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Nitric oxide scavenging modulates mitochondrial dysfunction induced by hypoxia/reoxygenation

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Abstract:
The objective of the present study was to delineate the role of excessive accumulation of mitochondrial nitrogen species contributing to oxidative stress induced by hypoxia/reoxygenation in isolated mitochondria. The present study shows that incubation of isolated rat heart mitochondria under hypoxic, but not anoxic conditions, followed by reoxygenation decreases the rate of mitochondrial oxygen consumption, mitochondrial membrane potential, and calcium retention capacity. These alterations were prevented, at least in part, by 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), a nitric oxide (NO) scavenger, N^G-nitro-L-arginine-methyl ester (L-NAME), a broad-spectrum NO synthase inhibitor, or tempol, a superoxide dismutase mimetic and catalytic scavenger of peroxynitrite-derived radicals. In conclusion, these findings suggest a crucial role for nitric oxide pathways in cardiac oxidative stress induced by hypoxia/reoxygenation.

Key words:
mitochondria, respiration, membrane potential, nitric oxide, peroxynitrite

Introduction

The inherent vulnerability of cardiomyocytes to oxygen deprivation and metabolic stress contributes to myocardial dysfunction in many heart diseases [16]. Experiments with isolated cardiac myocytes have demonstrated that hypoxia increases reactive species production. Excessive accumulation of reactive oxygen (ROS) and nitrogen species (RNS) and their uncontrolled oxidation of cellular components are referred to as oxidative stress [10, 16]. Mitochondria remain one of the main cellular sources of oxidative stress and play a crucial role in oxidative injury during hypoxia and reoxygenation [20]. The mitochondrial respiratory chain at complexes I and III has long been considered the major site of intracellular ROS production. Several studies have reported that exposing cells or tissues to hypoxic conditions increases oxidative stress [9]. Convincingly, mutant cells that lack mitochondrial respiration do not show this increase, indicating that mitochondrially generated ROS or RNS are responsible for this increase [8]. In hypoxic cells, this increase is mitigated by a defective cytochrome c or by inhibiting the expression of the Rieske iron-sulfur protein in complex III. Both condi-

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tions affect the oxidation of ubiquinol to ubisemiquinone, suggesting that the Q cycle in complex III contributes to the increased oxidative stress during hypoxia [8, 11].

Several studies suggest that mitochondria may produce nitric oxide (NO) *via* the mitochondrial NO synthase (mtNOS); however, the presence of a constitutively active mtNOS and the determination of mtNOS activity are still controversial [13]. Recently, another pathway for mitochondrial NO synthesis that uses the respiratory chain to reduce nitrite (NO_2) to NO has been revealed in several mammalian cells [5]. Because cytochrome oxidase produces NO from nitrite at low oxygen concentrations, it is possible that the mitochondrially generated oxidants for which concentration increases under hypoxic conditions are not only ROS, but also peroxynitrite ($ONOO^-$), which is formed from a reaction between mitochondrially generated superoxide anion and NO [7]. Within the mitochondrial matrix, peroxynitrite can irreversibly inhibit complexes I and II of the respiratory chain as well as ATP synthase. Peroxynitrite contributes to an increase in hydroxyl radical production, which in turn causes oxidation of lipids, proteins, and DNA [19].

Because mitochondria are the primary sites for oxidative and nitrosative stress within cardiac cells, it seems reasonable that targeting these organelles with nitric oxide and peroxynitrite scavengers could be a particularly effective strategy to protect the myocardium. Numerous compounds with these general characteristics have been synthesized and evaluated in a variety of *in vitro* and *in vivo* models of redox stress. In the present study, we tested whether 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), a NO scavenger, N^G-nitro-Larginine-methyl ester (L-NAME), a broad spectrum NO synthase inhibitor, or 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (tempol), a superoxide dismutase mimetic and catalytic scavenger of peroxynitrite-derived radicals, would reduce mitochondrial dysfunction induced by hypoxia and reoxygenation.

Materials and Methods

Animals used

Adult male (250–300 g) Sprague-Dawley rats (Charles River Lab, France) were used to prepare cardiac mitochondria. All experiments were conducted in accordance with the European Institute for Health guidelines for the use of laboratory animals.

Reagents

Carboxy-PTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide], a nitric oxide (NO) scavenger, was purchased from Cayman Chemical, Ann Arbor, MI, USA. Carboxy-PTIO reacts with NO to form nitric dioxide and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl (carboxy-PTI). N^G-nitro-L-arginine-methyl ester (L-NAME) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (tempol) were purchased from Sigma (Sigma, L'Isle d'Abeau-Chesnes, France). Suspensions of mitochondria were incubated with 10 μM carboxy-PTIO, 10 mM L-NAME, or 2.5 mM tempol.

Mitochondria preparation

As previously described [14], the heart was excised and then rinsed in buffer A (sucrose 300 mM, TES 5 mM, EGTA 0.2 mM, pH 7.2, at 4°C). After homogenization, a sample of 1 ml was first centrifuged at 800 × g for 10 min, and then the supernatant was centrifuged at 8,800 × g for 10 min. The pellet was suspended in buffer A and centrifuged at 8,800 × g for 10 min. The final mitochondria pellet was suspended either in respiration medium or in the medium used for mitochondrial membrane potential evaluation. The purity and integrity of isolated mitochondria were assessed by measuring the specific activities of NADPH-cytochrome c reductase, a reticulum-specific enzyme, and cytochrome c oxidase, an inner membrane enzyme [23].

In vitro hypoxia and near anoxia

To obtain hypoxic samples, 1 ml of aerated respiration medium MiR05 (sucrose 110 mM, EGTA 0.5 mM, $MgCl_2$ 3.0 mM, K-lactobionate 60 mM, KH_2PO_4 10 mM, taurine 20 mM, HEPES 20 mM, and 1.0 g/l BSA, pH 7.1, 25°C) was added to tightly sealed chamber thermostated at 25°C and equipped with an oxygen-sensitive sensor (Oxygraph 2k, Oroboros, Innsbruck, Austria) that continuously monitored the oxygen concentration. From the initial volume (1 ml) of the sealed chamber, a volume of 0.1 ml of the buffer was removed, and the remaining buffer was