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Changes in Antioxidant Status of Myocardium during Oxidative Stress under the Influence of Coenzyme Q_{10}

V. L. Lakomkin*, G. G. Konovalova, E. I. Kalenikova, I. V. Zabbarova, A. I. Kaminnyi, A. K. Tikhaze, V. Z. Lankin, E. K. Ruuge, and V. I. Kapelko

Russian Research and Productive Cardiology Complex, Ministry of Health of Russian Federation, 3-ya Cherepkovskaya ul. 15A, 121552 Moscow, Russia; fax: (7-095) 414-6699; E-mail: v.lakomkin@cardio.ru

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Abstract—Changes in myocardium were studied during oxidative stress induced by infusion of hydrogen peroxide in the coronary vessels of isolated rat heart. Moderate concentrations of H_2O_2 increased the heart rate but decreased the contractile force, whereas higher concentrations of H_2O_2 decreased both parameters and increased the end diastolic pressure. The effect of H_2O_2 was stable, cumulative, and was associated with disturbance in respiration of mitochondria, increased production of ROS in them, and decrease in activities of antioxidant enzymes in the myocardium. Changes in the antioxidant status of the myocardium induced by long-term addition of coenzyme Q_{10} into food was accompanied by decrease in the negative inotropic effect of H_2O_2, whereas the levels of superoxide dismutase and glutathione peroxidase after oxidative stress were virtually unchanged. The activities of these enzymes displayed a high positive correlation with the cardiac function. The findings suggest that coenzyme Q_{10} should increase resistance of the myocardium to oxidative stress not only by a direct antioxidant mechanism but also indirectly, due to increased protection of antioxidant enzymes.

Key words: isolated heart, antioxidant protection, oxidative stress, mitochondria, coenzyme Q_{10}, hydrogen peroxide

Reactive oxygen species (ROS) are normal metabolites, and many authors now consider them to be signal molecules [1-3]. Nevertheless, excess accumulation of ROS is specific for various diseases and seems to be dangerous because they can damage molecules of vitally important polymers, such as proteins and nucleic acids [4-9]. The state characterized by a dramatic increase in the level of ROS is usually called oxidative stress. Development of oxidative stress seems to be especially significant for functioning of organs with a high level of aerobic metabolism, such as the heart. Cells and tissues are protected against the toxic effect of ROS and organic free radicals by the antioxidant system of the cells, which includes antioxidant enzymes: superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px), and low-molecular-weight antioxidants (α-tocopherol, phenolic form of coenzyme Q_{10}, β-carotene, ascorbic acid, etc.) [3-5, 8, 10].

Modeling of oxidative stress by intracoronary injection of hydrogen peroxide is often used for assessment of the total antioxidant potential of myocardiocytes. Homolysis of H_2O_2 results in active hydroxyl radicals $^{\cdot}OH$ [11] capable of initiating free radical oxidation of unsaturated phospholipids and accumulation of lipoperoxides in membrane structures [12]. This is also associated with decrease in the contractile function of the myocardium, and this decrease is greater the higher the concentration of H_2O_2 [13, 14]. During early stages of the perfusion with H_2O_2, the negative inotropic effect is completely reversible [12, 15]; therefore, infusion of H_2O_2 seems to be a suitable approach for modeling oxidative stress in the myocardium [13]. Nevertheless, a biphasic effect of H_2O_2 on the heart [16-18] as well as the different ability of the myocardium for regeneration of function [16, 19] makes difficult the use of H_2O_2 for routine evaluation of the antioxidant status of the myocardium. Therefore, in the first part of the present work, we have tested various approaches for using H_2O_2, and the optimal approach has been chosen; in the second part of the work the decrease in the function was compared to changes in activities of the antioxidant enzymes, because their activities predetermine the resistance to oxidative stress [15, 20, 21]. The antioxidant status of the myocardium was changed by preliminary long-term peroral treatment of animals with a lipophilic proantioxidant, ubiquinone Q_{10} [22, 23].

* To whom correspondence should be addressed.

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MATERIALS AND METHODS

Experimental animals. Effects of H_2O_2 on functions were studied on Wistar rats with body weight of 300-350 g; in the second, mainly biochemical, part of the work rats of the WKY strain were used. During six weeks, about half of the WKY rats obtained the solubilized form of ubiquinone, Q_{10} (10 mg/kg; Akvion, Moscow), with water.

Biochemical studies. Ubiquinone and α-tocopherol were determined in blood serum by HPLC with electrochemical detection on a Coulochem II device (Environmental Sciences Associate, Inc., USA), and the results were processed using a computer program of the same firm.

Activities of the antioxidant enzymes were determined in supernatant fluids prepared by centrifugation of homogenates of the myocardium at 800*g* for 10 min. The protein content was determined in the specimens by the Lowry method. The activity of catalase was determined at 240 nm by the rate of H_2O_2 utilization using a Hitachi-557 recording spectrophotometer (Japan) [24]. The activity of Se-containing glutathione peroxidase was determined at 340 nm in a coupled glutathione reductase system by the rate of NADPH oxidation with *tert*-butyl hydroperoxide as a substrate, using an FP-901 chemical analyzer (Labsystems Oy, Finland) in the regime of kinetic operation [25]. The activity of Cu,Zn-superoxide dismutase (SOD) was determined by inhibition of reduction of Nitroblue Tetrazolium with the superoxide radical $O_2^{\cdot-}$ generated in the system of xanthine–xanthine oxidase, by kinetics of formazan production measured with the Hitachi-557 recording spectrophotometer at 560 nm [26]. The amount of catalase or glutathione peroxidase required for oxidation of 1 μmol H_2O_2 or GSH per min, respectively, was taken as the unit of the enzyme activity under conditions of determination. The amount of SOD required for 50% inhibition of reduction of Nitroblue Tetrazolium to formazan was taken as the unit of the enzyme activity under conditions of the experiment.

Study on contractile function of myocardium. The heart was isolated under urethane anesthesia (1.7 g/kg) and perfused at 37°C through the aorta with Krebs–Henseleit solution containing 11 mM glucose and saturated with carbogen (5% CO_2 + 95% O_2) at pH value of 7.40 ± 0.02. The heart was perfused using a perfusion pump at the rate of 14 ml/min and perfusion pressure of 65-70 mm Hg. Through the left auricle a latex balloon filled with physiological solution was introduced into the left ventricle of the heart. Pressure in the aorta and left ventricle and *dP/dt* were recorded with Gould Statham P23 Db electromanometers (USA) on a Gould Brush 2400 polygraph (USA). Under isovolumic regime, force of the myocardium contractions and expenditure of energy are mainly characterized by the developed pressure and contractile function intensity (CFI, product of the developed pressure and heart rate), which is proportional to oxygen consumption by the cardiac muscle. In various experimental series, these parameters were not lower than 130 and 536 mm Hg/sec, respectively. Hydrogen peroxide was introduced into the perfusate with a Sage Model 351 infusion pump (Division of Orion Res. Inc., USA) at a regulated rate providing constant concentration of H_2O_2.

In experiments with hearts from WKY rats and in some experiments on Wistar rats, the scheme of the experiment was changed: it included determination of the maximal contractile function intensity on gradual increase in the rate of perfusion to 22 ml/min. This was provided by additional activation of the contractile apparatus with Ca^{2+} entering through ion channels activated by distension [27]. Under these conditions, the maximal working capacity of the heart was observed on addition to the perfusate of 0.1 μM isoproterenol. At this perfusion rate the antioxidant system of the myocardium was assessed on addition into the perfusate of 70 μM H_2O_2 within 35 min, when CFI was decreased approximately twofold.

Study on oxidative metabolism of myocardium. From some of the hearts mitochondria were isolated and treated as described in [28]. In the mitochondria, the rate of oxygen consumption was determined in state 3 of the respiratory chain (in the presence of both oxidation substrates and ADP in the incubation medium) and in state 4 (in the presence of oxidation substrates but without ADP in the incubation medium). The rate of oxygen consumption was determined at 25°C with a Clark-type electrode using a YSI 53 polarograph (Yellow Spring Instruments, USA). The rate of generation by mitochondria of ROS, such as superoxide radicals $O_2^{\cdot-}$, was determined using the EPR signal of TIRON (4,5-dihydroxy-1,3-benzenedisulfonic acid, disodium salt monohydrate) spin scavenger. EPR spectra were recorded with an E-109E spectrometer (Varian, USA) at room temperature.

Reagents. Reagents from Sigma, Aldrich, ICN, Cambridge Isotope Labs (USA), and Serva (Germany) were used.

Data processing. The results were processed using *t*-test and ANOVA test, with attachments of the Origin 6.1 program (Microcal Software, Inc., USA). The results are presented as mean value ± mean error (M ± m).

RESULTS AND DISCUSSION

Effect of H_2O_2 on contractile function. In the first series of experiments (n = 9), the infusion of increasing concentrations of H_2O_2 (50-300 μM) for 10 min was accompanied by a nearly linear decrease in the developed pressure and +*dP/dt* (Fig. 1). The infusion of H_2O_2 up to the concentration of 150 μM increased the heart rate

(+15 ± 4%, $p < 0.01$), but higher concentration of H_2O_2 sharply decreased the heart rate, and in some experiments fibrillation appeared. The contractile function intensity retained its initial value only in the presence of the starting concentration of H_2O_2, but then steadily decreased concurrently with a sharp increase in the end diastolic pressure (Fig. 1). The perfusion pressure which at the constant rate of the flow characterizes resistance of the coronary vessels began to increase only in the presence of 200 μM H_2O_2 (+25%, $p < 0.01$). This is in agreement with the concept about the vasoconstrictory effect of increased concentration of H_2O_2 in blood vessels [29]. Our findings have shown that the developed pressure and heart rate change oppositely at moderate concentrations of H_2O_2. Similar changes are specific for the effect of caffeine [30], which is also associated with decrease in the level of Ca^{2+} in the sarcoplasmic reticulum and activation of the Na^+-Ca^{2+} exchange [31]. The concurrent increase in the level of myoplasmic Ca^{2+} [11, 32], along with decrease in contents of ATP and creatine phosphate [12, 14], underlie the increase in the final diastolic pressure under the influence of H_2O_2.

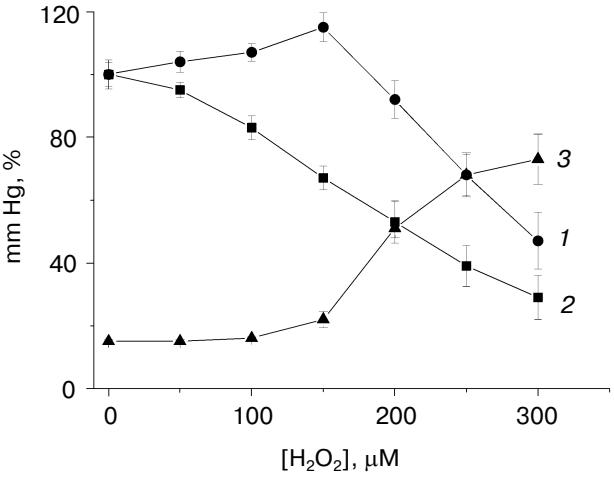

Fig. 1. Dependence of parameters of contractile function of isolated rat heart on concentration of hydrogen peroxide (μM) infused into coronary vessels. *1*) Heart rate; *2*) the maximal rate of pressure development (+*dP*/*dt*) in the left ventricle of the heart; *3*) end diastolic pressure in the left ventricle. Values of heart rate and +*dP*/*dt* are expressed in percent of the initial levels, and the end diastolic pressure is presented in mm Hg. All values are given as M ± m.

To determine the ability for recovery of function, in the second series of experiments 100 μM H_2O_2 was infused thrice, with 15-min intervals. The earliest reliable changes in the heart function clearly evident already after 5 min were decreases in the developed pressure by 11 ± 3% ($p < 0.02$) and its differentiated rates (±*dP*/*dt*) by 8-10%. In contrast, the heart rate significantly increased by 14 ± 4% ($p < 0.02$); therefore, the resulting parameter of the contractile function intensity was unchanged. Values of the initial and end diastolic pressure changed by not more than 1-2 mm Hg. After 10 and 15 min, these changes remained virtually at the same levels. Termination of the H_2O_2 infusion was not accompanied by a significant recovery of the contractile function; it remained on approximately the same level. The repeated infusion of H_2O_2 more significantly decreased the developed pressure and *dP*/*dt*, these parameters decreasing by 25-28% compared to the initial values. Concurrently, the end diastolic pressure and heart rate significantly increased. These changes progressed also after termination of the H_2O_2 infusion. The absence of recovery of parameters of the function seemed to be associated with continuous production of ROS that is confirmed by the entrance of reduced glutathione into the perfusate [19]. This also explains the deeper decrease in parameters of the function in response to the repeated infusion of H_2O_2. The complete recovery of the function after a single infusion of H_2O_2 required more than 50 min [13]. Thus, the effect of H_2O_2 on the contractile function of the heart is early, stable, and cumulative.

The third series of experiments performed on 4- and 6-month-old Wistar rats (9 and 11 animals, respectively) was designed to study response of the heart to oxidative stress during elevated load. The perfusion rate was increased stepwise from 14 to 26 ml/min concurrently with addition of 0.1 μM isoproterenol. The contractile function intensity, which under these conditions characterizes the energy expenditure [33], increased more than twofold compared to the initial level, and both the developed pressure and heart rate increased 1.4-1.5-fold. In spite of significant decrease in the content of creatine phosphate and in the ATP/ADP ratio, the high level of the function was retained [33]. The maximal value of the contractile function intensity in 6-month-old rats was 1006 ± 57 mm Hg and was significantly lower ($p < 0.05$) than in the group of 4-month-old rats (1317 ± 82 mm Hg) that corresponded to an age-related decrease in the level of the myocardium contractile function [19].

Preliminary experiments have shown that the long-term infusion of 100 μM H_2O_2 rapidly decreased the contractile function intensity, therefore, the concentration of 70 μM was chosen. On the background of continuous infusion of isoproterenol, H_2O_2 proportionally decreased the developed pressure and contractile function intensity, because the heart rate remained on the high level. The myocardium contractility decreased more steeply in 4-month-old rats, which displayed the higher initial level of the contractile function intensity; however, the relative decrease in the parameters was virtually the same. Thus, the value of the contractile function intensity in 4-month-old rats after 30 min decreased to 72 ± 7% and after 45 min to 40 ± 6%, whereas in 6-month-old rats the corresponding values were 74 ± 5% and 44 ± 5%, respectively. Consequently, the relative

effect of H_2O_2 did not depend on the initial level of the function. Nevertheless, 10 min after the infusion of H_2O_2 had been started, the decrease in the contractile function intensity was the same in these two groups (84 ± 5 and 90 ± 2%, respectively), and it did not differ from the decrease in the function under the influence of 100 μM H_2O_2 in the second series, i.e., in the presence of about 1.5-fold higher concentration of H_2O_2. This finding suggests that the more intensive oxygen metabolism should be associated with a decreased resistance of the myocardium to oxidative stress. This is also supported by data that infusion of H_2O_2 into hearts stopped by K^+ decreased the damage of the myocardium and loss of ATP [12] and by data on increased sensitivity to oxidative stress of hearts from rats with hyperthyroidism, which are characterized by constantly increased level of oxidative metabolism [34].

The myocardium of 4-month-old rats analyzed after 45-min infusion of 70 μM H_2O_2 displayed a very low rate of mitochondrial respiration in states 3 and 4: 10.8 ± 1.0 and 4.6 ± 0.8 nmol O_2/min per mg protein, respectively, and the value of the respiratory control was 2.2 ± 0.2. Mitochondria isolated from the hearts which were not perfused with H_2O_2 displayed significantly higher level of respiration: 87.4 ± 4.9 and 20.0 ± 1.6 nmol O_2/min per mg protein, respectively, and value of the respiratory control was 4.4 ± 0.2 ($p < 0.001$). These findings are consistent with the earlier found disturbance in the operation of the Krebs cycle under the influence of a similar dose of H_2O_2 [35]. The rate of succinate-dependent generation of superoxide radicals in mitochondria from the H_2O_2-treated hearts (in the presence of antimycin A) was 1.71 ± 0.63 nmol/min per mg protein, which was about twofold higher than in mitochondria from the control hearts: 0.8 ± 0.1 nmol/min per mg protein. Thus, the decrease in the contractility of the myocardium under the influence of H_2O_2 was associated with disorders in respiration and increased production of ROS.

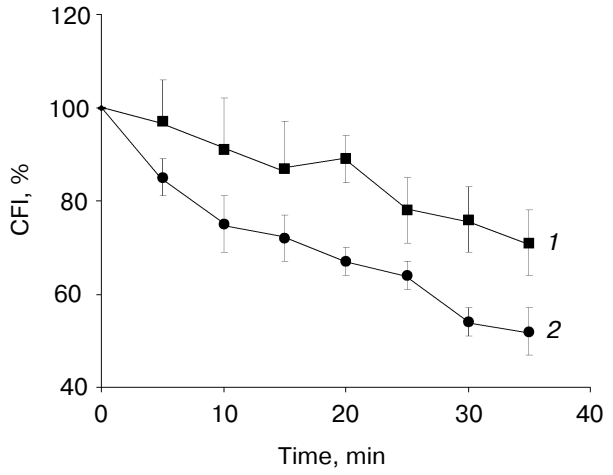

Fig. 2. Changes in the contractile function intensity (product of the heart rate and developed pressure) of isolated hearts from rats of WKY strain during continuous infusion of 70 μM H_2O_2. *1*) Hearts of WKY rats given ubiquinone for 6 weeks; *2*) hearts of control WKY rats. The values are presented as M ± m.

Table 1. Effect of long-term consumption of ubiquinone Q_{10} on contents of coenzymes Q_9, Q_{10}, and α-tocopherol in the blood plasma of rats

Group of rats	Coenzyme Q_9, ng/ml	Coenzyme Q_{10}, ng/ml	α-Tocopherol, μg/ml
WKY (n = 15)	210 ± 15	15.7 ± 1.5	2.95 ± 0.19
WKY + ubiquinone Q_{10} (n = 9)	150 ± 11*	154 ± 17**	3.99 ± 0.25*

* $p < 0.01$ compared to WKY rats not given Q_{10}.
** $p < 0.001$ compared to WKY rats not given Q_{10}.

Assessment of antioxidant status. In the second part of the work, H_2O_2 was added to the perfusate of isolated hearts from WKY rats at the constant concentration of 70 μM, and this resulted in gradual decrease in the contractile function intensity. The contractile function of the hearts from rats given ubiquinone Q_{10} decreased more slowly (Fig. 2), which is consistent with previous data [22, 23] and suggests an increase in the resistance to oxidative stress. Thirty-five minutes after starting the H_2O_2 infusion, the CFI of the hearts from rats treated with ubiquinone Q_{10} was 20% higher than in the control group. The effect of H_2O_2 in the rats treated with ubiquinone Q_{10} was accompanied by increase in the heart rate by 0.4 ± 0.1 Hz, whereas in the control group the heart rate was unchanged.

The level of α-tocopherol in the blood plasma of control rats was significantly higher than the level of coenzyme Q_{10} (Table 1), and this correlated with data from the literature. The prevalence of coenzyme Q_9 compared to coenzyme Q_{10} was also recorded. In the blood plasma of rats treated with ubiquinone Q_{10} the level of coenzyme Q_{10} was increased nearly tenfold and the level of α-tocopherol was also increased (Table 1). The content of coenzyme Q_{10} was shown to be 1.6-fold increased in the cardiac muscle of rats treated with ubiquinone Q_{10} [23], and a similar increase in the level of coenzyme Q_{10} was also recorded in another work [22].

Activities of the main antioxidant enzymes in the myocardium were significantly decreased after oxidative stress induced by intracoronary injection of H_2O_2 (Table 2). And activities of catalase and GSH-Px, which are directly responsible for utilization of H_2O_2, were decreased by 17-25%, whereas the activity of SOD was decreased by 38%. The activities of SOD or GSH-Px

decreased similarly under the influence of H_2O_2 or mixture of hypoxanthine–xanthine oxidase [15, 19], ischemia-reperfusion [36], or adriamycin [37]. Inactivation of SOD and GSH-Px under the influence of high concentrations of H_2O_2 or organic hydroperoxides has been shown in model systems [38, 39]. The inactivation is suggested to be due to rapid destruction of tryptophan residues in the enzyme molecule because of excess accumulation of H_2O_2 [38] due to possible complexing of copper with ROS.

The levels of all enzymes studied after the oxidative stress were significantly higher in the myocardium of animals which were for a long time given ubiquinone Q_{10} than in the myocardium of control rats (Table 2). It was shown earlier [23] that introduction of coenzyme Q_{10} by the same scheme was not associated with increase in activities of antioxidant enzymes. Based on these data combined with data of Table 2, it was suggested that the preliminary introduction into animals of coenzyme Q_{10} should promote the retention of antioxidant enzymes in the myocardium during oxidative stress. It is known that ROS are utilized not only by antioxidant enzymes but also by low-molecular-weight hydro- and lipophilic antioxidants. Contents of ascorbic acid and glutathione decreased under conditions of oxidative stress (reperfusion after ischemia), whereas the levels of lipophilic antioxidants were more resistant [40]. Therefore, in our work just the increased content of coenzyme Q_{10} in the myocardium protected antioxidant enzymes of the myocardium against the damage caused by ROS, in accordance to the reaction:

$$QH_2 + {}^{\cdot}OH \rightarrow {}^{\cdot}QH + H_2O.$$

Weak correlation was found after oxidative stress in the control group between the activities of GSH-Px and catalase and the level of CFI retained by termination of the H_2O_2 infusion (Table 3). Surprisingly, in the rats given ubiquinone Q_{10} a high positive correlation was found between the activities of GSH-Px and SOD and the level of CFI ($r > 0.9$). These findings suggest a crucial role of these enzymes in functioning of the myocardium during oxidative stress and are consistent with the concept that SOD and GSH-Px play the more important role in determination of antioxidant status of the myocardium [20-22] than catalase [41, 42].

Findings of the present work show that changes in the levels of components of the myocardial antioxidant system determine the degree of functional and metabolic disorders caused by ROS. Accumulation of coenzyme Q_{10} can increase the resistance of the myocardium to oxidative stress not only by the direct antioxidant mechanism but also indirectly, due to increased protection of antioxidant enzymes. Ubiquinone seems to be promising for improvement of resistance of the myocardium in diseases associated with development of oxidative stress.

Table 2. Effect of intracoronary infusion of H_2O_2 on contents of the main antioxidant enzymes in the myocardium of WKY rats, some of which were given ubiquinone Q_{10}

Enzyme	WKY		WKY + ubiquinone Q_{10}
	1	2	3
Superoxide dismutase, units per mg protein	46.9 ± 1.6	29.2 ± 3.2*	44.3 ± 1.1**
Glutathione peroxidase, units per mg protein	0.45 ± 0.01	0.34 ± 0.03*	0.46 ± 0.01**
Catalase, nmol/min per mg protein	121 ± 2	99 ± 6*	121 ± 12

Note: Group 1 ($n = 7$), before H_2O_2; groups 2 and 3 ($n = 11$), after H_2O_2.
* $p < 0.01$ compared to group 1.
** $p < 0.001$ compared to group 2.

Table 3. Correlation coefficients between activities of antioxidant enzymes (activity units per mg protein) and value of the contractile function intensity of the myocardium (in % of the initial value) during oxidative stress

Enzyme	WKY	WKY + ubiquinone Q_{10}
Superoxide dismutase	–0.01	0.91
Glutathione peroxidase	0.41	0.94
Catalase	0.38	0.09

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