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OXIDATIVE STRESS AND MYOCARDIAL INJURY: SPIN-TRAPPING AND LOW-TEMPERATURE EPR STUDY

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The aim of this study was to examine the effect of duration of ischemia and ischemic preconditioning on postischemic myocardial function, oxygen-derived free radical production by cardiac mitochondria and functional characteristics of mitochondrial electron carriers. Isolated Wistar rat hearts were perfused aerobically and then subjected to global ischemia of variable duration and reperfusion. The EPR spectra of freeze-clamped hearts were measured at 6-40 K and 243 K. Mitochondria were isolated after aerobic perfusion, ischemia of variable duration or reperfusion. TIRON was used as a spin trap to study the superoxide radical generation in mitochondria. The prolonged (30- and 45-minute) ischemia resulted in an essential enhancement of the superoxide-generating activity of the heart mitochondria, but both the relatively brief (15-minute) episodes of ischemia or ischemic preconditioning before more extended ischemia maintained the contractile function and energy metabolism of the postischemic heart. The ischemic preconditioning depressed the production of superoxide radicals after prolonged ischemia and changed the balance between the free radical intermediates of coenzyme Q differing in stability and contribution to the electron-transfer reactions in mitochondria.

INTRODUCTION

There has been considerable interest in the role of reactive oxygen species in the pathogenesis of myocardial injury under conditions of oxidative stress (Hess & Manson, 1984; McCord, 1984; Thompson & Hess, 1986; Ambrosio & Tritto, 1998; Lucchesi, 1998; Bolli & Marban, 1999). The strong evidence exists that oxygen-derived free radicals play an essential role in irreversible myocardial injury after extended periods of ischemia, but the details of their role in the processes of oxidative damage still remain controversial. During postischemic reperfusion, there are multiple mechanisms that enhance the production of oxygen radicals in ischemia-altered cardiac myocytes (Hearse, 1977; Opie, 1989; Ferrari, Ceconi, Curello, Alfieri & Visioli, 1993; Gross, Kersten & Warltier, 1999). It has been shown that the extent of the myocardial injury depends on the duration of ischemia (Henry, Archer, Nelson, Weir & From, 1993; Khatkevich, Dvoryantsev, Kapelko & Ruuge, 1999), and it is reasonable to expect that the rate of postischemic oxygen radical production in cardiac myocytes varies radically with the duration of ischemia.

In cardiac myocytes, the mitochondria have been identified as the main cellular source of oxygen-derived free radicals (Cadenas, Boveris, Ragan & Stoppani, 1977; Nohl, Jordan & Hegner, 1981; Otani, Tanaka, Inoue, Umemoto, Omomoto, Tanaka, Sato, Osako, Masuda, Nonoyama & Kagawa, 1984; Ledenev, Popova, Konstantinov & Ruuge, 1985; Das, George, Liu & Rao, 1989; Ide, Tsutsui, Kinugawa, Suematsu, Hayashidani, Ichikawa, Utsumi, Machida, Egashira & Takeshita, 2000; Paraidathathu, de Groot & Kehrer, 2001). The sites and the mechanisms of electron leakage from the mitochondrial respiratory chain have received much attention, but the attempts to identify the electron carriers responsible for oxygen radical generation have been of a contradictory nature (Nohl, 1993; Ernster & Dallner, 1995). Several research groups (Ksenzenko, Konstantinov, Khomutov, Tikhonov & Ruuge, 1983; Turrens, Alexandre & Lehninger, 1985; Ruuge, Ledenev, Lakomkin, Konstantinov & Ksenzenko, 1991; Ruuge, Kashkarov, Lakomkin, Timoshin & Vasil'eva, 1997; Ide, Tsutsui, Kinugawa, Utsumi, Kang, Hattori, Uchida, Arimura, Egashira & Takeshita, 1999; Timoshin, Pisarenko, Lakomkin, Studneva & Ruuge, 2000) have applied the methods of EPR spectroscopy and spin-trapping to detect metabolites of the mitochondrial respiratory chain involved in the production of reactive oxygen species in cardiac myocytes. These studies

demonstrated that the free radical intermediates of coenzyme Q in complex III, flavoenzymes and/or iron-sulfur proteins in complex I are the significant source of electron leakage, which takes place during mitochondrial respiration.

It has been shown that the brief periods of perfusate flow interruption before the prolonged ischemia (ischemic preconditioning) reduce essentially the extent of ischemia-associated myocardial injury (Murry, Jennings & Reimer, 1986; Yellon, Baxter, Garcia-Dorado, Heusch & Sumeray, 1998). Although the mechanisms underlying the protective effect are still not entirely understandable (Cohen, Baines & Downey, 2000), ischemic preconditioning is supposed to confer protection to myocardium through a signal-transduction pathway that may be divided into two phases: trigger phase, which occurs before the index ischemia, and the mediator-effector phase, which takes place during the prolonged ischemia. It has been assumed (Vanden Hoek, Becker, Shao, Li & Schumaker, 1998; Das, Engelman & Maulik, 1999) that oxygen free radicals generated in preconditioned cardiac myocytes are triggering the cascade of the protective mechanisms of ischemic preconditioning. There is a strong evidence that the end of the signal-transduction pathway is connected with opening of the mitochondrial ATP-dependent potassium (KATP) channels (Liu, Sato, O'Rourke O'Rourke & Marban, 1998). Once in the preconditioned state, the protein kinases in cardiac myocytes become activated and mediate protection of the ischemic heart. It was supposed that opening of mitochondrial KATP channels act as a trigger for the preconditioned state through the generation of superoxide radicals (Pain, Yang, Critz, Yue, Nakano, Liu, Heusch, Cohen & Downey, 2000).

The paramagnetic metabolites play distinctive role in electron flow in cardiac mitochondria and take important part in production of superoxide radicals by mitochondrial electron carriers. The experiments reported in this paper for isolated perfused rat heart and for isolated mitochondria concern the consequent changes that occur during ischemia and reperfusion in the contractile function of the cardiac muscle and the functional characteristics of the mitochondrial respiratory chain. The methods of low-temperature EPR spectroscopy and spin-trapping allowed us to determine the effects of the duration of ischemia and ischemic preconditioning on the rate of oxygen free radical production in cardiac mitochondria and on the redox equilibrium between mitochondrial electron carriers — coenzyme Q, flavoenzymes and iron-sulfur proteins.

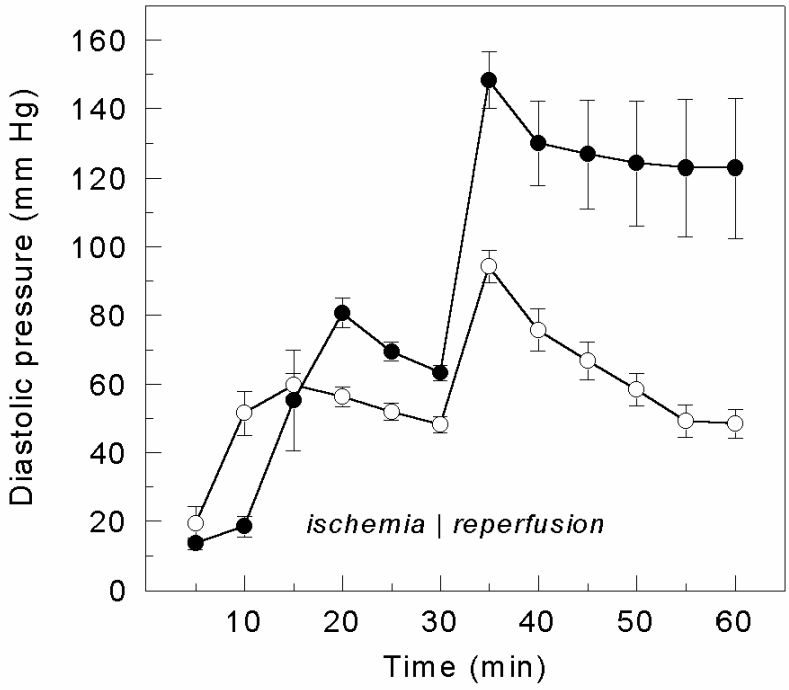

Fig. 1. Time course of the diastolic pressure of the rat hearts during 30-minute global ischemia and following reperfusion in the control group (solid circles) and in the group subjected to ischemic preconditioning (open circles).

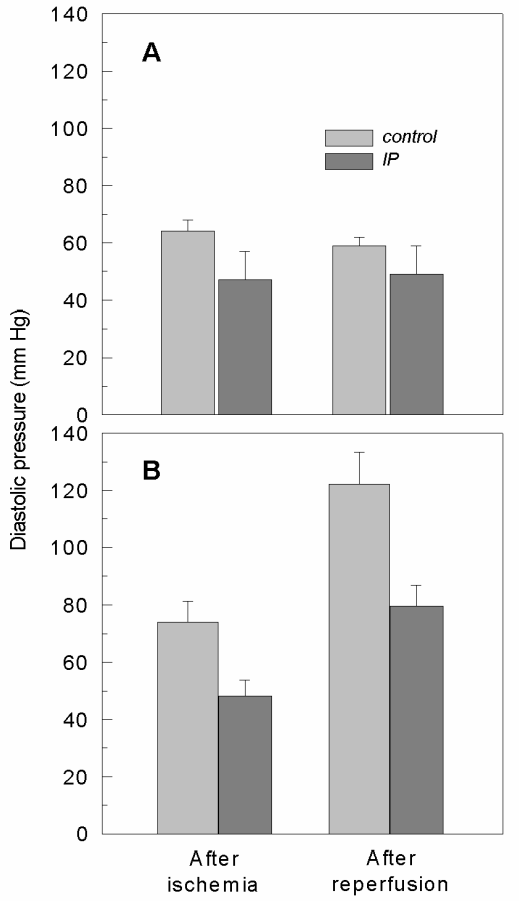

Fig. 2. Diastolic pressure of the control hearts (light gray columns) and preconditioned hearts (gray columns) after global ischemia and reperfusion. (A) 15 min of global ischemia. (B) 30 min of global ischemia.

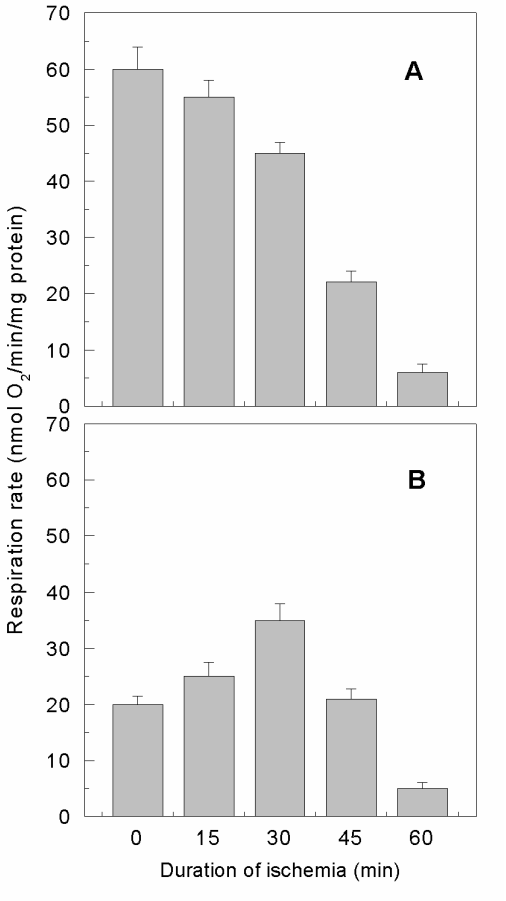

Fig. 3. Respiration rate of mitochondria isolated from the hearts after global ischemia of various duration in state 3 (A) and state 4 (B) of the respiratory chain. Oxygen consumption was measured at a temperature of 25ºC.

MATERIALS AND METHODS

Isolated heart model

Male Wistar rats (250-350 g) maintained on a standard diet were used for this study. Anesthesia was induced and maintained with urethane (1.8-2.0 g/kg), after which heparin (500 U/kg) was administered intravenously. The hearts were removed and perfused normothermically at 37°C with modified Krebs-Henseleit medium by the Langendorff procedure or by the Neely ("working heart") procedure, as described previously (Timoshin, Lakomkin & Ruuge, 1993). Immediately after mounting on the cannula, hearts were perfused for a 30-minute equilibration period. The preconditioned group of hearts was then subjected to four cycles of 5-minute ischemia and 5-minute reperfusion, while the control group of hearts was perfused for an identical time as for the preconditioning protocol. Duration of global, non-flow ischemia at 37°C in EPR experiments with frozen myocardial tissue was 25 min, duration of reperfusion – 3 min and 20 min. At the stated times in the perfusion-ischemia-reperfusion sequence, hearts were freeze-clamped with Wollenberger tongs that had been precooled in liquid nitrogen.

Frozen myocardial tissue was processed for spectroscopic analysis by chopping the wafer of tissue under liquid nitrogen to produce small fragments. These fragments were immediately transferred to the standard EPR sample tube. EPR measurements were performed at 6-40 K or 243 K with X-band E-109E spectrometer (Varian, USA), upon use of microwave power 0.01-200 mW and modulation amplitude 0.4 mT. The standard EPR sample „weak pitch" (10^{13} spins/cm) was used to determine the intensities of free radical signals. The EPR data were compared with the physiological parameters of both control and preconditioned hearts.

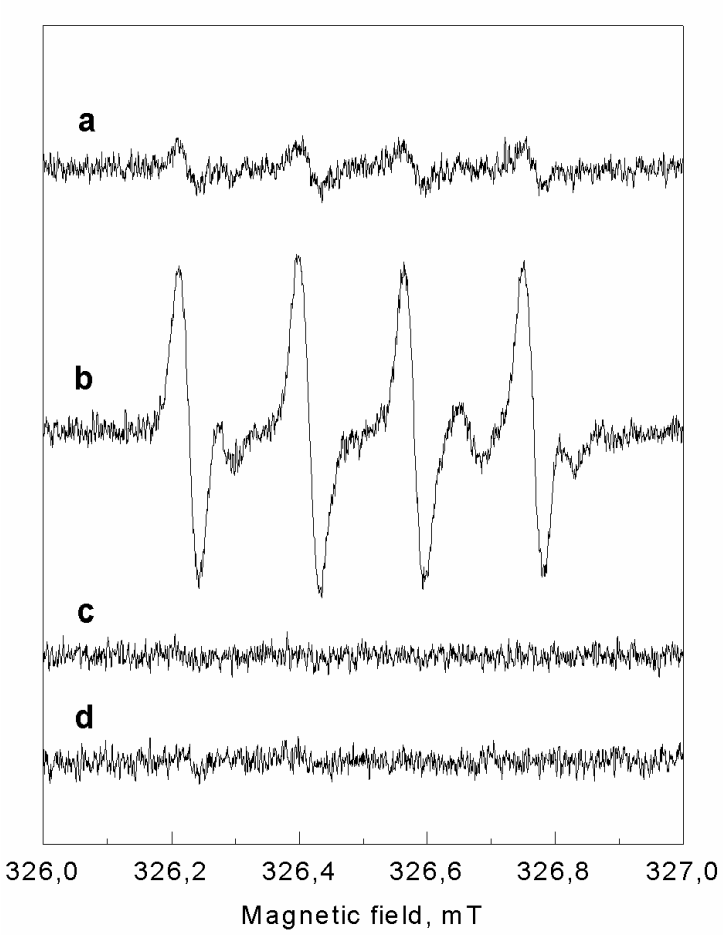

Fig. 4. EPR spectra of TIRON in the suspension of heart mitochondria without inhibitors (a), in presence of antimycin A (b), myxothiazol (c) and superoxide dismutase (d). The spectra were recorded at a temperature of 25ºC, a microwave power of 5 mW and a modulation amplitude of 0.05 mT under conditions of continous aeration after 10 min of adding of mitochondria into incubation medium.

Experiments with isolated mitochondria

The mitochondria were isolated from rat hearts, which were perfused at 37°C by the Langendorff method isovolumically at perfusion pressure 60-80 mm Hg. The perfusion medium contained 118 mM NaCl, 5.9 mM KCl, 3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.5 mM EDTA, 20 mM $NaHCO_3$ and 11 mM glucose (pH 7.4, saturated with 95% O_2 and 5% CO_2). The hearts were divided into two groups: control hearts and hearts subjected to ischemic preconditioning. The hearts that were to be preconditioned were exposed to four episodes of 5 min of global ischemia separated by 5 min of reperfusion. After aerobic perfusion or preconditioning, both group of hearts were exposed to 15, 30, 45 or 60 min of global, no-flow ischemia and then to 30-45 min of reperfusion. At the end of initial perfusion or preconditioning, prolonged ischemia or reperfusion, hearts were quickly removed from the cannula and placed into ice-cold incubation medium.

Mitochondria were isolated from the precooled heart muscle, according to procedure described in (Korkina & Ruuge, 2000). The isolation medium contained 0.3 M sucrose, 10 mM HEPES and 0.25 mM EDTA (pH 7.4). The final mitochondrial precipitate was suspended in the isolation medium with BSA (30-35 mg protein/ml) and stored on ice. Protein content was measured by the biuret method. The incubation medium contained 0.25 M sucrose, 20 mM HEPES, 1 mM EGTA, 4 mM KH_2PO_4 and 3 mM $MgCl_2$ (pH 7.4). The concentration of mitochondrial protein in the mixture was about 1 mg/ml. Respiration was initiated upon the addition of 5 mM succinate (in the majority of experiments) or 5 mM glutamate plus 5 mM malate, and the reaction was started by addition of mitochondria. The rate of oxygen consumption was measured using Clark electrode and YSI 53

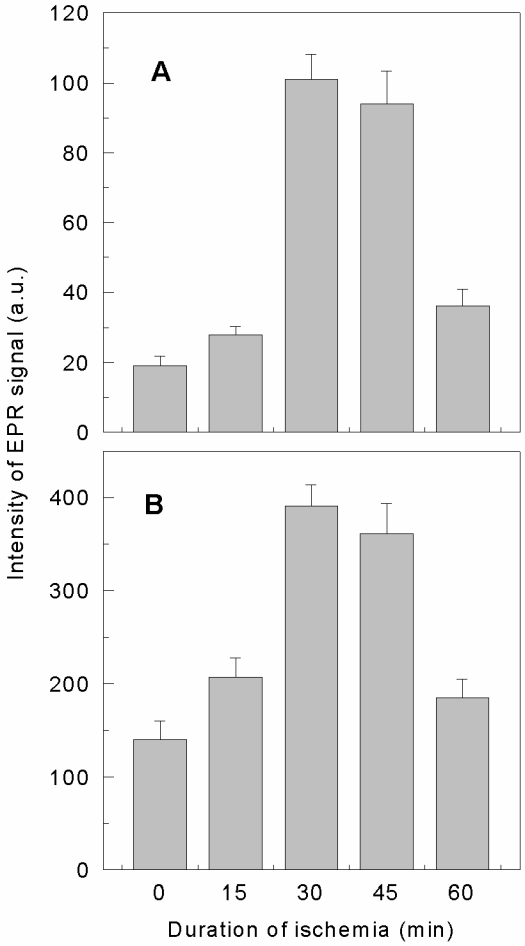

Fig. 5. Intensity of the EPR signals of TIRON in the suspension of mitochondria isolated from the hearts after global ischemia of various duration. (A) without inhibitors; (B) in presence of antimycin A. The EPR spectra were recorded at a temperature of 25ºC under conditions of continous aeration after 10 min of addition of mitochondria into incubation medium.

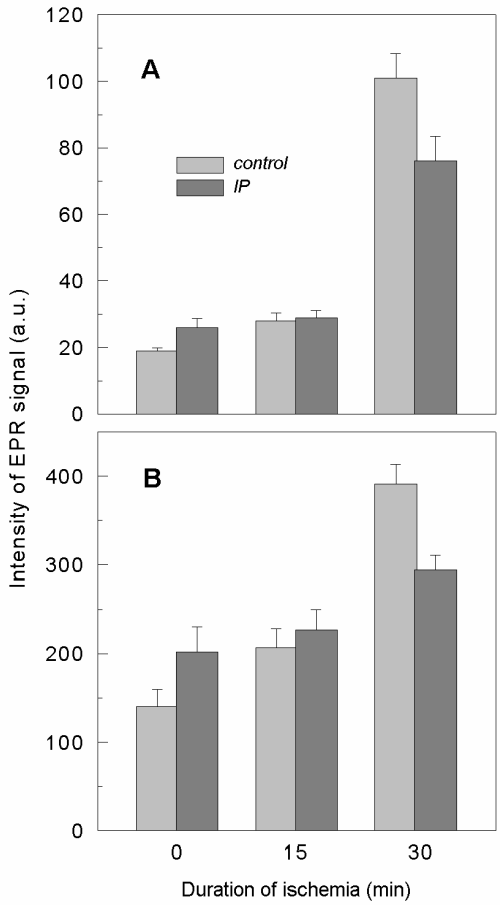

Fig. 6. Intensity of the EPR signals of TIRON in the suspension of mitochondria isolated from the control hearts (light gray columns) and preconditioned hearts (gray columns) after global ischemia. (A) without inhibitors; (B) in presence of antimycin A. The EPR spectra were recorded at a temperature of 25ºC under conditions of continous aeration after 10 min of addition of mitochondria into incubation medium.

oxymeter (Yellow Spring Instruments Inc., USA). Before respiration or EPR measurements, the following reagents were selectively added: 20-250 μM ADP, 5 μM antimycin A, 2 μM rotenone, 5 μM myxothiazol, 3 mM KCN, 0.2-0.4 μM CCCP.

The rate of the superoxide radical production was determined from the EPR spectra of TIRON (4,5-dihydroxy-1,3-benzene-disulfonic acid), an oxygen free radical scavenger (Grigolava, Ksenzenko, Konstantinov, Tikhonov, Kerimov & Ruuge, 1980). The samples were placed into a gas-permeable thin wall Teflon capillary (Norell Inc., USA) and kept during EPR measurements under conditions of continuous aeration. The oxygen content in the incubation medium was determined from the widths of the components of the EPR spectra of TEMPONE-D-^{15}N (4-oxo-2,2,6,6-tetramethyl-piperidine-D16-1-oxyl-^{15}N) (James, Grinberg, Michaels & Swartz, 1995). The EPR spectra were recorded at 25°C with X-band E-109E spectrometer (Varian, USA), upon use of microwave power 5 or 0.5 mW (for TIRON and TEMPONE-D-15N, respectively) and modulation amplitude 0.05 mT. It was shown in our previous study (Ledenev, Konstantinov, Popova & Ruuge, 1986) that the rate of superoxide radical generation in TIRON-containing medium was proportional to the square of the height of the EPR signal of TIRON free radicals. The absolute values of the rate of superoxide production by mitochondria were found using the xanthine oxidase-xanthine model system. The kinetic characteristics of the xanthine oxidase-xanthine system were calibrated

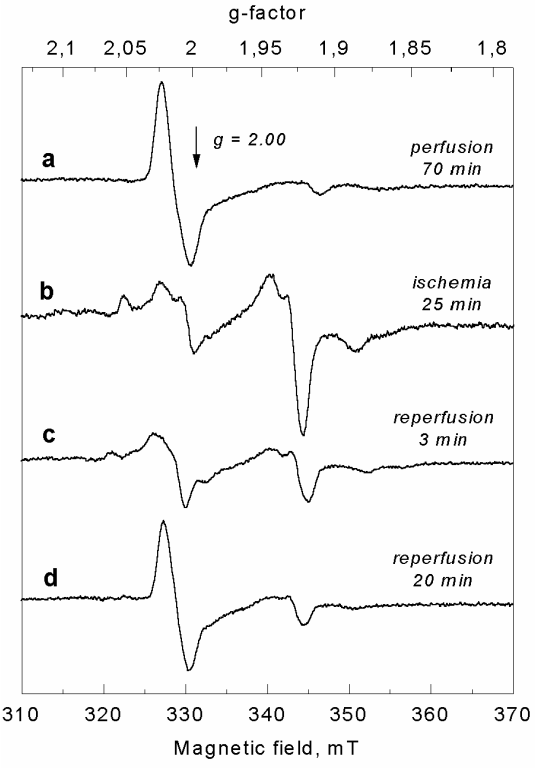

Fig. 7. EPR spectra of the hearts freeze-clamped after min of aerobic perfusion (a), 25 min of global ischemia (b), 3 min of reperfusion (c) and 20 min of reperfusion (d). The spectra were recorded at a temperature of 12.5 K, a microwave frequency of 9.15 GHz, a microwave power of 1 mW and a modulation amplitude of 0.4 mT.

with DU-8B spectrophotometer (Beckman, USA) by measuring the rate of cytochrome c reduction by superoxide radicals.

The reagents used in the experiments were produced by Aldrich (USA), ICN (USA), Serva (FRG) and Sigma (USA).

Statistics

Data are presented as means ± SE. The two-population Student's t-test and the one-way ANOVA test were used for comparison of data. A value of $p < 0.05$ the differences between the experimental data were considered statistically significant. The data in Fig. 9 were fitted using Origin 6.0 (MicroCal, USA) program.

RESULTS AND DISCUSSION

Cardiac function

After the onset of global ischemia, the left ventricle diastolic pressure, which in isovolumic mode reflects the degree of myocardial fiber tension, began to increase and reached its maximum in the control group within 15-20 min (Fig. 1). The end-ischemic level of the diastolic pressure depended on the duration of ischemia, and after 15 min of ischemia its value was significantly lower than after 30 min of ischemia (Fig. 2). At the initial period of reperfusion, a notable rise in the diastolic pressure was observed after 30 min of ischemia, and the increased level of the diastolic pressure was measured during whole period of reperfusion (Fig. 1). At the end of reperfusion, its value was higher than in case of 15-minute ischemia (Fig. 2).

The process of ischemic preconditioning did not modify the functional parameters of the heart. The left ventricle diastolic and developed pressures, heart rate and aortic perfusion pressure in the preconditioned group did not differ significantly from parameters in the group not subjected to ischemic preconditioning. After 15 min of ischemia, no crucial functional difference between the control group and the group subjected to ischemic preconditioning was observed during reperfusion. The hearts in both groups resumed a normal rhythm, and the values of the degree of recovery of developed pressure, diastolic pressure and aortic perfusion pressure were alike (data not shown). The functional difference between the groups became prominent in case of more extended (30-minute) ischemia. The diastolic pressure began to increase in the preconditioned group sooner than in the control group and reached its maximum at 10 min, when it still continued to raise in the control group (Fig. 1). By the end of 30-minute ischemia and during reperfusion, the level of diastolic pressure in the hearts subjected to ischemic preconditioning was noticeably lower than in the control group.

It has been shown by us and other investigators (Khatkevich *et al.*, 1999; Moolman, Genade, Winterbach, Williams & Lochner, 1995) that for the isolated rat hearts the duration of the ischemic episode from 15 to 30 min is critical for initiation of the irreversible myocardial injury. In our experiments, ischemic preconditioning exerted its protective effect that was shown by considerably higher recovery of contractile function, reduced level of post-perfusion contracture and better restoration of energy metabolism only after 25-30 min of global ischemia.

Functional activity of mitochondria

The dependence of the mitochondrial respiration rate on the duration of global ischemia of rat hearts is shown in Fig. 3. The data given in Fig. 3 clearly demonstrate that the duration of ischemia had substantial effect on the rate of oxygen consumption by the mitochondria isolated from the post-

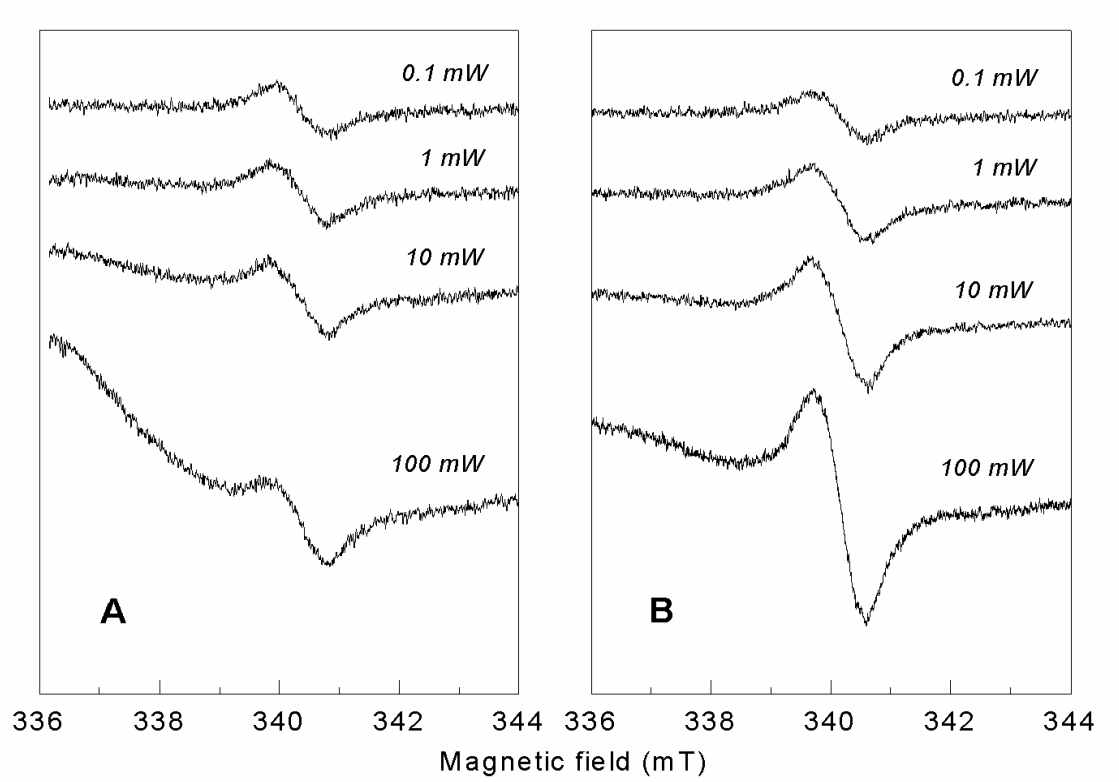

Fig. 8. Free radical EPR signals of the control hearts freeze-clamped after aerobic perfusion (A) and hearts subjected to ischemic preconditioning (B). The spectra were recorded at various microwave power levels and at a temperature of 40.5 K.

postischemic myocardial tissue. The slight changes in the rate of oxygen consumption by the mitocondria isolated after 15 min of ischemia versus control mitochondria were not statistically significant. The further increase of the ischemic period (30, 45 and 60 min in Fig. 3) resulted in the considerable reduction of the respiration rate in the state 3 (in case of 45- and 60-minute ischemia also in the state 4) and brought to the complete loss of the respiratory control.

Ischemic preconditioning considerably preserved the normal mitochondrial function after prolonged, 30-minute myocardial ischemia, but it did not have notable effect on the respiration rate in both state 3 and state 4 of the mitochondrial respiratory chain in case of less extended, 15-minute ischemia (data not shown).

Superoxide radical formation by mitochondria

It has been shown (Grigolava *et al.*, 1980) that TIRON can be oxidized by superoxide radicals into a relatively stable free radicals. Previously, we demonstrated that the rate of superoxide anion-radical formation in a sample was proportional to the square of the intensity of the EPR signal of TIRON (Ledenev *et al.*, 1986). Fig. 4 displays the typical EPR spectra of TIRON in the suspension of heart mitochondria. At baseline conditions, only small EPR signals appeared in the presence of succinate as a substrate for the mitochondrial fraction obtained from the normal heart after aerobic perfusion (Fig. 4, spectrum a). The mitochondria oxidizing succinate in state 4 continued to generate superoxide radicals throughout the whole experiment, if the mitochondrial suspension was within a gas-permeable Teflon capillary in the air atmosphere. When mitochondria were treated with antimycin A, which selectively blocks the electron transport at the specific site in the "protonmotive Q cycle" of complex III (Trumpower, 1990), the intensity of the EPR signal of TIRON increased remarkably (Fig. 4, spectrum b). At the same time, the recordable EPR signal disappeared completely in the presence of myxothiazol (Fig. 4, spectrum c) – another specific inhibitor of the Q cycle. In the presence of succinate as a substrate, the rate of superoxide generation by isolated cardiac mitochondria was 0.01 ± 0.004 nmol $O_2^{\bullet -}$/min/mg protein at a temperature of 25ºC. If the electron transport in the cytochrome bc_1 segment (complex III) was inhibited by antimycin A, the rate of succinate-dependent superoxide radical formation in mitochondria was much higher and equaled to 0.51 ± 0.07 nmol $O_2^{\bullet -}$/min/mg protein. To further

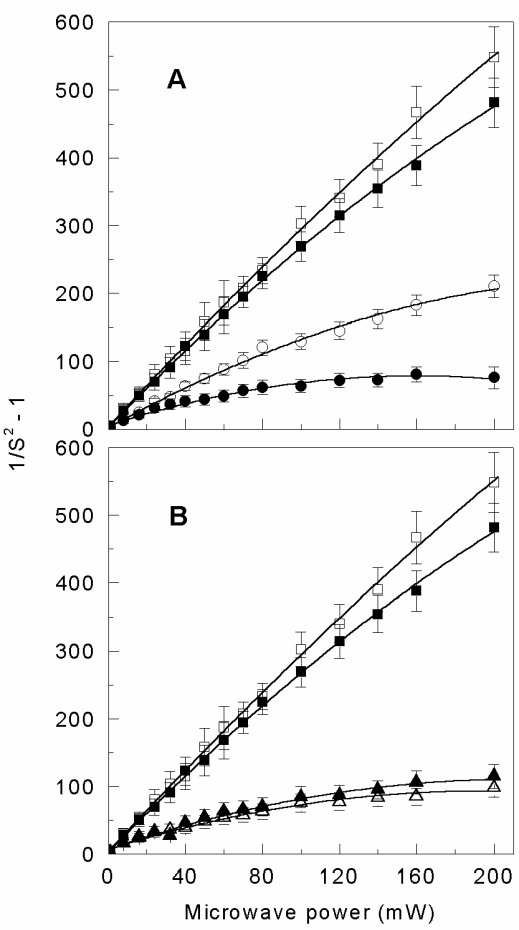

Fig. 9. Dependence of the expression $(1/S^2 - 1)$ on the microwave power level for the saturation factor S (Timoshin et al., 1993) of the free radical EPR signals. The control hearts (open symbols) and preconditioned hearts (solid symbols) were freeze-clamped before global ischemia (circles), after global ischemia (squares) and reperfusion (triangles). The spectra were recorded at various microwave power levels and at a temperature of 243 K.

confirm the succinate-dependent generation of $O_2^{\bullet-}$ by electron carriers of complex III, the mitochondria were treated with superoxide dismutase. Fig. 4 (spectrum d) shows that the EPR signal of TIRON was entirely (by 90-95%) attenuated in the presence of superoxide dismutase. These results indicate that the functional block of complex III is capable of producing $O_2^{\bullet-}$, which is consistent with the findings of previous studies (Ksenzenko *et al.*, 1983; Turrens *et al.*, 1985; Ide *et al.*, 1999). Taken together with the results of other investigations (Takeshige & Minakami, 1979; Turrens & Boveris, 1980; Ruuge, Ledenev, Lakomkin, Konstantinov & Ksenzenko, 1991; Ide *et al.*, 1999), it is reasonable to conclude that both complex I and complex III of the mitochondrial respiratory chain are important sources of highly reactive oxygen free radicals in cardiac myocytes.

To determine whether the superoxide radical production by mitochondrial electron carriers is enhanced after prolonged ischemia, the isolated mitochondria were reacted with succinate (without inhibitors and in presence of antimycin A), and the corresponding EPR spectra of TIRON were recorded. The dependence of the intensity of the EPR signals of TIRON on the duration of myocardial global ischemia is shown in Fig. 5. Comparatively small EPR signals appeared for mitochondria isolated from the control hearts and mitochondria from the hearts subjected to 15 min of ischemia. In mitochondria isolated after 30 and 45 min of ischemia, the magnitude of $O_2^{\bullet-}$ production was much higher (Fig. 5). The superoxide radical production by complex III was maximally enhanced in case of 30-minute ischemia and equal to 0.26 ± 0.02 and 3.95 ± 0.2 nmol $O_2^{\bullet-}$/min/mg protein in the absence and presence of antimycin A, respectively. As in case of the study of mitochondrial respiration (see above), more extensive, 60-minute ischemia turned up to be critical for the isolated rat hearts and caused a substantial decrease of the rate of superoxide radical generation in mitochondria.

The process of ischemic preconditioning, which preserved the contractility and mitochondrial function after prolonged, 30-minute myocardial ischemia (see above), considerably reduced the rate of superoxide radical production by mitochondria isolated after 30 min of ischemia (Fig. 6). However, we did not find out a beneficial effect of ischemic preconditioning on recovery of contractile function and energy metabolism, when the duration of myocardial ischemia was limited to 15 min. As it is shown in Fig. 6, the rate of superoxide radical generation in mitochondria isolated after 15 min of ischemia from the preconditioned hearts did no differ significantly from the rate of superoxide formation in mitochondria isolated from the control hearts. At the same time, we established a small but statistically significant increase of the rate of superoxide radical production by heart mitochondria after ischemic preconditioning. It has been assumed (Vanden Hoek, *et al.*,1998; Das *et al.*, 1999; Pain, Yang, Critz, Yue, Nakano, Liu, Heusch, Cohen & Downey, 2000) that superoxide radicals generated in preconditioned cardiac myocytes are triggering the cascade of the protective mechanisms of ischemic preconditioning.

Paramagnetic metabolites in mitochondria

The low-temperature EPR spectra of the rat hearts that were freeze-clamped after aerobic perfusion, global non-flow ischemia or restoration of oxygenated perfusate flow are shown in Fig. 7. The microwave power, 1 mW, and temperature, 12.5 K, were appropriate for recording the EPR spectra of the mitochondrial iron-sulfur proteins (Beinert, 1978; Ruuge *et al.*, 1997). However, these conditions were no suitable for measuring the EPR signals of the free radical intermediates of coenzyme Q and flavoproteins, and higher temperatures of 40 K (Fig. 8) and 243 K (Konstantinov & Ruuge, 1977; Ruuge *et al.*, 1991) were additionally used in our experiments. It is obvious from Fig. 7 and Fig. 8 that the low-temperature EPR spectra of the hearts freezed after aerobic perfusion, ischemia or reperfusion are superpositions of spectra from different free radical centers and iron-sulfur clusters in mitochondria. The overlapping low-temperature EPR spectra could be successfully resolved by changing both the microwave power and temperature (Beinert, 1978), and a detailed analysis of the values of g-factor, line-widths and relaxation parameters (data not shown) allowed us to assign the components of the low-temperature spectra to distinctive Fe-S centers of complexes I, II and III of the mitochondrial respiratory chain and free radical intermediates of coenzyme Q and flavoproteins. As seen in Fig. 7, the EPR spectra of the hearts fixed in aerobic conditions (during initial perfusion and reperfusion) display a predominant signal from the S3 high-potential iron-sulfur protein of succinate-coenzyme Q reductase (complex II), exceptionally paramagnetic in oxidized state. At the same time, the EPR spectra of the oxygenated myocardium also contained rather weak signals from other Fe-S centers of the respiratory chain that could be paramagnetic only in reduced form. The hearts that were fixed during aerobic perfusion and reperfusion demonstrate free radical EPR signals with a line-width of 0.84 ± 0.02 mT and a gaussian line-shape (Fig. 8; Ruuge *et al.*, 1997), which are typical for the free radicals of coenzyme Q. It means that the myocardial free radical signal in aerobic conditions seems to be mainly due to coenzyme Q.

The global ischemia caused principal changes in the redox state of the EPR-detectable mitochondrial electron carriers – coenzyme Q, flavoproteins and Fe-S centers. The free radical EPR signal of the ischemic heart became saturated at essentially lower microwave power level than the free radical signal of the oxygenated myocardial tissue (Fig. 9). Its line-width became equal to 1.28 ± 0.03 mT, which was characteristic for flavine free radicals (Ruuge *et al.*, 1991; Timoshin *et al.*, 1993). The ischemia essentially increased the contribution of flavoproteins to the free radical signal and radically changed the redox state of the iron-sulfur proteins in cardiac mitochondria. It is evident from Fig. 7 that the EPR spectrum of the ischemic myocardium contains overlapping signals from the majority of mitochondrial Fe-S clusters paramagnetic in reduced form. At the same time, it appears that some amount of the S3 centers of complex II still remained oxidized.

It was logical to assume that considerable changes in the redox equilibrium of coenzyme Q and other electron carriers in ischemia-altered mitochondria might have a straight link with an enhanced oxygen-derived free radical production in reperfused myocardium. It has been shown (Ksenzenko *et al.*, 1983; Nohl, 1993; Ernster & Dallner, 1995) that one of the mechanisms of superoxide radical generation in cardiac mitochondria is the auto-oxidation of the semiquinones of coenzyme Q formed in the "protonmotive Q cycle" in the bc_1 segment (complex III) of the respiratory chain. The mitochondrial NADH-coenzyme Q reductase (complex I) is also involved in superoxide formation (Cadenas *et al.*, 1977; Ide *et al.*, 1999), but the electron carriers of complex I that are capable to reduce the molecular oxygen are not yet undoubtedly identified.

The free radical intermediates of coenzyme Q ($Q^{\bullet-}$ and $QH^{\bullet}$) play exceptional role in coupling of electron flow and oxidative phosphorylation in mitochondria and take important part in production of superoxide radicals by mitochondrial electron carriers. The experimental data presented above clearly demonstrated the protective effect of ischemic preconditioning on the contractile function and energy metabolism of the hearts subjected to prolonged ischemia and reperfusion. It was shown that mitochondria isolated after prolonged ischemia from the preconditioned hearts produced much less superoxide radicals than mitochondria isolated from the control hearts. At the same time, ischemic preconditioning initiated a slightly higher superoxide production by mitochondria, which was supposed to trigger the cascade of protective mechanisms in cardiac myocytes (Van den Hoek *et al.*,1998; Das *et al.*, 1999; Pain *et al.*, 2000). Fig. 8 presents the typical free radical EPR signals of the control heart and the preconditioned heart that were recorded at different levels of microwave power at a temperature of 40 K. The corresponding microwave saturation curves for control and preconditioned hearts measured at 243 K are shown in Fig. 9. It is evident from Fig. 8 and 9 that ischemic preconditioning changes significantly the

relaxation parameters of the myocardial free radical EPR signal, which in aerobic conditions is mainly due to the semiquinones of coenzyme Q. Previously it has been shown (Ruuge *et al.*, 1991) that in oxygenated myocardium part of coenzyme Q free radicals forms a complex with paramagnetic S3 high-potential iron-sulfur protein of complex II, and, as a result, the relaxation rate of this fraction of free radical species remarkably increases. It means that the process of ischemic preconditioning is capable to modify the equilibrium between different fractions of semiquinones of coenzyme Q, which apparently enhances the direct interaction of coenzyme Q free radicals with oxygen.

Conclusions

Oxidative stress has been found to be an important etiological factor in the pathogenesis of the cardiovascular diseases. Because the mitochondria are the source of reactive oxygen species and play a central role in energy metabolism and apoptosis (Cook & Poole-Wilson, 1999), it has been expected that mitochondrically generated oxygen free radicals are a necessary cause of the oxidative damage of cardiac myocytes. This hypothesis is supported by our results on isolated perfused rat hearts and mitochondria, which have shown the severe effects of myocardial ischemia on cardiac function, energy metabolism and oxygen radical generation in mitochondria. In this study, ischemia-induced losses in contractile function and mitochondrial respiration were found to be linked with significantly enhanced level of oxygen free radicals and changes in redox equilibrium of mitochondrial electron carriers. The ischemic preconditioning preserved the normal mitochondrial function during prolonged ischemia and reduced the rate of production of reactive oxygen species, which might play an important role in the recovery of contractile function and energy metabolism of the postischemic heart. The results suggest that TIRON may be useful for the detection of oxygen radicals in isolated cardiac mitochondria.

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