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REDOX STATUS OF CARDIAC CELLS. FERRITIN, REACTIVE OXYGEN AND NITROGEN SPECIES

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It has been demonstrated that ferritin stimulates the free-radical oxidation of rat heart mitochondria induced by tert-butyl hydroperoxide, at the same time, the dinitrosyl-iron complexes (DNIC) and combination of S-nitrosoglutathione with reduced glutathione inhibit effectively lipid peroxidation cascades in mitochondrial membranes. The antioxidant effect of DNIC was confirmed by their interaction with tert-butyl alkoxyl and alkylperoxyl radicals, superoxide radicals and peroxynitrite. In condition of tert-butyl free radical generation, DNIC were rapidly destructed, the ferritin inhibiting this process. The destruction of DNIC could be also observed, when superoxide radicals were generated by mitochondrial respiratory chain or xanthine-xanthine oxidase system. DNIC effectively inhibited the destruction of β-carotene induced by peroxynitrite. The nature of destruction of DNIC under the action of peroxynitrite indicates on the possibility of formation of intermediates. Evidently, the mitochondrial enzymatic systems are able to contribute to the regeneration of these intermediates into initial nitrosyl form. Their formation in the reaction system containing mitochondria, ferritin, S-nitrosoglutathione with reduced glutathione points to the interconnection of the metabolism of iron, superoxide and DNIC. Apparently, the balance between antioxidant and prooxidant reactions in cardiac cells depends on the equilibrium between DNIC, ferritin and "free" Fe ions.

INTRODUCTION

Reactive oxygen and nitrogen species (ROS and RNS) have been implicated in the heart tissue injury that follows myocardial ischemia and reperfusion, atherosclerosis and neurodegenerative diseases (Lucas & Szweda, 1998; Droge, 2002; Ruuge, Zabbarova, Korkina, Khatkevich, Lakomkin & Timoshin, 2002; Ferdinandly & Schulz, 2003; Becker, 2004; Lankin, Antonovsky & Tikhase, 2004; Leonard, Harris & Shi, 2004; Kruszewski, 2004; Schulz, Kelm & Heusch, 2004). Imbalance between production of ROS and RNS and antioxidant defense can result in oxidative stress. However, the mechanisms for the enhanced ROS and RNS generation under the conditions of oxidative stress as well as subcellular targets of their attack remain unclear. It is now obvious that the signal transduction pathways that control cell growth, differentiation and death are responsive to changes in the redox status of the cell. It has been shown that reactive oxygen and nitrogen species play an important role in signal transduction and participate in phenomenon what is known as "redox cell signaling" (Leonard *et al.*, 2004; Brookes, Levonen, Shiva, Sarti & Darley-Usmar, 2002; Droge, 2002; Kim, Ohshima, Pediaditaks & Lemasters, 2004; Schulz *et al.*, 2004). The metals ions of variable valence, especially "free" iron and iron in numerous physiological complexes, play an important role in production of ROS – superoxide, hydroxide and lipid free radicals. It is also known that the metabolism of iron in cells is largely regulated by nitric oxide (NO), and the dinitrosyl-iron complexes with thiol ligands – $(RS^-)_2$-Fe^+-$(NO^+)_2$ – play a special role in this regulation (Kennedy, Antholine & Beinert, 1997; Watts & Richardson, 2002; Kruszewski, 2004). The dinitrosyl-iron complexes (DNIC) represent a metabolic form of NO, which assure its stabilization and transport in organism (Vanin, Stukan & Manukhina, 1996**;** Ueno, Suzuki, Fujii, Vanin & Yoshimura, 2002). It has been shown that DNIC can participate in energy-dependent export of nonheme iron from cells (Watts & Richardson, 2002). Inactivation of the aconitase – an iron-sulfur protein – under the action of nitric oxide leads to formation of DNIC and inhibition of the ferritin synthesis on the translation level (Kennedy *et al.*, 1997). At the same time, NO, glutathione and superoxide promote release of iron from ferritin – the main depot of iron in the cells (Biemond, van Eijk, Swaak & Koster, 1984; Boyer, Grabil & Petrovich; 1988; Reif & Simmons, 1990; Watts & Richardson, 2002). On the other hand, the participation of "labile iron pool", thiols and superoxide radicals in Haber-Weiss and Fenton reactions can stimulate the processes of free-radical derived oxidation (Reif, 1992; Minotti, Mancuso, Frustaci, Mordente, Santini & Calafiore,

1996; Arosio & Levi, 2002; Becker, 2004; Lankin *et al.*, 2004; Leonard *et al.*, 2004).

The interaction of glutathione and other thiols with reactive nitrogen species brings to the formation of S-nitrosothiols, which produce DNIC as a result of the reaction with Fe^{2+} ions (Vanin *et al.*, 1996; Shumaev, Petrova, Zabbarova, Vanin, Topunov, Lankin & Ruuge, 2003). In different model systems, it has been established that both mononitrosyl- and dinitrosyl-iron complexes and S-nitrosoglutathione (GSNO) possess antioxidant properties (Rubbo, Radi, Trujillo, Telleri, Kalyanaraman, Barnes, Kirk & Freeman, 1994; Gorbunov, Yalowich, Gaddam, Thampaatty, Ritov, Kisin, Elsauyed & Kagan, 1997; Vanin, Huisman, Stroes, de Ruijter-Heijstek, Rabelink & van Faassen, 2001; Shafer, Wang, Kelley, Cueno, Martin & Buetter, 2002; Shumaev, Ruuge & Vanin, 2004), while the other NO metabolites – peroxynitrite ($ONOO^-$) and nitroxyl anion (NO^-) – expose cytotoxic action (Beckman & Koppenol, 1996; Ma, Gao, Liu, Lopez, Christopher, Fukuto, Wink & Feelish, 1999; Valdez, Alvarez, Arnaiz, Schopfer, Carreras, Poderoso & Boveris, 2000; Lankin *et al.*, 2004). The dual role of reactive nitrogen species was clearly demonstrated in the study of the influence of RNS on the ischemic injury of myocardium – nitroxyl anions increased, but GSNO essentially decreased the damage of the myocardial tissue (Ma *et al.*, 1999). It is necessary to outline that the decisive importance of ROS, RNS and Fe ions as cytotoxic and regulatory agents was demonstrated actually in the case of ischemia and subsequent reperfusion of the myocardium (Zeltcer, Berenstein, Kitrossky, Chevion & Samuni, 2001; Brookes *et al.*, 2002; Ferdinandly & Schulz, 2003; Becker, 2004; Kruszewski, 2004; Schulz *et al.*, 2004; Kim *et al.*, 2004).

In mitochondria, electrons from reduced substrates are passed through the complexes of the electron transport chain to oxygen, the proton motive force set up by proton pumping drives protons back through the ATP synthase forming ATP. However, there are side reactions that are relevant here – electrons may leak from the respiratory chain and react inappropriately with oxygen to form superoxide. In cardiomyocytes, mitochondria are the main source of reactive oxygen species (Cadenas & Davies, 2000; Ruuge *et al.*, 2002). Mitochondria contain NO synthase (mtNOS) and ferritin, which serves as a source of iron for a number of mitochondrial Fe-S proteins (Arosio & Levi, 2002; Giulivi, 2003; Kanai & Peterson, 2004). In literature, different mechanisms of GSNO formation in mitochondria have also been considered (Steffen, Sarkela, Gybina, Steele, Trasseth, Kuehl & Giulivi, 2001). Due to simultaneous generation of $O_2^{\bullet-}$ and NO in mitochondria, highly toxic peroxynitrite is formed what causes the damage of these organelles (Valdez, Alvarez, Arnaiz, Schopfer, Carreras, Poderoso & Boveris, 2000; Riobo, Clement, Melani, Boveris, Cadenas, Moncada & Poderoso, 2001). It seems reasonable to suppose that the intensity of the processes of free-radical mediated oxidation depends on the rate of superoxide and nitric oxide generation in mitochondria, in that way regulating exchange of iron and formation or destruction of DNIC.

The experiments reported in this paper concern the effects of DNIC, GSNO and ferritin on the lipid peroxidation of biological membranes and the interaction of dinitrosyl-iron complexes with reactive oxygen species.

MATERIALS AND METHODS

Reagents

In this study, we used reduced glutathione from Calbiochem (USA), sodium nitrite, HEPES, ferritin from horse spleen, metmyoglobin from horse heart, xanthine and xanthine oxidase from Sigma (USA), DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide) from OXIS (USA), β-carotene from BASF (Germany) and tert-butyl hydroperoxide from Merck (Germany). Other reagents used were from Sigma (USA).

Synthesis of GSNO, dinitrosyl-iron complexes and peroxynitrite

GSNO were synthesized by mixing equimolar amounts of $NaNO_2$ and GSH in an acidic medium at a molar ratio 3:2 as it was described earlier (Shumaev *et al.*, 2004). Dinitrosyl-iron complexes with glutathione in diamagnetic dimeric form were obtained by treating the $FeSO_4$ and GSH solutions with gaseous NO in a Thunberg tube in 10 mM HEPES buffer (pH 7.4) at a molar ratio 1:2 as described by Vanin *et al.* (2001). To synthesize peroxynitrite, cooled 1 M $NaNO_2$ and 1 M H_2O_2 solutions in 0.3 M H_2SO_4 were mixed, and an equal volume 1.4 M NaOH was added to the mixture obtained (Shumaev *et al.*, 2003). GSNO, DNIC and peroxynitrite preparations were stored by snap freezing of aliquots (50 μl) in liquid nitrogen. The concentrations of GSNO and peroxynitrite were estimated by optical absorbance at 338 and 302 nm, respectively. The concentrations of DNIC were determined by EPR spectroscopy. Since dimeric form of DNIC used in this study is not EPR detectable, it was converted into the monomeric paramagnetic form. For that, cysteine was added to

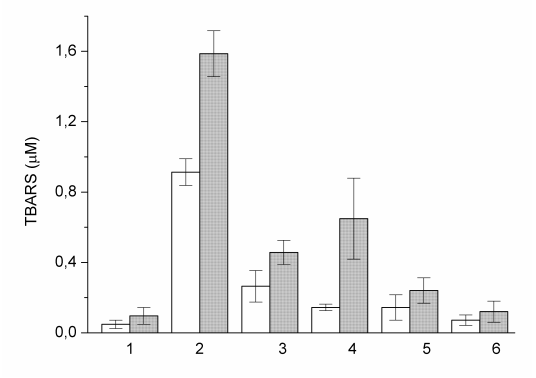

Fig. 1. Accumulation of TBARS during free-radical mediated oxidation of rat heart mitochondria. Reaction mixture contained: mitochondria (0.5 mg protein/ml), 100 mM phosphate buffer (pH 7.4) and 400 μM tert-butyl hydroperoxide. (1) – without additions; (2) – (1) + 100 μM metmyoglobin; (3) – (2) + 200 μM GSH; (4) – (2) + 100 μM GSNO; (5) – (2) + 50 μM DNIC; (6) – (2) + 100 μM GSNO and 200 μM GSH. Gray colomns: + 2.5 μg/ml ferritin.

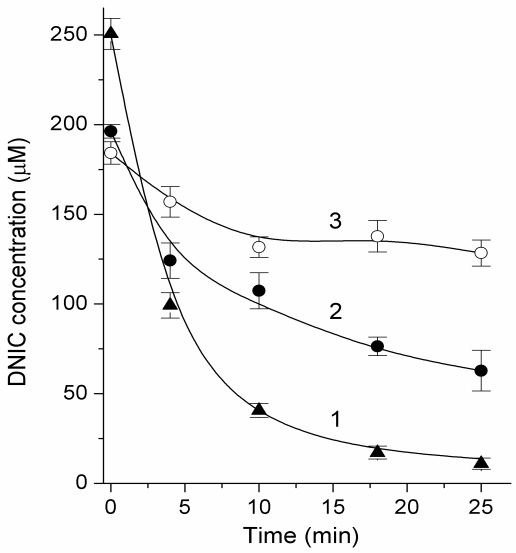

Fig.2. Kinetics of the destruction of DNIC monitored by changes in the EPR signal intensity. Reaction mixture contained: 250 μM DNIC, 400 μM tert-butyl hydroperoxide and 50 μM metmyoglobin (1); 250 μM DNIC, 50 μM metmyoglobin and 1.5 μg/ml ferritin (2); 250 μM DNIC and 1.5 μg/ml ferritin (3). Before the EPR spectra were recorded, 5 mM cysteine was added to the samples.

added to the studied solutions in the molar ratio DNIC/cysteine = 1:25, and this resulted in formation of monomeric EPR detectable dinitrosyl-iron complexes with cysteine.

Isolation of mitochondria

Mitochondria were isolated from the precooled rat hearts, according to procedure described earlier (Ruuge et al., 2002). The isolation medium contained 0.3 M sucrose, 10 mM HEPES buffer (pH 7.4) and 0.25 mM EDTA. The final mitochondrial precipitate was suspended in the isolation medium with 2 mg/ml BSA and stored on ice. Protein content was measured by the biuret method.

Peroxidation of mitochondrial preparations

The mitochondria (0.5 mg protein/ml) were incubated at 37°C with the 0.4 mM tert-butyl hydroperoxide in 100 mM phosphate buffer (pH 7.4). The free-radical mediated peroxidation of mitochondrial lipids was initiated by addition of 0.1 mM metmyoglobin and/or 2.5 μg/ml ferritin. The samples (1 ml) were incubated during 2 hours at temperature 37ºC. After that, peroxidation was stopped by adding into the samples 100 μM DTPA (diethylenetriaminopentaacetic acid) and 10 μM butylated hydroxytoluene.

Estimation of the level of mitochondrial membrane oxidation

Lipid peroxidation products were determined by reaction with 2-thiobarbituric acid as thiobarbituric acid reactive substances (TBARS). Trichloroacetic acid (2.5 ml of 20% solution) and 2-thiobarbituric acid (1 ml of 0.67% solution) were added to 0.5 ml of incubation medium containing mitochondria. The color of the complexes of 2-thiobarbituric acid with TBARS was developed in a water bath at 100°C for 30 min. After cooling with tap water to room temperature, 4 ml of n-butanol was added, and the sample was shaken vigorously. After centrifugation, the color of the butanol layer was measured at 535 nm (ε=1.56×10^5 M^{-1}cm^{-1}).

EPR Measurements

The EPR spectra were recorded using a E-109E spectrometer from Varian (USA) under the following conditions: temperature ~25°C, microwave power 10 mW, microwave frequency 9.15 GHz, modulation amplitudes 0.1 or 0.4 mT for DEPMPO spin-adducts or DNIC, respectively. Before measurements, most samples were placed into glass capillaries (inside diameter ~0.8 mm). In experiments, which involved generation of superoxide, the samples were placed into gas-permeable capillaries PTFE 22 (inside diameter 0.635 mm, wall thickness 0.051 mm) from Zeus Industrial Products, Inc. (USA) and aerated during EPR measurements.

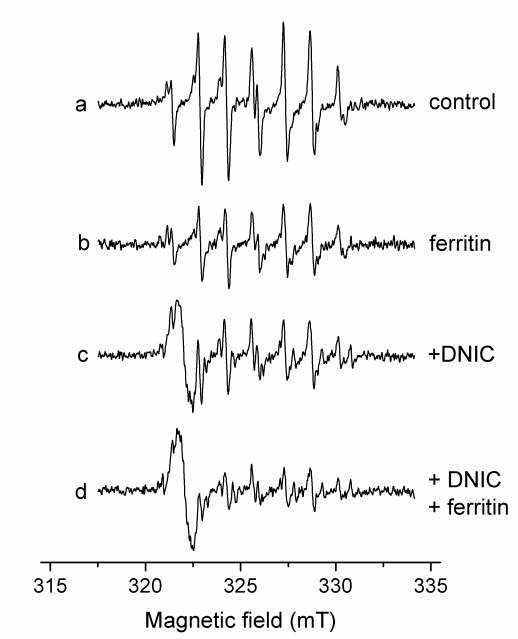

Fig.3. Effect of ferritin and DNIC on the formation of thiyl radical. The reaction mixture contained: 1.5 mM GSH, 10 mM DEPMPO, 100 μM metmyoglobin, 1.5 mM tert-butyl hydroperoxide and 100 mM phosphate buffer (pH 7.4). EPR spectra of DEPMPO spin adducts with thiyl radicals were measured: (a) – without additions; (b) – 1.5 μg/ml ferritin; (c) – 0.5 mM DNIC; (d) – 1.5 μg/ml ferritin and 0.5 mM DNIC.

Statistical analysis

Data are expressed as means ± SE. The one-way analysis of variance (ANOVA) was used for comparison of data. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Effect of ferritin, GSNO and DNIC on peroxidation of mitochondrial membranes

In Fig. 1, an accumulation of malondialdehyde during free radical mediated oxidation of rat heart mitochondria is shown. Ferritin in combination with tert-butyl hydroperoxide gave rise to a slight oxidation of mitochondria (Fig. 1, 1). At the same time, ferritin stimulated the mitochondrial membrane peroxidation induced by tert-butyl and metmyoglobin (Fig. 1, 2). It was found that GSH, GSNO and DNIC inhibited free-radical peroxidation of mitochondria both in presence and absence of ferritin (Fig. 1, 3-6). The formation of the lipid peroxidation products was most effectively suppressed in presence of DNIC (Fig. 1, 5) and also in case of combined action of GSNO and GSH (Fig. 1.6). It was of the essence that, in those experiments, the differences in the degree of the mitochondrial membrane peroxidation in presence and absence of ferritin were not significant.

In reactions between metmyoglobin and organic hydroperoxides, alkoxyl ($RO^{\bullet}$) and alkylperoxyl ($ROO^{\bullet}$) radicals and oxoferrylmyoglobin (Mb-Fe^{IV}=O) can be formed:

$$\text{Mb-Fe}^{2+}\text{-OH} + \text{ROOH} \rightarrow \text{Mb-Fe}^{4+} = \text{O} + \text{RO}\bullet + \text{H}_2\text{O} \quad (1)$$

$$\text{Mb-Fe}^{3+} + \text{ROOH} \rightarrow \text{Mb-Fe}^{2+} + \text{ROO}\bullet + \text{H}+ \quad (2)$$

$$\text{Mb-Fe}^{2+} + \text{ROOH} \rightarrow \text{Mb-Fe}^{3+} + \text{RO}\bullet + \text{OH}- \quad (3)$$

At the same time, free Fe ions contained in mitochondrial preparation and released from ferritin can take part in reactions (2) and (3). The Fe ions can also induce generation of $O_2^{\bullet-}$ radicals in Haber-Weiss reaction, which brings further in Fenton reaction to the formation of hydrogen peroxide and hydroxyl radicals:

$$\text{Fe}^{2+} + \text{O}_2 \rightarrow \text{Fe}^{3+} + \text{O}_2^{\bullet-} \quad (4)$$

$$\text{Fe}^{2+} + \text{O}_2^{\bullet-} + 2\text{H}^+ \rightarrow \text{Fe}^{3+} + \text{H}_2\text{O}_2 \quad (5)$$

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^{\bullet} + \text{OH}^- \quad (6)$$

Due to the fact that superoxide is able to promote the release of iron from ferritin, a redox cycle constantly producing ROS can appear. The intensification of the peroxidation in reaction mixture containing tert-butyl hydroperoxide, myoglobin and ferritin may be also connected with oxidative degradation of ferritin leading to more intensive release of Fe ions (Ruderic, Volk, Sitte & Grune, 2000).

Interaction of DNIC with tert-butyl hydroperoxide. Effect of ferritin

Under the action of DNIC, the inhibition of the mitochondrial membrane peroxidation induced by metmyoglobin and tert-butyl hydroperoxide presumes the interaction of the dinitrosyl complexes with intermediates of reactions (1-3). Indeed, in the reaction mixture containing metmyoglobin and tert-butyl hydroperoxide, a quick decrease of the concentration of DNIC was observed (Fig. 2, 1). In these experiments, ferritin significantly decreased the rate of the DNIC destruction (Fig. 2, 2). The effect of ferritin points to the probable antioxidant action of Fe ions released into the medium. Presumably, Fe^{2+} ions can reduce ferrylmyoglobin – a powerful oxidant. However, earlier we have demonstrated that ferrylmyoglobin in concentration used practically did not caused degradation of DNIC (Shumaev *et al.*, 2004). It is known that Fe ions were able to neutralize free radicals and in-

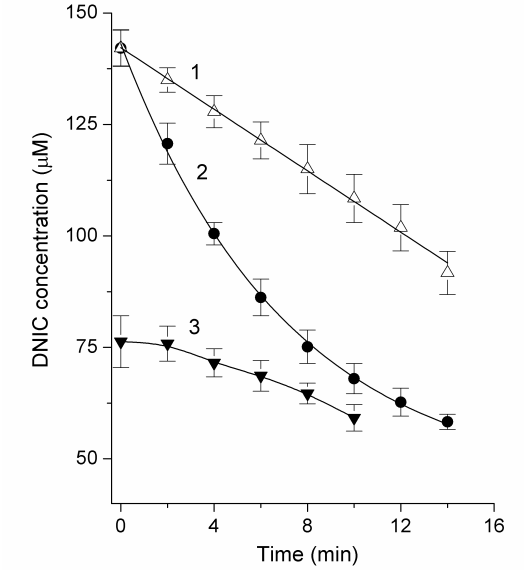

Fig.4. Kinetics of the destruction of 150 μM DNIC in the reaction mixture containing mitochondria (10 mg protein/ml), 10 mM succinate, 250 μM antimycin A and 5 mM cysteine (2 and 3). (3) – DNIC and cysteine was added into the reaction mixture after 12 min of incubation of mitochondria with succinate and antimycin A. (1) – the destruction of 150 μM DNIC in the presence of 10 mM succinate and 250 μM antimycin A in the mitochondria isolation medium.

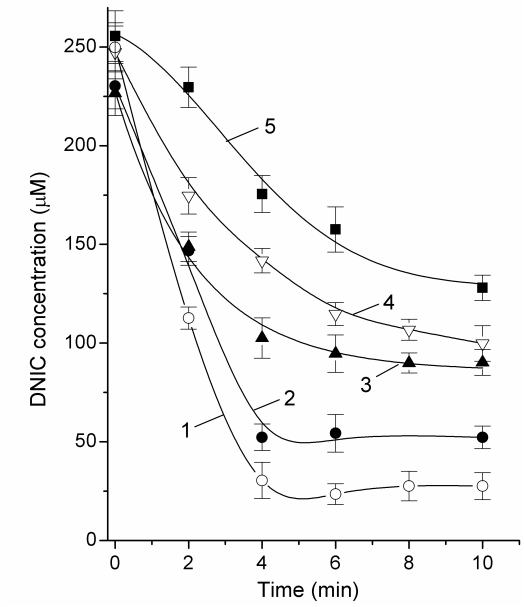

Fig.5. Kinetics of the destruction of 250 μM DNIC incubated with 0,2 unit/ml xanthine oxidase and 1 mM xanthine (1); in the presence of 1.5 μg/ml ferritin (2 and 3); 600 units/ml catalase (3-5) and 50 units/ml superoxide dismutase (5).

hibit lipid peroxidation (Minotti *et al*., 1996). Nevertheless, ferritin itself in combination with tert-butyl hydroperoxide stimulates destruction of DNIC although notably slighter than in the system containing metmyoglobin (Fig. 2, 3). It permits to propose that free Fe ions or ferritin can take part in the DNIC regeneration from the products of their decomposition. The decrease of the intensity of EPR signal of dinitrosyl-iron complexes in the presence of ferritin (Fig. 2, 2,3) indicates to the interaction of ferritin itself with DNIC. Although glutathione is known as an antioxidant, its thiyl radicals are able to participate in $O_2^{\bullet-}$ radical generation (Leonard, Harris & Shi, 2004):

$$Fe^{3+} + GSH \rightarrow Fe^{2+} + GS^{\bullet} \quad (7)$$

$$GS^{\bullet} + RSH \rightarrow RSSR^{\bullet-} + H^{+} \quad (8)$$

$$RSSR^{\bullet-} + O_2 \rightarrow RSSR + O_2^{\bullet-} \quad (9)$$

It seems interesting to point out that both ferritin and DNIC decrease the concentration of DEMPO spin-adducts with glutathione radicals in the reaction mixture containing metmyoglobin and tert-butyl hydroperoxide (Fig. 3a-c). Considerable decrease of the thiyl radical concentration under the combined action of ferritin and DNIC allows assuming the existence of the antioxidant synergism of these compounds (Fig. 3d).

Interaction of DNIC with superoxide in different $O_2^{\bullet-}$ generating systems

In addition to reactions (4) and (9), ubisemiquinone and other electron carriers of the respiratory chain, which are capable of one-electron oxygen reduction, can serve as an origin of superoxide radicals in our model system used for the oxidation of mitochondrial membranes:

$$Q_{10}^{\bullet-} + O_2 \rightarrow Q_{10} + O_2^{\bullet-} \quad (10)$$

In Fig. 4, there is presented a kinetics of the destruction of DNIC in conditions of intensive $O_2^{\bullet-}$ generation in bc_1 segment (complex III) of the mitochondrial respiratory chain. DNIC destruction took also place during enzymatic $O_2^{\bullet-}$ generation in xanthine-xanthine oxidase system (Fig. 5). At the same time, both superoxide dismutase and catalase lowered the rate of DNIC decomposition (Fig. 5, 1-5). The action of catalase shows that, besides superoxide, the hydroxyl radicals took part in DNIC destruction. The latter, as it was already mentioned above, appears in reaction between Fe^{2+} ions and hydrogen peroxide formed from $O_2^{\bullet-}$ according to reaction (6). However, without catalase, ferritin did not stimulate and even slowed down DNIC decomposition (Fig. 5, 2). Possibly, this effect has the same reasons as the protective action of ferritin in

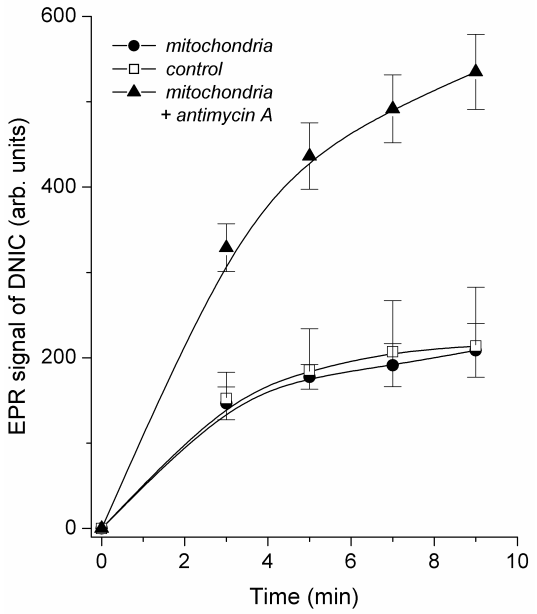

Fig.6. Effect of mitochondria (7.5 mg protein/ml) or mitochondria with 250 μM antimycin A on the formation of DNIC in systems containing 3 mM GSNO and 2.5 μg/ml ferritin.

conditions of tert-butyl free radical generation. At the same time, DNIC and ferritin can compete for superoxide. On the other hand, ferritin slightly stimulated the DNIC destruction in presence of catalase, although in these conditions, an intensification of its protective action could be expected (Fig. 5, 3). The data obtained demonstrate that, in conditions of $O_2^{\bullet-}$ generation, ferritin is capable of both acceleration and inhibition of the DNIC destruction. The protective action of ferritin is apparently connected with neutralizing of hydrogen peroxide and hydroxyl radicals. When hydrogen peroxide has been removed by catalase, the equilibrium shifts to the destructive action of ferritin.

Formation of DNIC in conditions of $O_2^{\bullet-}$ generation by mitochondria

It is shown in Fig. 6 that stimulation of $O_2^{\bullet-}$ generation by rat heart mitochondria in reaction mixture containing GSNO, ferritin and thiols brings to the increase of the rate of DNIC formation. It seems reasonable to propose that this rise is connected with increase in the release of Fe ions from ferritin during its interaction with superoxide radicals (Biemond *et al.*, 1984). Indeed, the addition of DTPA – a Fe ion chelator – into the reaction mixture resulted in inhibition of the DNIC formation (data not shown). It is worth mentioning that considerable stimulation of the DNIC formation was not observed in earlier experiments, when xanthine-xanthine oxidase as an $O_2^{\bullet-}$ generating system was used (Shumaev *et al.*, 2003). The absence of the DNIC destruction in those conditions is an evidence of constant DNIC regeneration under the excess of GSNO and Fe ions. Probably, the DNIC regeneration intensifies in presence of mitochondria, and the high efficiency of the antioxidant action of the combination of GSNO and GSH during free radical derived oxidation of mitochondria (Fig. 1, 6) can be explained by the formation of DNIC (Fig. 6).

Interaction of DNIC with peroxynitrite Effect of mitochondria

Generation of $O_2^{\bullet-}$ in the reaction mixture containing NO donors in high concentration must lead

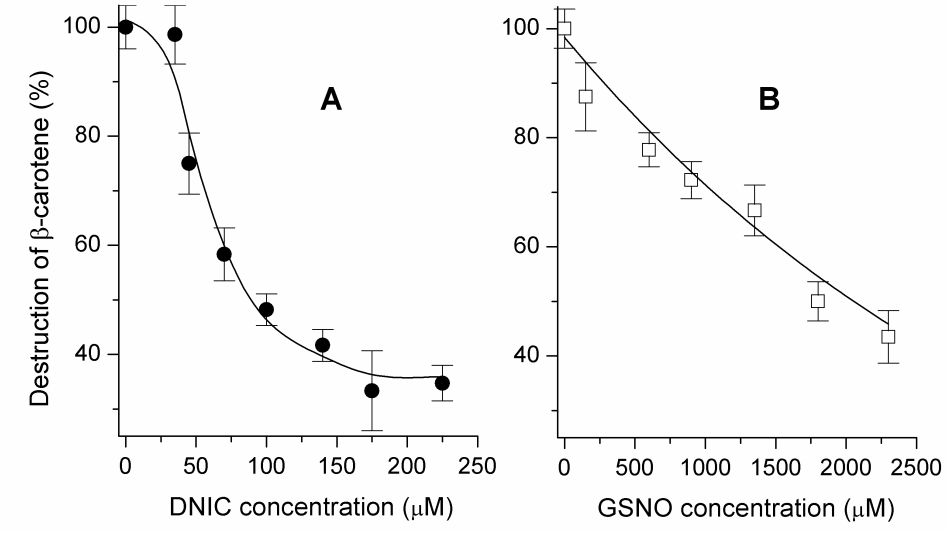

Fig.7. Effect of various concentrations of DNIC (A) or GSNO (B) on the β-carotene oxidation by peroxynitrite. The reaction mixture contained: 15 μM β-carotene hydrosol, 150 μM peroxynitrite, 200 mM phosphate buffer (pH 7.4).

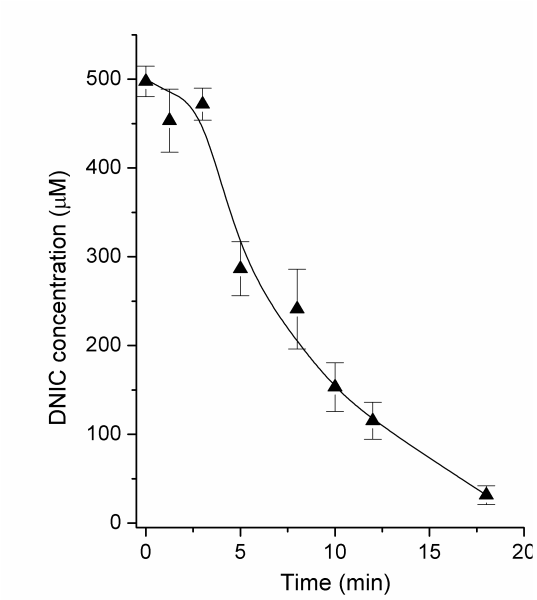

Fig.8. The destruction of DNIC by peroxynitrite. The reaction mixture contained: 0,5 mM DNIC, 2 mM peroxynitrite, 200 mM phosphate buffer (pH 7.4).

to the formation of peroxynitrite – a powerful oxidant:

$$NO + O_2^{\bullet -} = ONOO^- \qquad (11)$$

It is possible that the relatively low antioxidant activity of GSNO during the mitochondria oxidation in system containing myoglobin, ferritin and tert-butyl hydroperoxide (Fig. 1, 4) is connected with peroxynitrite formation.

In our experiments, DNIC effectively inhibited peroxynitrite-induced oxidation of lipophilic antioxidant – β-carotene, the antioxidant action of DNIC being essentially higher than that of GSNO (Fig. 7). Probably, the both nitrosyl and thiol components of DNIC are able to interact with peroxynitrite, what explains the high antioxidant efficiency of DNIC. In presence of $ONOO^-$ and also other oxidants studied, the DNIC destruction took place (Fig. 8). Low, as compared with decomposition of peroxynitrite itself, rate of the DNIC decay points to the possibility of formation of intermediate compounds capable to regenerate into initial nitrosyl form. In this case, the kinetics of the decrease of DNIC concentration shows the degradation of these intermediate complexes. The data given above allow to propose that mitochondria can participate in regeneration of nitrosyl-iron complexes. In fact, administration of the mitochondria into reaction mixture even after peroxynitrite-induced complete DNIC destruction results in regeneration of the nitrosyl-iron complexes (Fig. 9A, B). It is worthy of note that in case of incubation of DNIC, which was treated preliminary with peroxynitrite, with mitochondria in presence of succinate, both dinitrosyl- and mononitrosyl-iron complexes are formed. In absence of the substrates, only mononitrosyl-iron complexes are formed (Fig.

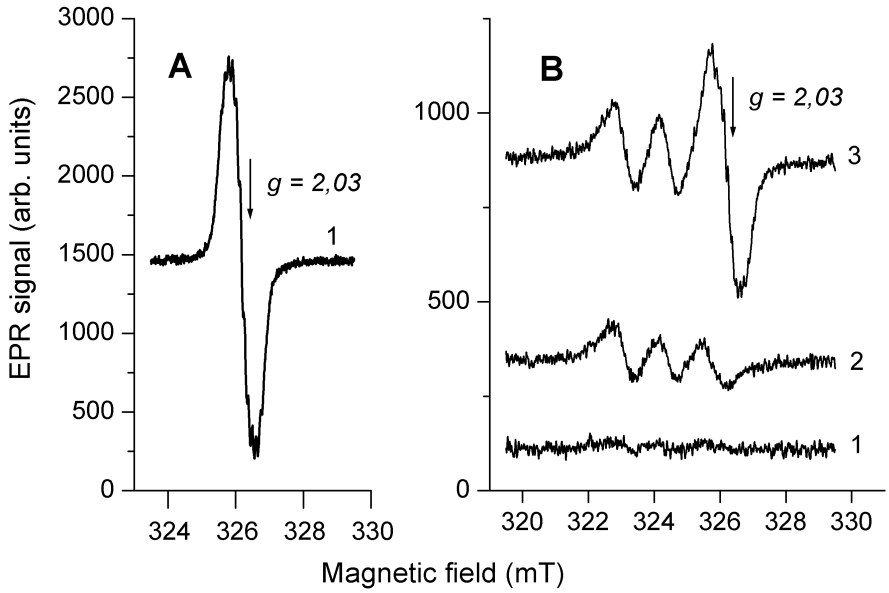

Fig.9. Effect of mitochondria on destruction of DNIC by the action of peroxynitrite. (A): EPR spectrum of the initial solution of 250 μM DNIC. (B): (1) – EPR spectra of the solution of DNIC after 3 min incubation with 3 mM peroxynitrite; 2 – the same as (1) but after addition of mitochondria (7.5 mg protein/ml); (3) – the same as (1) but after addition of mitochondria (7.5 mg protein/ml) and 10 mM succinate.

9B).

Reaction between peroxynitrite and DNIC is of doubtless interest as mechanism of antioxidant defense of mitochondria. It is known that peroxynitrite reacts with several low- and macromolecular mitochondrial components, it also irreversibly inhibits complex I of the respiratory chain, causes lipid peroxidation and activates processes, which lead to apoptosis (Riobo *et al.*, 2001; Brookes *et al.* 2002; Droge, 2002). However, if NO reacts not with $O_2^{\bullet-}$, but with Fe ions and reduced thiols, as a result appear dinitrosyl-iron complexes that neutralize peroxynitrite.

Ferritin, which regulates iron homeostasis, plays an important role in antioxidant processes as an original buffer, by reducing the labile pool of redox-active iron. Our results demonstrate that DNIC may take part in these processes. It is known that NO inhibits lipid peroxidation by interaction with lipid free radicals, and, as a result, nitro- and nitrosoderivatives that do not participate in consequent chain reactions of free-radical oxidation are formed (Rubbo *et al.*, 1994; Shafer *et al.*, 2002). Under conditions, when alkoxyl and alkylperoxyl radicals are formed, the antioxidant properties of DNIC can also be connected with interaction of NO or nitrosyl groups of DNIC with these radicals or with tert-butyl hydroperoxide:

$$(GS^-)_2\text{-}Fe^+\text{-}(NO^+)_2 + ROO^\bullet(RO^\bullet) \rightarrow GSNO + Fe^{2+} + ROONO(RONO) \quad (12)$$

Because of feroxidase reaction, ferritin should rapidly oxidize Fe^{2+} ions, which are formed during DNIC decomposition, into Fe^{3+} ions, and these ions will further be bound with ferritin. As a result, ferritin can protect DNIC against free-radical destruction, which may be catalyzed by the products of their decomposition. In this case, nitrosyl group may remain bound with GSNO or with NO-binding sites of ferritin (Lee, Arosio, Cozzi & Chasteen, 1994). In presence of thiols or $O_2^{\bullet-}$, Fe ions should come from ferritin into the medium, and DNIC regeneration should take place. It cannot be excluded that DNIC is formed with participation of FeNO groups, which are bound with ferritin itself (Lee *et al.*, 1994). On the other hand, it seems to be probable that DNIC protect ferritin from oxidative degradation, thereby preventing the escalation of the processes of free-radical mediated oxidation.

From this it follows that metabolic pathways of iron and reactive oxygen and nitrogen species are closely connected, and interaction of ferritin and DNIC with intermediates of free-radical reactions may function as a trigger in regulation of the balance between antioxidant and prooxidant processes in a cell. Maintenance of this balance has special significance in conditions of ischemia/reperfusion, which are accompanied by increasing the rate of ROS synthesis, stimulating NO synthesis and rising intracellular concentration of "free" iron (Droge, 2002).

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