Free Radical Biology and Medicine 89 (2015) 300–310

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

The cytoprotective effect of nitrite is based on the formation of dinitrosyl iron complexes

Peter Dungel, Martin Perlinger, Adelheid Weidinger, Heinz Redl, Andrey V. Kozlov*

Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, AUVA Research Center, Austrian Cluster for Tissue Regeneration, A-1200 Vienna, Austria

ARTICLE INFO

Article history:
Received 27 March 2015
Received in revised form
26 August 2015
Accepted 28 August 2015
Available online 28 September 2015

Keywords:
Mitochondria
Rats
Iron ions
Nitrite
Cytoprotection
Lipid peroxidation
Cytochrome c

ABSTRACT

Nitrite protects various organs from ischemia–reperfusion injury by ameliorating mitochondrial dysfunction. Here we provide evidence that this protection is due to the inhibition of iron-mediated oxidative reactions caused by the release of iron ions upon hypoxia. We show in a model of isolated rat liver mitochondria that upon hypoxia, mitochondria reduce nitrite to nitric oxide (NO) in amounts sufficient to inactivate redox-active iron ions by formation of inactive dinitrosyl iron complexes (DNIC). The scavenging of iron ions in turn prevents the oxidative modification of the outer mitochondrial membrane and the release of cytochrome c during reoxygenation. This action of nitrite protects mitochondrial function. The formation of DNIC with nitrite-derived NO could also be confirmed in an ischemia–reperfusion model in liver tissue. Our data suggest that the formation of DNIC is a key mechanism of nitrite-mediated cytoprotection.

1. Introduction

For decades nitrite was considered to be a stable end product of nitric oxide (NO) metabolism, but it has recently been rediscovered as a key reservoir for NO [1–3]. Nitrite is formed in blood via the reaction between endogenous NO and ceruloplasmin [4]. Under physiological conditions, nitrite is present in the blood in the nanomolar range [5], but it can reach micromolar levels in the blood of highlanders [3,6], suggesting a particular role of nitrite under hypoxic conditions. In fact, it has been documented in a number of publications that nitrite protects tissues against hypoxia/ischemia-induced injury in organs such as the brain, liver, and heart [7–10]. Moreover, in all these studies, nitrite was protective if given before the onset or before the end of hypoxia/ischemia, but not after the onset of reoxygenation. Scavenging of NO abolished the cytoprotective effects of nitrite [11], suggesting NO as the active intermediate of nitrite action. Mitochondria [12], hemoglobin (Hb) [13,14], xanthine oxidoreductase (XOR) [15–17], and myoglobin (Mb) [18–20] have been shown to possess efficient nitrite reductase activity. The reduction of nitrite by Hb, Mb, and XOR has been extensively implicated in the regulation of hemodynamics, and a solid body of evidence confirms the important contribution of these proteins to cGMP-mediated vasodilation [21]. In contrast to vasodilatory actions, the mechanism of nitrite/NO-mediated cytoprotection is still not understood. However, it has been suggested that it targets reactive oxygen species (ROS) and mitochondrial dysfunction typically occurring during reperfusion [20,22]. This is associated with the opening of the mitochondrial permeability transition pore (MPTP) [20] and the activation of AMP-kinase [23] in a ROS-dependent manner. Mitochondria are the major intracellular generators of superoxide anions. Superoxide by itself is not very toxic, but gives rise to hydrogen peroxide, a catalytically neutral molecule, which, however, can yield very toxic hydroxyl radicals [24] in a reaction with iron ions. In fact, transition metal ions such as iron and copper ions execute the most toxic reactions, causing oxidative damage. Elevated levels of intracellular iron ions have already been associated with tissue damage at the mitochondrial level during reperfusion or reoxygenation [25], inflammation [26,27], and other conditions. Based on these data, we aimed to prove that the protective effect of nitrite upon hypoxia is based on a mechanism controlling iron-mediated impairment of mitochondrial function.

2. Materials and methods

2.1. Isolation of mitochondria

Rats were anesthetized with 2.5% isoflurane and sacrificed by decapitation. The livers were excised and immediately placed in

* Corresponding author. Fax: +43 059393 41982.
E-mail address: andrey.kozlov@trauma.lbg.ac.at (A.V. Kozlov).

http://dx.doi.org/10.1016/j.freeradbiomed.2015.08.012
0891-5849/

ice-cold preparation buffer containing 0.25 M sucrose, 10 mM Tris-HCl, 0.5 mM EDTA, and 0.05% essentially fatty-acid-free bovine serum albumin at pH 7.2. After removal of fat and connective tissue the livers were washed, chopped into pieces, and homogenized in preparation buffer using a Potter–Elvehjem tissue grinder. Mitochondria were then isolated by four consecutive centrifugation steps, each for 10 min at 4 °C. First, the homogenate was centrifuged at 900 *g*, the pellet discarded, and the supernatant centrifuged at 11,400 *g*. The pellet was resuspended in 50 ml preparation buffer containing 500 μM EDTA and again centrifuged at 11,400 *g*. Two identical consecutive washing steps followed with preparation buffer containing 100 μM EDTA. The final pellet was resuspended in 1 ml preparation buffer (100 μM EDTA). The protein concentration of the suspension of isolated mitochondria was determined using a bicinchoninic acid (BCA) protein determination assay (Micro BCA Protein Assay Kit, Thermo Scientific, Austria) according to the manufacturer's instructions. For further experiments, mitochondrial suspensions were diluted with preparation buffer to a final protein concentration of 50 mg/ml.

2.2. Experimental setup: mitochondrial model of hypoxia/reoxygenation

The objective of this study was to clarify whether nitrite-mediated protection of mitochondria is based on the mitigation of redox-active ferrous iron released under ischemic conditions. To test our hypothesis, we developed a model of hypoxia/reoxygenation injury in isolated rat liver mitochondria. For the development of this model we assumed several important issues: (1) The concentration of mitochondria in in vitro experiments had to be substantially lower than the content of mitochondria in tissue. More highly concentrated suspensions of mitochondria could hardly be reoxygenated because of the high oxygen consumption rate. (2) The major damage to mitochondria occurs during reperfusion, but (3) the protective mechanisms require a hypoxic phase for the formation of necessary precursors. Taking into account these issues, we developed the following model.

During the hypoxic phase, the high oxygen consumption rate of isolated mitochondria (50 mg/ml), in addition to the substitution of nitrogen by oxygen in the reaction vessels, guaranteed hypoxic conditions in the samples. For efficient reoxygenation, the mitochondrial suspension was strongly diluted in incubation buffer equilibrated with air oxygen. The iron concentration is in line with our recently published data showing in an in vitro model in the liver that 1 h hypoxia results in the release of 20 μM ferrous ions [27]. The concentrations of ferrous ions and nitrite during hypoxia were much higher than those occurring/used for treatment in vivo (Supplemental Table 1), but reached these levels after dilution during reoxygenation (Supplemental Fig. 1). The high concentrations of iron and nitrite did not affect mitochondrial function (Complexes II, III, IV, V) during the hypoxic phase. The data presented in Supplemental Fig. 1 show that immediately after onset of reoxygenation, mitochondrial respiration on succinate did not differ from that of normoxic controls. The impairment of mitochondrial function developed later during reoxygenation. However, some changes, such as inhibition of basal respiration and electron transport via complex I, already occurred during the hypoxic phase, but were not sensitive to the presence of nitrite. During hypoxia, mitochondria were incubated with or without ferrous iron and with or without nitrite for 15 min. Nitrite was applied during hypoxia, as this is in line with in vivo models, where nitrite was also applied during or minutes before the end of ischemia [9] and has been shown to activate nitrite-mediated protection. The impact of iron and nitrite on isolated mitochondria was investigated after 15 min hypoxia (HOX) and 15 min reoxygenation (ReOX) and compared to mitochondria incubated under normoxic (NOX) conditions (Supplemental Fig. 1).

Incubation under hypoxic condition was performed in glass vials sealed with a septum. A 50 μl suspension of mitochondria was mixed with either 10 mM succinate or 5 mM glutamate and 5 mM malate, as well as nitrite and/or iron. In selected experiments, the highly specific iron chelator desferrioxamine B (Desferal, 20 μM) was used instead of nitrite. The glass vials were flushed with nitrogen and mitochondria were incubated for 15 min under hypoxia at room temperature (RT). For determination of DNIC, samples were drawn directly from the glass vial at the end of hypoxia. For all other analyses mitochondria were transferred to a high-resolution oxygraph (Oroboros, Innsbruck, Austria) diluting the samples 1:100 in incubation buffer, at which point reoxygenation started. Respiratory activity was measured immediately at the onset of reoxygenation, as well as at the end of the 15 min reoxygenation period, as described below. Alternatively, samples for ROS and TBARS were drawn at given time points.

2.3. Mitochondrial respiration

Mitochondrial respiration was determined using a high-resolution respirometer (Oxygraph-2 k, Oroboros Instruments, Innsbruck, Austria). Isolated mitochondria were added to incubation buffer containing 105 mM KCl, 5 mM KH_2PO_4, 20 mM Tris-HCL, and 5 mg/ml fatty acid-free bovine serum albumin (pH 7.2, 25 °C) at a final concentration of 0.5 mg/ml. Mitochondrial respiration was analyzed according to the method of Chance and Williams [28]. Basal respiration was defined as respiration not linked to ATP synthesis and is represented by respiration states 2 (before addition of ADP) and 4 (after complete consumption of ADP). Mitochondrial respiration at state 2 was determined using 5 mM glutamate/5 mM malate as substrates for complex I or 1 ng/ml rotenone/10 mM succinate as substrate for complex II. State 3 respiration was determined after application of adenosine diphosphate (ADP, 62.5 μM). Uncoupling of mitochondrial respiration was induced by application of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 0.5 μM). Integrity of the outer mitochondrial membrane was investigated by adding exogenous cytochrome c (cytochrome c, 2.5 μM). Mitochondrial respiratory activity was determined (i) before hypoxia, (ii) after 15 min of hypoxia immediately at the onset of reoxygenation, (iii) at the end of the 15 min reoxygenation phase. Iron and nitrite were added to the respective groups as solutions of $FeSO_4$ and $NaNO_2$ before the onset of hypoxia.

2.4. Effect of nitric oxide on the rate of lipid peroxidation in liver homogenate

Direct effects of NO on lipid peroxidation were determined in an in vitro model of liver homogenate. Briefly, rat liver homogenate was prepared as described. Samples of 1 mL were treated with 10 μM myxothiazol, a specific inhibitor of mitochondria, in order to directly analyze the effects of NO on lipid peroxidation. NO was added as 50 μL of NO-saturated saline (2 mM). Lipid peroxidation was induced by 20 μM $FeSO_4$ and 200 μM sodium ascorbate. Controls were left untreated. Samples were incubated for 30 min at 37 °C and TBARS determined as described below.

2.5. Lipid peroxidation

The accumulation of TBARS, a lipid peroxidation product, induced by iron in mitochondria is well established [29]. Mitochondrial suspensions were treated as described above and samples were drawn at different time points during reoxygenation. Samples were immediately frozen in liquid nitrogen and stored at -20 °C until further procedures. For analysis, 100 μl of