the control. However, the sensitivity of the method, it must be admitted, did not allow O_2^- generation to be observed in either control or ischemic samples in the presence of antimycin. An uncoupler of oxidative phosphorylation (CCCP) stimulated the formation of superoxide radicals in control samples but had no effect on O_2^- generation by ischemic mitochondria. This result is evidence that energization of the membrane influences the superoxide-forming activity of mitochondria. Ischemic mitochondria are virtually completely uncoupled (Table 1, respiratory control 1.5), and for that reason the addition of CCCP in this case caused no change in the rate of O_2^- generation.

The increase in the rate of O_2^- generation by ischemic mitochondria thus revealed can be explained not only by de-energization of the inner mitochondrial membrane, but also by the following causes: injury to the membrane and associated increased likelihood of interaction between ubisemiquinone and oxygen and (or) reduced activity of mitochondrial superoxide dismutase, on account of which most of the O_2^- under normal physiological conditions is rapidly converted into hydrogen peroxide [4]. Some help with the elucidation of the contribution of each of these factors may be given by the study of O_2^- generation by mitoplasts, washed to remove superoxide dismutase. The rate of formation of superoxide radicals by mitochondria *in vivo* ought to be lower than values determined in the presence of antimycin *in vitro* (Table 1), for antimycin, as has already been mentioned, stimulates O_2^- generation [11]. However, considering the accumulation of free fatty acids, capable of inhibiting the respiratory chain, in the heart cell that is characteristic of ischemia, the fall in activity of "antioxidant" enzymes (superoxide dismutase, glutathione peroxidase, etc.) [13], and also the high concentration of mitochondria in the heart, it can be postulated that these intracellular organelles make a significant contribution to the increase in concentration of free oxygen radicals in the myocardium during ischemia and subsequent reperfusion.

LITERATURE CITED

1. I. V. Grigolava, M. Yu. Ksenzenko, A. A. Konstantinov, et al., Biokhimiya, 45, 75 (1980).
2. A. Boveris and B. Chance, Biochem. J., 134, 707 (1973).
3. A. Boveris, E. Cadenas, and A. O. M. Stoppani, Biochem. J., 156, 435 (1976).
4. B. Chance, H. Sies, and A. Boveris, Physiol. Rev., 59, 527 (1979).
5. G. Gauduel and M. A. Duvelleroy, J. Mol. Cell. Cardiol., 16, 459 (1984).
6. E. F. Hartree, Anal. Biochem., 48, 422 (1972).
7. G. H. Hogeboom, Methods Enzymol., 1, 16 (1955).
8. H. Jaarsveld and A. Lochner, Basic Res. Cardiol., 77, 388 (1982).
9. R. B. Jennings, K. A. Reimer, M. L. Hill, et al., Circ. Res., 49, 892 (1981).
10. S. R. Jolly, W. J. Kane, M. B. Bailie, et al., Circ. Res., 54, 277 (1984).
11. M. Ksenzenko, A. A. Konstantinov, G. B. Khomutov, et al., FEBS Lett., 155, 19 (1983).
12. M. Ksenzenko, A. A. Konstantinov, G. B. Khomutov, et al., FEBS Lett., 175, 105 (1984).
13. F. Z. Meerson, V. E. Kagan, V. P. Kozlov, et al., Basic Res. Cardiol., 77, 465 (1982).
14. P. S. Rao, M. V. Cohen, and H. S. Mueller, J. Mol. Cell. Cardiol., 15, 713 (1983).
15. W. Rouslin, Am. J. Physiol., 244, H743 (1983).