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The role of myoglobin degradation in nephrotoxicity after rhabdomyolysis

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ABSTRACT

The fate of myoglobin in renal cells was explored in an animal model of rhabdomyolysis known as the pathology highly related to oxidative stress resulting in impairment of renal functioning. The working hypothesis was that the proper degradation of myoglobin in rhabdomyolytic kidney can activate the reparative processes in the tissue. We found that incubation of myoglobin with kidney cells causes its accumulation in the cytoplasm. In rhabdomyolytic rats, the level of heme and free iron in cytoplasm and mitochondria of kidney cells is remarkably increased while inhibition of proteolysis results in further elevation of myoglobin content in the renal tissue. Heme oxygenase and ferritin levels were found to be increased in the kidney tissue at rhabdomyolysis and simulating conditions performed by i/v injection of myoglobin. In addition, the level of peroxidized lipids was high in rhabdomyolytic kidney and became even higher after inhibition of proteolysis by aprotinin. Elevated levels of carbonylated proteins were also observed after rhabdomyolysis, however, if prior to induction of rhabdomyolysis the injection of myoglobin was done, the level of carbonylated proteins dropped versus unprimed kidney tissue thus affording protection to the kidney against oxidative stress. Injection of myoglobin to the rat results in impairment of renal functioning and inhibition of myoglobin degradation in the rhabdomyolytic animal aggravates acute renal failure, demonstrating that degradation of myoglobin is somehow beneficial although it may result in undesired release of free iron which can participate in toxic redox cycling.

1. Introduction

Myoglobin is suggested to be the main nephro-pathogenic factor in rhabdomyolysis. After release from damaged muscle tissues into circulating blood, it is absorbed by the kidney where it forms deposits in the lumen of the renal tubules. Such myoglobin deposition associates with impairing the primary urine flow and reabsorption, apparently through induction of oxidative stress with subsequent nephron cell death [1]. Recent data show that myoglobin induces lipid peroxidation of cellular membranes ultimately producing malondialdehyde as well as isoprostanes [2] (products of arachidonic acid oxidation which are very potent vasoconstrictors), causing further impairment of the tissue functioning burdened by resultant ischemia. The heme moiety of myoglobin is thought to play a key role in oxidative injury to the tissue. The iron in the heme may change its valence from Fe^{+2} (ferrous) to Fe^{+3} (ferric) state and back depending on the intracellular redox state thus forming a redox cycle as a result of the heme iron autoxidation and reduction [3].

The mechanism of the myoglobin effect on renal tubule cells remains unclear. Earlier, we have shown that mitochondria are deeply involved in oxidative stress associated with myoglobinuria [4]. After rhabdomyolysis induction, the respiratory control in mitochondria isolated from rat kidneys is remarkably suppressed and, conversely, malondialdehyde level is elevated. The key role of oxidative stress and mitochondria in acute kidney injury in rhabdomyolysis has also been demonstrated using mitochondria-targeted antioxidants which effectively prevented oxidative renal damage and dysfunction [5].

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Mechanisms of myoglobin interaction with kidney cells, in particular, those of its degradation and utilization by renal tubule cells need further detailed analysis. It has been hypothesized that endocytosis receptors megalin and cubilin may be involved in myoglobin absorption by tubule cells [6] and indeed, knocking-out of the megaline gene in mice has been shown to significantly reduce accumulation of myoglobin in tubule cells and its nephrotoxicity [6]. Obviously, after being absorbed by a tubule epithelium cell, myoglobin may undergo modifications, for example, through proteolysis by lysosomal enzymes, which has been supported by the fact that lysosomal cathepsins B and L activity increases after myoglobin injection [7].

In the present study, we investigated the possible role of the proteolytic cleavage of myoglobin in the development of its toxic effects on renal cells.

2. Methods

2.1. Modelling of rhabdomyolysis in animals

The study was performed on male outbred white rats weighing from 200 to 250 g kept in vivarium on a standard diet. Experimental procedures were conducted in accordance with the European Community Council directives 86/609/EEC and the study was approved by the Animal Ethics Committees of the Moscow State University (the Protocol Registration number 4/13 from April 8, 2013). Rhabdomyolysis was induced according to a standard protocol by administering 50% glycerin water solution into the muscles of the rats' paws, 10 ml per kg, a half-dose into each paw [8]. Before administration, the animals were kept on a normal diet, but deprived of water for 24 h. At different time points after the induction, the rats were euthanized by decapitation and their blood and kidneys were collected for determination of biochemical parameters.

In some experiments, the animals received horse myoglobin (Sigma, USA) intravenously in a saline 10 μmol per kg as a single dose or 30 μmol per kg three times with 30 min intervals between injections (total dose: 90 μmol per kg).

2.2. Malondialdehyde (MDA) and carbonylated protein detection in rat kidney tissue

MDA was detected using thiobarbituric acid (TBA) following a standard protocol [9]. The incubation mixture contained 1% H_3PO_4, 0.8% TBA and analyzed sample in the 3:1:0.9 proportion. The sample was dissolved to the final concentration of 1.5–6 mg protein/ml in 10 mM EDTA with 1.15% KCl. The mixture was boiled for 45 min, cooled, and centrifuged at 12, 000 g for 10 min. The optical density of the supernatant was measured at 532 nm with a Hitachi-557 spectrophotometer. 1′,3,3'-tetraethoxypropane (Sigma, USA) solution was used as reference.

The quantitative detection of oxidized proteins in the samples was performed using the commercial OxyBlot™ kit (Millipore, USA). The method is based on the detection of carbonyl groups joint to the side chains of amino acids (lysine, arginine, proline, and threonine) in oxidative modifications mediated by reactive oxygen species or other radicals. Carbonyl groups specifically interact with 2,4-dinytrophenylhydrazine resulting in formation of dinitrophenylhydrazone (DNP) groups in proteins. After such modifications the proteins were separated using the standard protein electrophoresis method, transferred onto a PVDF membrane and incubated with anti-DNP-antibodies. The protein loci of interest on the membrane were detected using ECL chemiluminescent substrate.

2.3. Immunoblotting

The proteins were separated by tris-tricine electrophoresis in 16.5% PAGE according to Laemmli in denaturating conditions in a BioRad chamber and transferred onto a PVDF membrane (Amersham Pharmacia Biotech, Rainham, UK). The transfer was performed with a semi-dry method for 30 min at 300 mA using a BioRad transblot chamber in a transfer buffer 48 mM Tris-HCl, 39 mM glycine, 0.0375% sodium dodecyl sulfate, 20% ethanol, pH 8.3). After the transfer the membrane was blocked overnight in 3% dry milk, dissolved in PBS with 0.1% twin-20, washed in PBS three times and incubated for one hour at 37 °C with the primary antibodies against: myoglobin (Santa-Cruz Biotechnology, USA), heme oxygenase (Abcam, UK), ferritin (FTH1, CellSignaling, USA). The membrane was washed in PBS-Tween and incubated for one hour with secondary antibodies conjugated with horseradish peroxidase (anti-rabbit 1:12000, (Sigma, USA)). The detection was performed using the ECL kit (Amersham Pharmacia Biotech, Rainham, UK) with the exposure to the Kodak film.

2.4. Renal tubular cells culture

Kidneys were excised aseptically from 3 to 7 day old rats, renal cortex was homogenized and placed in balanced Hanks solution at pH 7.4. After several washes the dispensed tissue was placed in 0.1% collagenase and incubated for 20–30 min at 37 °C. The final suspension was centrifuged for 3 min at 50 × g. The pellet was resuspended in ~10 ml of DMEM/F12 medium supplemented with 10% fetal calf serum (FCS) and kept for 2 min, after which the supernatant was transferred to another tube and the pellet was repeatedly resuspended. After 10 min, the renal tubules were pelleted, and dissociated cells remaining in the suspension were discarded. The pellet was resuspended in DMEM/F-12 containing 10% FCS and seeded in culture plates and glass-bottom dishes. Myoglobin solution was added to the cultured cells to concentration 500 μM and incubated for 60 min at 37 °C in DMEM/F-12 medium containing 10 mM Hepes-NaOH. After immunocytochemical staining, cells were analyzed in glass-bottom dishes with an LSM510 inverted confocal microscope (Carl Zeiss Inc., Jena, Germany).

2.5. Detection of heme and iron ions

Heme was detected by spectroscopy as described earlier [10]. The technique is based on the formation of a pyridine-hemochrome complex: the heme-pyridine complex optical density gap between wavelengths 556 nm and 540 nm is proportional to the heme concentration. To define the heme concentration, 0.2 ml of mitochondrial or cytoplasm sample (previously dissolved 5–10 times in 50 mM potassium phosphate buffer) is added to 0.8 ml of 20% pyridine on 50 mM NaOH. The hemochrome was first oxidized by 100 mM $K_3Fe(CN)_6$ (reference value) and then reduced by sodium dithionite. For the reduced hemochrome, a spectrum from 520 nm to 600 nm was acquired, with a characteristic peak at 556 nm and minimum at 540 nm. The heme concentration was calculated through the formula (D(556)-D(540))/20.7.

The free iron ions were detected using the mordant blue technique [11] which in the presence of cetyltrimethyl-ammonium bromide form a complex with an absorbance maximum at 637 nm. The method allows to detect ferrous and ferric ions. The commercial Iron Chromazurol (EliTech, France) agent was used in compliance with the manufacturer's recommendation. After 15-min incubation, the optical density was measured using a Hitachi 557 spectrophotometer. A standard iron chloride solution was used as control.