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Myoglobin causes oxidative stress, increase of NO production and dysfunction of kidney's mitochondria

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ABSTRACT

Rhabdomyolysis or crush syndrome is a pathology caused by muscle injury resulting in acute renal failure. The latest data give strong evidence that this syndrome caused by accumulation of muscle breakdown products in the blood stream is associated with oxidative stress with primary role of mitochondria. In order to evaluate the significance of oxidative stress under rhabdomyolysis we explored the direct effect of myoglobin on renal tubules and isolated kidney mitochondria while measuring mitochondrial respiratory control, production of reactive oxygen and nitrogen species and lipid peroxidation. In parallel, we evaluated mitochondrial damage under myoglobinurea *in vivo*. An increase of lipid peroxidation products in kidney mitochondria and release of cytochrome *c* was detected on the first day of myoglobinuria. In mitochondria incubated with myoglobin we detected respiratory control drop, uncoupling of oxidative phosphorylation, an increase of lipid peroxidation products and stimulated NO synthesis. Mitochondrial pore inhibitor, cyclosporine A, mitochondria-targeted antioxidant (SkQ1) and deferoxamine (Fe-chelator and ferryl-myoglobin reducer) abrogated these events. Similar effects (oxidative stress and mitochondrial dysfunction) were revealed when myoglobin was added to isolated renal tubules. Thus, rhabdomyolysis can be considered as oxidative stress-mediated pathology with mitochondria to be the primary target and possibly the source of reactive oxygen and nitrogen species. We speculate that rhabdomyolysis-induced kidney damage involves direct interaction of myoglobin with mitochondria possibly resulting in iron ions release from myoglobin's heme, which promotes the peroxidation of mitochondrial membranes. Usage of mitochondrial permeability transition blockers, Fe-chelators or mitochondria-targeted antioxidants, may bring salvage from this pathology.

1. Introduction

Rhabdomyolysis (muscle fiber dissolution) is the injury-induced pathological lysis of skeletal muscle tissue, leading to the release of intracellular components into the extracellular fluid and the circulation. This process explains approximately 10 to 15% of all acute renal failure (ARF) cases. The significance of such pathology may be clear from the fact that the major cause of rhabdomyolysis is the crush factor, i.e., myolysis due to traumatic compression of muscle as a result of accidents or disasters. There is a number of case reports on the development of rhabdomyolysis associated with drug use such as cocaine and ecstasy [1,2], venom poisoning [3] and alcohol abuse [4]. Interestingly, while 0.1% of all patients with coronary artery disease using statins to lower blood cholesterol suffer from severe rhabdomyolysis that apparently results from altering the production of small proteins necessary for myocyte maintenance [5], 1–5% of all statin users complain on myalgias and muscle weakness [6].

Many researchers suggest the main toxic element to be myoglobin, an 18,800 Da oxygen carrier muscle cell protein. Myoglobin is quickly filtered by the glomeruli and then resides in the tubules, where it may cause obstruction and renal failure. The mechanisms of kidney injury during rhabdomyolysis are still not completely understood. There is a lack of knowledge on the participation of mitochondria as well as of reactive oxygen and nitrogen species (ROS and RNS correspondingly) in the development of myoglobinuric renal dysfunction, whereas its importance in some other renal pathologies, such as kidney ischemia and others is well established [7].

While in earlier reports it was a widely held belief that the general cause of myoglobinuric ARF is the formation of myoglobin aggregates and renal tubular obstruction, leading to impairment of primary urine flow and reabsorption [8–10], recent studies have shown the development of oxidative stress under the conditions of myoglobinuria, accompanied by the increased production of ROS and lipid peroxidation [11,12]. The heme of a myoglobin molecule is thought to play the key role in this process. In one study the oxidative damage was caused by the direct effect of heme without releasing iron from the heme [13]. However, the presence of hemoxygenase and ferrochelatase in the cell [14,15] may potentially result in the appearance of free iron in the cell with further ignition of Fenton

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reaction. At the same time, under rhabdomyolysis and myoglobin accumulation in tubules, their epithelial cells show elevated expression of hemoxygenase [16] which makes such process of iron release highly probable. We must admit that earlier reports suggested that myoglobin may release free ferrous (Fe^{2+}) ions, which could induce lipid peroxidation as a result of the generation of hydroxyl radicals via Fenton's reaction [17]. An alternative mechanism could involve the heme moiety of myoglobin absorbed by a renal tubule cell [13, 18]. It can be realized either through endocytotic absorption of myoglobin [19] by the tubular cells similar to what happens with albumin [20] or through the direct transport of heme (for example, by a specific transporter HCP1 (heme carrier transporter) which is known to facilitate heme transport into the cell and specifically participates in heme capture in kidney cells [21]. Indirect evidence of the activation of hemoxygenase after myoglobin accumulation in the tubules [16] is in support of such speculation, however, there is no doubt that the expression of this protein can be determined by other factors including oxidative stress [22].

In renal tubules, oxyferrous myoglobin would undergo autooxidation to the ferric form (MetMb, Fe^{3+}), which is catalytically competent to promote a lipid peroxidation reactions. The redox cycle occurs between the ferric (Fe^{3+}) and ferryl ($[Fe=O]^{2+}$) myoglobin oxidation states, and the latter can directly initiate lipid oxidation [13,18].

Until now, most researchers have proposed development of oxidative stress during rhabdomyolysis, after detecting the presence of lipid peroxidation products either in serum or in renal tissue. There is a consensus that one of the basic regulators of ROS production and cell death are mitochondria [23] but the knowledge on these organelles functioning under myoglobinuria is still not complete. There are limited evidences of mitochondria participation in development of pathological processes during rhabdomyolysis [12,24,25].

Moreover, very little is known about the role of RNS in myoglobinuria. NO is an important modulator of oxidative stress and may be a predisposing as well as a protective factor. This issue has taken on special significance after the discovery of mitochondrial NO-synthase (NOS) [26], which enables mitochondria to directly regulate their ROS production [27].

Our study was focused on mitochondrial functioning, development of oxidative stress and increase of NO production in a glycerol-induced myoglobinuria in the rat as well as the study of myoglobin effect on isolated rat kidney mitochondria. The goal of this work was to investigate the mitochondrial status during myoglobin-induced ARF, to model the mitochondria dysfunction caused by myoglobin *in vitro* and to study the role of mitochondrial NOS in this pathology.

2. Materials and methods

2.1. Modeling of glycerol-induced rhabdomyolysis in rat

Experiments were performed on outbred white male rats (180–200 g) fed *ad libitum*. Animal protocols were approved by the institutional review ethical committee. Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Animals in the rhabdomyolysis group were deprived of water for approximately 36 h and then injected with glycerol (10 ml/kg of a 50% water solution) intramuscular in two legs [28]. Control animals were untreated. At regular intervals after rhabdomyolysis induction, blood samples were collected for measurement of biochemical parameters and kidney were excised for the mitochondria isolation.

2.2. Isolation of mitochondria from rat kidney

Kidney mitochondria were isolated by homogenization and differential centrifugation in a medium containing 250 mM sucrose, 20 mM HEPES–KOH, 1 mM EGTA, 0.1% BSA, pH 7.4. Mitochondria were suspended in incubation solution (250 mM sucrose, 10 mM HEPES–KOH, 2 mM KH_2PO_4, 10 μM rotenone, pH7.4) and energized by adding of 5 mM K-succinate. Total mitochondrial protein was determined by a bicinchoninic acid protein assay kit (Sigma Aldrich, USA).

Mitochondrial oxygen uptake was measured with a Clark-type oxygen electrode Oxygraph-2k (Oroborus, Paar, Austria). Reaction components were added to the chamber containing 2 ml of incubation buffer at 25 °C. In classical terms, one of the most important mitochondrial functional indices is non-phosphorylated (state 3 after ADP supplement or state 3^u after uncoupler supplement) and phosphorylated (state 4) respiration rate of isolated mitochondria [29]. Proper ratio (V_3/V_4), also called respiratory control index defines proton fluxes through the inner mitochondrial membrane under the state of the ATP synthesis (V_3) versus the resting state (V_4). Mitochondrial uncoupling was induced by adding 2,4-dinitrophenol (DNP) to a final concentration of 100 μM. Oxygen consumption rate for state 3 and 4 was calculated per amount of mitochondrial protein in the sample.

2.3. Measurement of MDA products and nitric oxide in serum and mitochondria of rat kidney

Malonic dialdehyde was determined in the sample by thiobarbituric acid assay according to the method of [30] with minor modification. Each sample was mixed with 0.8% thiobarbituric acid and 1% H_3PO_4 in the ratio 0.9:1.0:3.0. The mixture was boiled for 45 min and centrifuged after cooling at 15,000 *g* for 10 min. Absorbance of the resulting supernatant at 532 nm was measured by a Hitachi 557 spectrophotometer. 1,3,3-tetraetoxipropan (Sigma, USA) was used as a standard solution.

Nitric oxide was measured by colorimetric method of Griess (Griess reagent, Sigma-Aldridge, USA). To remove protein the samples were mixed with an equal volume of icy methanol, incubated for 5 min at 4 °C and centrifuged at 20,000 *g* for 10 min. The resulting supernatant was combined with an equal volume of Griess reagent and incubated for 30 min at 4 °C. The absorbance at 540 nm was measured using a plate reader (LabSystems Multiscan).

2.4. Incubation of mitochondria with myoglobin in vitro

Mitochondria were diluted to a final concentration of 4 mg/ml with the incubation buffer containing 250 mM sucrose, 10 mM HEPES, 1 mM EGTA, 0.1% BSA, pH 7.4. 5 mM K-succinate and 10 μM rotenone were added to initiate the respiration. Myoglobin was added to the sample to final concentrations of 50, 100, 500 μM, accordingly. The control sample was supplemented either with the same amount of BSA or the incubation medium. Samples were incubated for 1 h at room temperature with gentle shaking then some aliquots of mitochondria were used for oxygen consumption measurements and another were centrifuged (10,500 *g*) for 10 min. The pellet was suspended in 1.15% KCl with 10 mM EGTA and was used for MDA measurements. Supernatant was used for nitrite determination.

2.5. Western blot analysis

Samples of serum or mitochondria, normalized for total protein content (50 mg/lane), were separated by Tris–tricine electrophoresis into 16.5% PAAG and transferred onto a PVDF membrane (Amersham Pharmacia Biotech, Rainham, UK). Membranes were blocked with 3% (wt/vol) non-fat milk in PBS with 0.1% (v/v) Tween 20, subsequently incubated with primary antibodies: mouse monoclonal anti-cytochrome *c* 1:1000 (BD Biosciences Pharmingen, USA), mouse monoclonal anti-porin 1:1000 (BD Biosciences Pharmingen, USA) or rabbit monoclonal myoglobin 1:500 (Santa Cruz, USA) and stained with anti-mouse IgG or anti-rabbit IgG conjugated with horseradish peroxidase (Calbiochem, USA). The detection was made using ECL Western blotting kit (Amersham Pharmacia Biotech, Rainham, UK).

Spots density analysis in the film was done by ImageJ software (NIH, Bethesda, MD, USA) after scanning.

2.6. Measurement of mitochondria membrane potential and ROS production in isolating tubules

Kidneys of 3–7 days old rats were excised and dissociated with 0.1% collagenase solution (20 min at 37 °C). Large fragments of tissue were removed, and tubules were precipitated by gentle centrifugation (50 *g*) for 3 min. The pellet was suspended in DMEM/F-12 medium without bicarbonate supplemented with 20 mM HEPES. All further steps were done at 37 °C.

The myoglobin solution was added to the tubules suspension to final concentration of 500 μM and incubated for 1 h. Then tubules were washed out of myoglobin and incubated with fluorescent probes TMRE (200 nM) and 2′,7′-dichlorodihydrofluorescein diacetate (DCF, 10 μM, Invitrogen, USA). Renal tubules were imaged with an LSM510 inverted confocal microscope (Carl Zeiss Inc., Jena, Germany) in a multi-channel mode as appropriate for the dyes loaded. Analysis of fluorochrome incorporation was performed in glass-bottom dishes with the excitation at 488 nm (for DCF) and 543 nm (for TMRE), and emission collected at 500–530 nm and >560 nm, respectively. To minimize the contribution of photo-induced mitochondria/cell damage in the relative fluorescence intensities we analyzed images used by averaging first four scans only. Images were processed by ImageJ software (NIH, Bethesda, MD, USA).

2.7. Statistics

All the experiments were performed more than in triplicate. All data are mean ± SEM. Comparisons within groups were made by Student *t* test, and *P* value less than 0.05 was taken to indicate statistical significance.

3. Results

3.1. Mitochondrial damage in myoglobin-induced ARF

This study of ARF development under rhabdomyolysis demonstrates that the activities of principal enzyme-reporters of the tissue damage (such as lactate dehydrogenase (LDH) and alanine aminotransferase (ALT)) increased greatly in the blood within the first 2 days (Fig. 1B). A basic index of severe ARF development was the increase in the blood of nitrogen metabolism products (urea and creatinine) in the blood, which reached its maximum at 1–2 days (Fig. 1A). Further, most parameters returned to a normal level that allows suggesting functional recovery of kidneys.

We found that myoglobin deposits in the kidney tubules become already detectable during very first hours after rhabdomyolysis induction (Fig. 1C). In control kidney, myoglobin was not detected (Fig. 1C). Myoglobin appeared in the renal tissue 3 h after glycerol injection, and its maximal concentration was observed after 15 h, and went down to a non-detectible level after 24 h. The latter may be due to the possibility that the protein moiety of myoglobin molecule was at least partially degraded, resulting in the failure of specific antibodies to detect the antigen. Also myoglobin could be rapidly excreted in the urine already at first day of rhabdomyolysis.

We used the release of cytochrome *c* from kidney mitochondria as a marker of their damage [31]. There is evidence that at cell apoptotic death, cytochrome *c* not only is released from mitochondria into the cytoplasm, but also appears in the extracellular space. It seems that blood plasma cytochrome *c* level reflects the degree of tissue injury. In fact, we revealed that cytochrome *c* was detected in the blood of rhabdomyolytic rats (Fig. 1D). This demonstrated the extensive damage of renal cells and the induction of their apoptotic or necrotic death. Cytochrome *c* was not found in control serum.

More conclusive proof of apoptotic launching and mitochondria injury in renal cells was obtained by western blotting of mitochondria isolated from kidneys on different periods of rhabdomyolysis. We

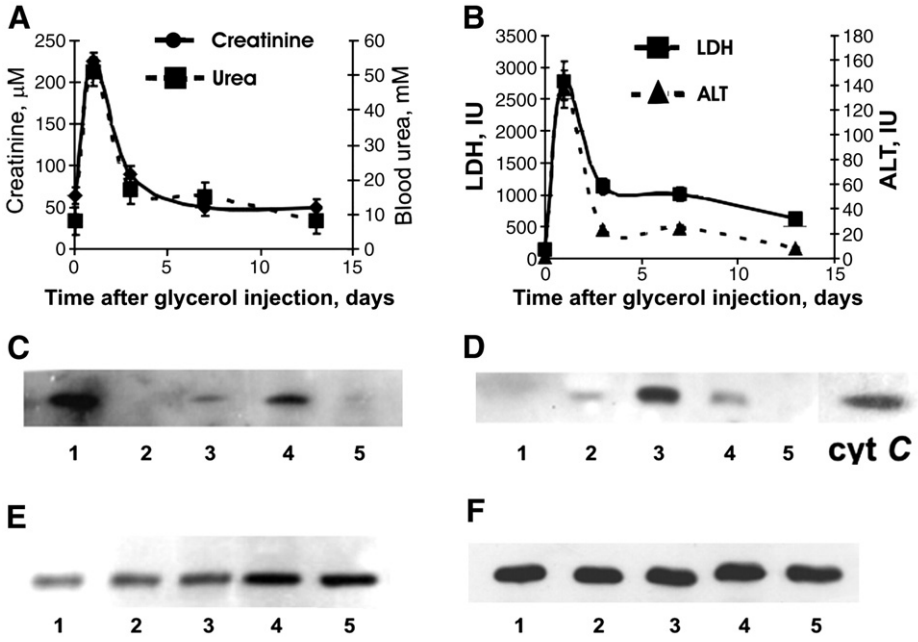

Fig. 1. ARF development and the damage of kidney mitochondria in rhabdomyolytic rats. The rise of blood urea and creatinine level (A), and ALT and LDH activity (B) are indices of ARF development on the first days after rhabdomyolysis induction. Appearance of myoglobin in the blood correlates with its accumulation in the kidney (C) western blotting of kidney homogenate with anti Mb antibodies where lane 1, rat skeletal muscle; lane 2, control kidney; lanes 3–5, the kidney 4, 15 and 24 h after induction of rhabdomyolysis correspondingly). Cytochrome *c* appears in the blood (D) western blotting of blood serum with anti-cytochrome *c* antibodies where lane 1, control serum; lanes 2–5, 5, 16, 24 and 48 h after induction of rhabdomyolysis correspondingly) with a corresponding loss of cytochrome *c* in isolated kidney mitochondria (E, lanes 1–4, 4, 16, 24, and 48 h, and lane 5, control kidney mitochondria. (F) The same samples as in E with anti-VDAC antibodies.

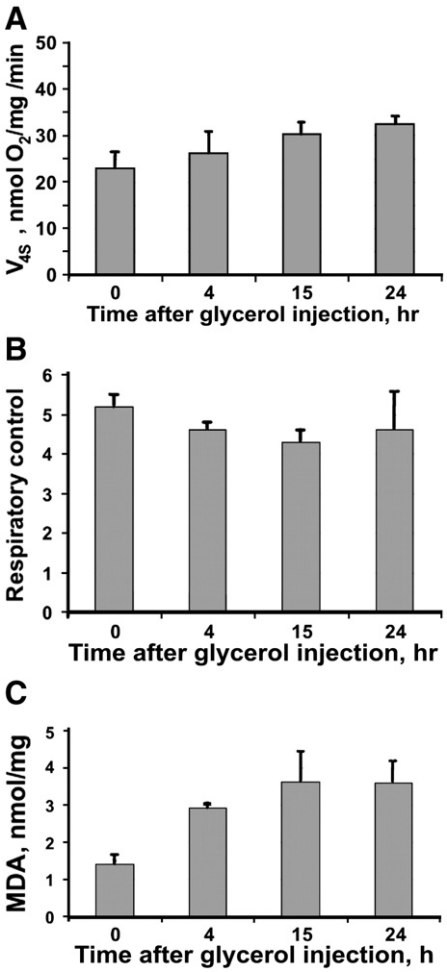

Fig. 2. Dysfunction of kidney mitochondria and lipid peroxidation during rhabdomyolysis. Oxygen consumption rate in state IV (A), respiratory control (B) and concentration of MDA products (C) in mitochondria isolated from kidney of rats at various times after induction of rhabdomyolysis are shown.

found that the amount of cytochrome *c* in mitochondria was decreased and maximum of cytochrome *c* release was observed at 4 h after rhabdomyolysis induction.

3.2. Oxidative stress and dysfunction of mitochondria in rhabdomyolytic rats

We analyzed basic functional parameters of mitochondria isolated from kidney at different periods after rhabdomyolysis induction. In a first turn, the oxygen consumption rates with succinate in state 3 and 4 (V_3^U and V_4^S) and MDA levels were measured and V_3^U/V_4^S ratio was determined in mitochondria. We revealed an essential increase of MDA level in mitochondria on the very first day of myoglobinuria (Fig. 2A), which demonstrated that a significant oxidative stress evolves, leading to a lipid peroxidation in mitochondria. The greatest rise was observed after 4 h when MDA level rose twice as compared with control values. After 15 h of rhabdomyolysis, MDA concentration in mitochondria stabilized and later on the same day began to drop.

Twenty-four hours after glycerol injection the oxygen consumption rate (at state 4) increased (Fig. 2B). This indirectly indicates some loss of a coupling between oxidation and phosphorylation in mitochondria, possibly due to a change of permeability in the inner mitochondrial membrane to ions. Although the state 3 respiration rate (with the uncoupler) did not change much (data not shown), the ratio V_3^U/V_4^S in rhabdomyolytic mitochondria was significantly diminished.

3.3. In vitro model of myoglobin influence on mitochondria functioning

For a more comprehensive study of myoglobin effect on mitochondria, we used an *in vitro* model, where myoglobin directly interacts with isolated renal mitochondria. Apart from the consideration that the renal tubule cells intake mainly heme or heme iron [12], the probability of absorption by the cells of intact myoglobin molecules from tubules lumen cannot be ruled out. Such transport is typical for most proteins as they are filtering into primary urine from blood plasma following by reabsorption in renal tubules [32].

To study the direct effect of myoglobin on kidney mitochondria, the mitochondrial suspension was supplemented with a commercially available form of myoglobin, metmyoglobin (Fe^{3+}). It is known that mitochondria can reduce the iron in metmyoglobin molecule (Fe^{3+}) to a ferrous form (Fe^{2+}) using succinate as a respiratory substrate [33], resulting in the presence of both forms of myoglobin in the incubation medium. Therefore, this ability of mitochondria has no need to artificially reduce myoglobin's heme, and apparently, the system more closely corresponds to the situation in kidney *in vivo*, where both forms of myoglobin are present.

When studying the interaction of myoglobin with mitochondria *in vitro* we analyzed the mitochondrial oxygen consumption rate after incubation with myoglobin and the behavior of markers of oxidative and nitrosyl stress, because these parameters were found to change *in vivo*. In a control system, we substituted myoglobin for appropriate concentration of BSA. Data on mitochondria function impairment are presented in Table 1.

We found that mitochondrial respiration rate in state 4 increases twice and to about three-fold as referred to control values at myoglobin concentrations 100 and 500 μM correspondingly 1 h after preincubation with myoglobin at room temperature. Incubation with BSA did not affect the respiration rate, showing that the previous effect was not due to a change of oncotic pressure caused by the presence of protein in the incubation medium. The uncoupling respiration rate (state 3^u) almost did not change during incubation with either myoglobin or BSA, which correlates with our data for function of mitochondria isolated from the kidneys of rats suffering myoglobinuria. The low respiratory control of mitochondria incubated with myoglobin may be explained by that on average the population of isolated mitochondria is less coupled and apparently there is a link between relative uncoupling and the presence of peroxidated lipids, suggesting a lipid peroxide-induced distortion of the ion permeability of the inner mitochondrial membrane. Further experiments revealed that 100 nM cyclosporine A (CsA) protects from the myoglobin-induced drop of the respiratory control (Table 1). These results can be interpreted in the way that Mb causes the induction of the permeability transition in some part of the population of isolated mitochondria (potentially resulting in the absence of the membrane potential in these mitochondria and maximal activation of their respiration), which is misleading by giving an average lowering of the respiratory control with the tentative wrong interpretation of mitochondrial uncoupling.

Table 1
The influence of incubation with myoglobin on functional characteristics of renal mitochondria: the respiration rates in state 3 (V_3^s) and 4 (V_4^u) and the respiratory control.

Sample	V_4^S (nmol O_2 min^{-1} mg^{-1})	V_3^U (nmol O_2 min^{-1} mg^{-1})	Respiratory control
Control	11.5 ± 2.1	55.1 ± 5.3	4.8 ± 0.6
100 μM Mb	22.7 ± 1.7	54.5 ± 4.2	2.4 ± 0.3
500 μM Mb	32.5 ± 5.4	51.0 ± 3.6	1.6 ± 0.6
100 μM Mb + 2 mM DFO	16.6 ± 1.2	53.2 ± 6.8	3.2 ± 0.2
500 μM Mb + 100 nM SkQ1	38.6 ± 4.6	54.1 ± 6.8	1.4 ± 0.2
100 μM Mb + 100 nM CsA	12.3 ± 2.8	52.9 ± 10.3	4.3 ± 0.5
100 μM BSA	12.6 ± 1.3	52.1 ± 9.1	4.1 ± 0.3
500 μM BSA	11.85 ± 1.7	54.3 ± 11.3	4.5 ± 0.1

The effects of mitochondria-targeted antioxidant SkQ1, iron ions chelator DFO and PTP inhibitor cyclosporin A are presented.

Incubation of mitochondria with 100 μM myoglobin in the presence of 2 mM chelator of iron ions, deferoxamine (DFO) showed that this chelating significantly although not fully restored the respiratory control value (Table 1). When compared with the data on the prevention of the myoglobin-induced changes of mitochondrial functional parameters, these data show that chelation of iron ions does not prevent the mitochondrial permeability transition caused by myoglobin supplement. The administration of mitochondria-targeted antioxidant SkQ1 also did not improve the functional characteristics of uncoupled mitochondria (Table 1). Both DFO and antioxidant greatly reduced the generation of MDA products and generation of nitrite as well (see below).

However we cannot exclude the possibility that the inhibitory effect of DFO is due to its ability to reduce ferryl myoglobin [34,35] which is known to promote lipid peroxidation. We tried to add reducing equivalents (i.e. ascorbate, not shown) and observed lower amount of MDA products in isolated mitochondria after incubation with myoglobin. However we cannot exclude that ascorbate can significantly shift the entire redox state of mitochondrial matrix towards more reduced state thus increasing the antioxidative capacity of the system. Unfortunately, all possible experimental designs cannot provide the clean discrimination between these two possibilities.

3.4. Oxidative stress and NO production in mitochondria caused by myoglobin

The suggestion that the failure of the permeability barrier by the inner mitochondrial membrane after myoglobin exposure is due to lipid peroxidation of mitochondrial membranes was confirmed by the data that MDA products in mitochondria are doubled during incubation with 100 μM myoglobin for 1 h and almost tripled with 500 μM (Fig. 3A). Simultaneous incubation with both myoglobin and 100 nM CsA reduced the MDA production markedly, however, the control values were not achieved. This implied that myoglobin-induced PTP induction is at least partially responsible for oxidative stress and lipid peroxidation. Essential antioxidative effect was noticed for DFO, although again, as in case of cyclosporine A, full recovery was not observed (Fig. 3). The beneficial effect of SkQ1 was modest but significant (Fig. 3). It is reported that this new mitochondria-targeted antioxidant accumulates in mitochondria and efficiently prevents ROS production and mitochondria dysfunction in different types of oxidative stress [36–43]. Thus, it looks probable, that free iron released from the myoglobin's heme significantly contributed to the overall damaging effect of myoglobin on mitochondria, inducing lipid peroxidation, apparently mediated by a Fenton reaction. Mitochondria-targeted antioxidants apparently replenished mitochondrial antioxidative defense and significantly hampered deleterious Fe-mediated oxidative lipid damage somehow going with the involvement of the mitochondrial permeability transition.

Finally, we studied the change of NO production during myoglobin-induced oxidative damage of mitochondria. The prerequisites for such a study were our findings of the rise of a nitrite (product of NO oxidation) level in the blood plasma of rhabdomyolytic rats. On the second day after onset of myoglobinuria, the concentration of nitrite in serum was 12.0 ± 1.5 μM, whereas it was 4.5 ± 0.5 μM in the control sample. *In vitro* model also revealed the growth of the nitrite concentration in the incubation medium of isolated mitochondria (Fig. 3B). Although the increase of nitrite level at 100 μM myoglobin was not significant, it reached almost 7-fold at 500 μM of myoglobin referred to a level observed in the control sample. We proposed that myoglobin activates the mitochondrial NOS, resulting in the rise of NO level and received indirect support of this idea from the fact that the supplement of the system with NOS substrate L-arginine (5 mM) significantly enhanced the effect of myoglobin alone (Fig. 3B). Conversely, NOS inhibitor L-nitroarginine decreased the nitrite concentration to the control level. DFO reduced the myoglobin-induced growth of nitrite concentration at such conditions as well. The tentative explanation of these data is that myoglobin supplement causes the activation of ROS production (possibly superoxide because it may be scavenged by hydrophobic mitochondria-targeted antioxidant SkQ) which in turn activates NOS. In this case, the rise of MDA products (possibly originating from the elevated ROS level) goes in parallel with the rise of nitrite (apparently originating from activated NOS).

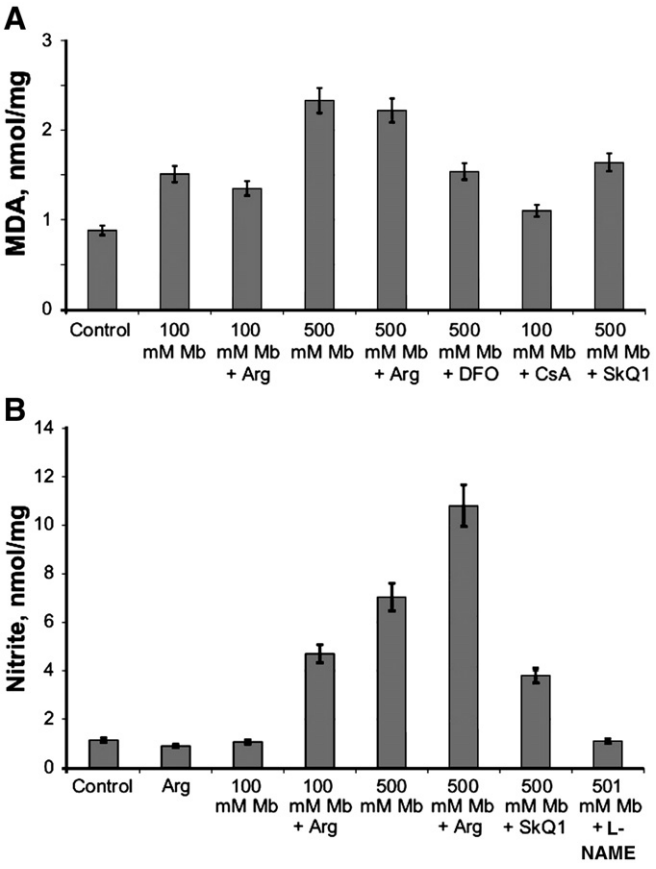

Fig. 3. Oxidative stress and NO production in rat kidney mitochondria incubated with myoglobin. Generation of MDA products (A) and nitrite (B) in mitochondria, incubated with different concentrations of myoglobin supplemented with: 5 mM of NOS substrate L-arginine; 2 mM of iron chelator, DFO; 100 nM of PTP inhibitor, CsA; 100 nM mitochondria-targeted antioxidant SkQ1, and 5 mM of NOS inhibitor L-NAME.

3.5. Oxidative stress and dysfunction of renal tubules mitochondria induced by myoglobin

Apparent signs of mitochondrial alterations induced by myoglobin found in isolated mitochondria were confirmed by using confocal microscopy of isolated renal tubules. The incubation of tubules with 100 μM myoglobin resulted in a rise of DCF fluorescence signal in tubules cells (Fig. 4A,B) pointing to the induced increase of ROS production in these cells. At the same time, we revealed a significant reduction of TMRE fluorescence signal, pointing to the drop of the mitochondrial membrane potential (Fig. 4C,D). These facts support the conclusion that myoglobin causes the distortion of barrier function of the inner mitochondria membrane and provokes the development of oxidative stress. Interestingly, the simultaneous staining of tubules with DCF and TMRE revealed good colocalization of these probes, which points to dominating generation of ROS in mitochondria.

4. Discussion

In a given study, we pursued a goal to ascertain the link between provoked ARF development and changes in renal mitochondria

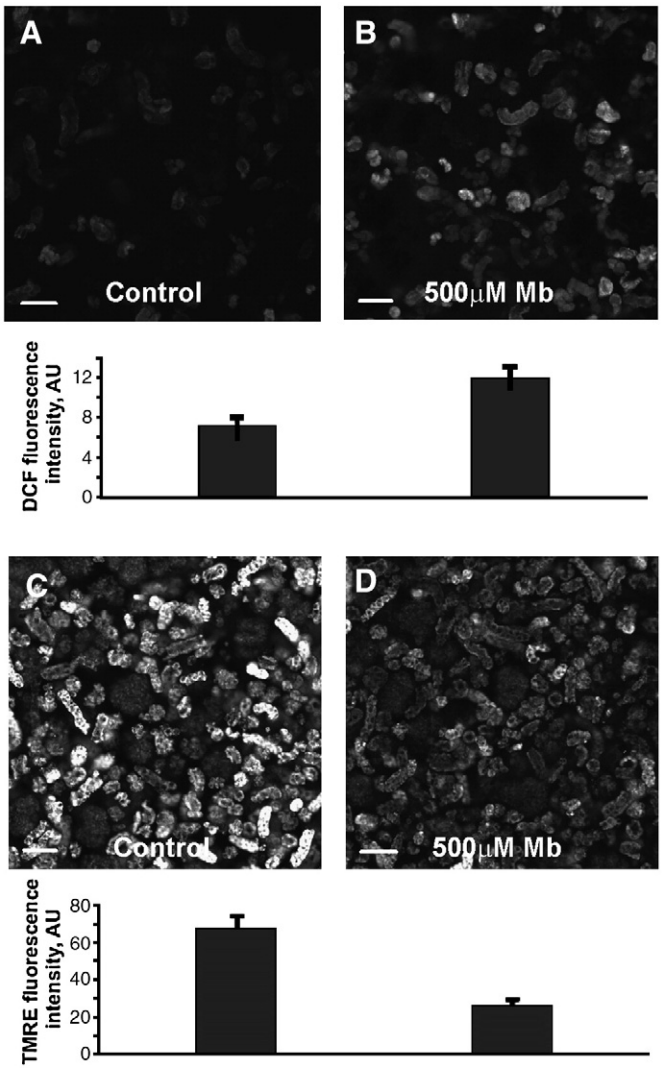

Fig. 4. ROS generation and disruption of mitochondrial potential in renal tubules cells incubated with myoglobin. Incubation of renal tubules with 500 μM myoglobin for 1 h causes significant increase of ROS production (B) and the drop of the membrane potential as indicated by the rise of DCF (B) and TMRE (D) fluorescence. (A, C) Control renal tubules stained with DCF and TMRM correspondingly. Diagrams indicate average fluorescence intensity through 10 confocal images in each sample. Bar 100 μm.

functioning after rhabdomyolysis induction. We found that this pathology proceeds with peroxidation of mitochondrial lipids pointing to the involvement of the oxidative stress. It appears conceivable that such mitochondrial damage is one of the principal causes of kidney dysfunction. The dynamics after the crush onset show that already during the first hours after muscle degradation myoglobin becomes detectable in renal tubules, and kidney mitochondria malfunctioning can be observed. Induced pathological changes in kidney resulting in the loss of mitochondrial cytochrome *c* and its appearance in urine, possibly associated with the induction of mitochondrial permeability transition and apoptotic challenge were indicated in earliest reports [31,44].

It is possible that these changes were accompanied by development of oxidative stress, peroxidation of mitochondrial membranes and uncoupling of oxidative phosphorylation in mitochondria. Stimulation of these processes was proved by accumulation of MDA products in kidney mitochondria and falling of the respiratory control within very first hours after the induction of rhabdomyolysis. Kidney malfunctioning, increase of uremia and azotemia occur either simultaneously with these events or with some delay (Fig. 1), and is likely a consequence of mitochondrial dysfunction and oxidative stress.

Such set of abnormalities is typical for ARF provoked by ischemia/reperfusion [7]. The mitochondrial dysfunction and hyperproduction of ROS tightly correlate with ischemic damage often resulting in a kidney cells death and further ARF. However, apparently under myoglobinuria another scenario may come into play also with the involvement of oxidative stress. In this case, redox-modified myoglobin and/or iron released from its heme are most likely the primary sources of radicals, and the damage of mitochondria could be a secondary process [13,18]. According to this scenario, mitochondria can not only transmit but also amplify the transmission of oxidative damage [45]. But the detailed mechanism of myoglobin influence on mitochondria remained unresolved. In two studies, renal tubules [24] or cultivated cells [25] were exposed to myoglobin, and mitochondrial functioning was evaluated through indirect effect of mitochondrial inhibitors on MDA products formation and viability of cells. We still cannot exclude the possibility that the primary target for myoglobin being in a native or modified form and for iron released from its heme may be upstream of mitochondria with further signaling to mitochondria.

Apparently, in such complex system as a cell, exogenous myoglobin may lead to a change in different pathways and mitochondrial contribution might be significantly obscured. No doubt that cellular model being more difficult in interpretation still is more advanced being more close to *in vivo* situation but more simplified model of direct effect of myoglobin on mitochondria was needed. We were aware of that quite often the data received *in vitro* (with isolated mitochondria) and *in situ* (with isolated cells) are conflicting. Particularly in the above mentioned study, when using cells, the inhibition of a respiratory chain by rotenone led to increase of MDA products generation, while antimycin A caused their reduction, although in both cases the death of kidney cells virtually did not change. However, it is known that these inhibitors have the opposite effects on isolated mitochondria, for example rotenone decreases ROS generation by blocking formation of superoxide in the complex I of respiratory chain [46] and antimycin increases this generation by blocking a respiratory chain at Q-cycle level [46]. On the other hand, some effectors (i.e. hypoxia/reoxygenation) tested in isolated mitochondria [47–49] demonstrate the responses similar to those observed in the cells with some peculiar distinctions. In addition, it has been shown that myoglobin in its native form can be uptaken by the renal cells by endocytosis [19] thus getting a direct access to intracellular mitochondria. Considering this, the study of interaction of myoglobin with isolating mitochondria made good sense.

In fact, using isolated mitochondria we found that myoglobin induces the same changes in mitochondrial functions as was found for myoglobinuria *in vivo*. The rise of respiration rate in state 4 and the drop of respiratory control were arguments in proof of mitochondrial uncoupling and accumulation of MDA directly pointed to that deleterious effect of myoglobin apparently involved lipid peroxidation. Also, the development of oxidative stress and the loss of mitochondria potential in tubule cells loaded with myoglobin were observed as well. The key role in mitochondrial lipid peroxidation probably belongs to iron ions released from the myoglobin heme since this process was sensitive to Fe-chelator DFO (Fig. 3A). The lack of mitigating effect of DFO and antioxidant SkQ1 on the mitochondrial coupling after incubation with myoglobin was quite unexpected. Both compounds causing elevation of MDA products apparently via induced lipid peroxidation did not restore the respiration control, decreased during myoglobin action. Due to the ability of DFO to reduce ferryl myoglobin, we still cannot exclude the possibility that since lipid peroxidation does occur in this system, this might suggest that ferryl myoglobin is generated and the presence of DFO lowers the toxicity of myoglobin through its reducing capacity. Unfortunately, the usage of other reducing agents (such as ascorbate which lowered lipid peroxidation) seems to be not optimal since we cannot exclude the general shift of the redox state in the entire system (mitochondrial

matrix) towards more reduced one which may affect the antioxidative capacity of the system.

We found that PTP inhibitor, cyclosporine A, prevents myoglobin-induced mitochondrial damage. It not only restored the respiratory control, being felt during incubation with myoglobin, but also reduced the accumulation of MDA products. Based on these facts one can suppose two tentative pathways of myoglobin effect on mitochondria. From one side, it could promote the lipid peroxidation and on the other hand, it could induce mitochondrial permeability transition and these processes within certain limits apparently proceed quite independently. On the other hand, the inhibition of PTP partially reduced the lipid peroxidation, proving the pore to be one of the key players in myoglobin-mediated oxidative stress in mitochondria. But based on the absence of restoring effects of DFO and antioxidant SkQ1 on the mitochondrial coupling we conclude that suppression of lipid peroxidation cannot prevent the mitochondria dysfunction.

Another important finding was the demonstration of NO synthesis activation in mitochondria exposed to myoglobin. NO participation in regulation of damage during rhabdomyolysis was suggested in earlier reports [13,18] with later evidence of positive influence of NO-donors on kidney functional recovery after rhabdomyolysis [50]. In our experiments, we revealed the increase of nitrite level in the blood of animals after rhabdomyolysis with the non-specific inhibitor of NOS, L-NAME being protective from this rise which was explained by the enhanced generation of NO by NOS under rhabdomyolysis. NO is known to be an important regulator of mitochondria functions, with respiratory components as primary targets possibly attenuating ROS generation [27]. Earlier we demonstrated the rise of NO synthesis in mitochondria of the kidney exposed to ischemia/reperfusion [7]. Since in this study the nitrite abundance was blunted by DFO and specific mitochondria-targeted antioxidant SkQ1, we can speculate that myoglobin itself, iron ions released from it and induced ROS generation as well activate mitochondrial NOS.

In conclusion, we found mitochondria to play an important role in the development of kidney dysfunction under rhabdomyolysis with the involvement of ROS and RNS. In this case, mitochondria are not only the target for myoglobin and iron ions released from it but also a transmitter and induced source of these reactive molecules. All of these factors affect mitochondrial respiration, which apparently contributes greatly to the kidney pathological changes, possibly resulting in kidney cells death and ARF development. Lipid peroxidation of mitochondrial membranes may become a crucial factor of myoglobin-induced damage of mitochondria, which can be developed after direct interaction of mitochondria with myoglobin. Participation of mitochondrial NOS in these processes opens new horizons in the system of renal cell interactions with myoglobin, which needs further investigation.

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