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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

purged with N_2. Once the O_2 reached the desired concentration, N_2 was stopped, and the mitochondria sample (0.5 mg in 0.1 ml) was added to the chamber. After 15 min of stirring, the chamber cap was gently lifted, and the suspensions were reoxygenated with air for 15 min and referred to as hypoxia/reoxygenation. In another series of experiments, near anoxia was reached within seconds, as mitochondria consumed all available oxygen in the chamber. Throughout the anoxic period, the oxygen concentration was at zero within the closed chamber that contained the mitochondrial suspensions. After 15 min of anoxia, suspensions were exposed to room air for 15 min and referred to as anoxia/reoxygenation.

Mitochondrial respiration

Mitochondria were suspended in the respiration medium MiR05 at a concentration of 500 µg per 100 µl. The following respiration parameters were evaluated with high-resolution Oxygraph 2k (Oroboros, Innsbruck, Austria), as previously described [14]: state 2 respiration rate (oxygen uptake with glutamate 5 mM and malate 2 mM in the absence of exogenous ADP) and state 3 respiration rate (oxygen uptake with 5 mM, malate 2 mM, and ADP 500 µM).

Mitochondrial membrane potential measurement and calcium retention capacity

In separate experiments, isolated mitochondria (1 mg/ml proteins) were suspended in buffer B (sucrose 250 mM, Tris-MOPS 10 mM, glutamate-Tris 5 mM, malate-Tris 2 mM, Pi-Tris mM, EGTA-Tris 0.02 mM, pH 7.4, at 25°C) in a multiport measurement chamber (NOCHM-4, WPI, Aston, UK) equipped with Ca^{2+}, tetraphenylphosphonium (TPP^+)-selective microelectrodes, and reference electrodes (WPI, Aston, UK), as previously described [6, 12, 14].

First, mitochondria were gently stirred for 1.5 min in buffer C containing 1.5 µM TPP^+ (Sigma, Saint Quentin, France). Calibration of the TPP^+ electrode was performed following the manufacturer's recommendations in "Determination of Membrane Potential with TPP^+ and an Ion Selective Electrode System" at http://www.oroboros.at/index.php?id=protocols_tpp-membranepotential. Mitochondrial transmembrane potential ($\Delta\Psi m$) was estimated according to the transmembrane distribution of TPP^+ [6] and calculated as $59\log(v/V) - 59\log(10^{\Delta E/59} - 1)$, where v is the mitochondrial matrix volume (1.1 µl/mg mitochondrial protein), V is the volume chamber (1 ml), and ΔE is the voltage difference (mV) between measurements before and after 2,4-dinitrophenol (DNP) treatment (50 µM), which was used to fully induce $\Delta\Psi m$ dissipation [12, 14].

In another series of experiments, mitochondria calcium retention capacity was assessed, as previously described [4, 14]. In brief, mitochondrial were gently stirred for 1.5 min in buffer B, and 20 µM $CaCl_2$ was added every 90 s using a microsyringe injector adapted to a Micro4 Pump Controller (UMPII and Micro4, WPI, Aston, UK). Each 20-µM $CaCl_2$ pulse was detected as a peak of extramitochondrial Ca^{2+} concentration. Ca^{2+} was then rapidly taken up by the mitochondria, resulting in a return of extramitochondrial Ca^{2+} concentration near baseline levels.

Statistics

The results were analyzed using ANOVA procedures. When a significant difference was found, the specific differences between groups were tested using a sequentially rejective Bonferroni procedure. A value of $p < 0.05$ was considered statistically significant.

Results and Discussion

Effects of *in vitro* hypoxia and near anoxia in mitochondrial preparations

Under normoxic conditions (control), the rate of oxygen consumption (state 2) was 105 ± 25 pmol oxygen/s/mg. Addition of ADP increased oxygen consumption (state 3) to 880 ± 135 pmol oxygen/s/mg. During normoxia, the mitochondrial membrane potential at steady state was –216 ± 5 mV, and the calcium retention capacity was 175 ± 30 nmol/mg mitochondrial protein. After 15 min of hypoxia (2–4 mmHg) followed by reoxygenation, the rate of mitochondrial oxygen consumption, mitochondrial membrane potential, and calcium retention capacity markedly decreased; near anoxia conditions had no effect on these mitochondrial parameters (Tab. 1). These results are consistent with previous studies showing that hypoxia/reoxygenation, but not anoxia, may induce injury to cardiac mitochondria, character-

Tab. 1. Mitochondrial parameters in response to hypoxia and near anoxia followed by reoxygenation

	State 2	State 3	Membrane potential	Ca^{2+} retention capacity
Control	105 ± 25	1020 ± 65	–216 ± 5	175 ± 30
Hypoxia	80 ± 5*	845 ± 35*	–195 ± 3*	145 ± 30*
Anoxia	100 ± 12	1175 ± 60	–219 ± 1	195 ± 35

The rates of respiration are given in picomoles of O_2 per second per milligram wet weight (pmol O_2/s/mg). The state 2 respiration rate (state 2) was determined with glutamate (5 mM) + malate (2 mM) without ADP. The state 3 respiration rate (state 3) was determined in the presence of ADP (500 μM) with mitochondrial substrates added to the preparation. Mitochondrial membrane potential is expressed in mV, and Ca^{2+} retention capacity is expressed in nmol/mg mitochondrial protein. The data are expressed as the mean ± SEM. The results were analyzed with one-way ANOVA and Bonferroni's multiple comparison *post-hoc* adjustment (n = 10 in each group; * indicates $p < 0.05$)

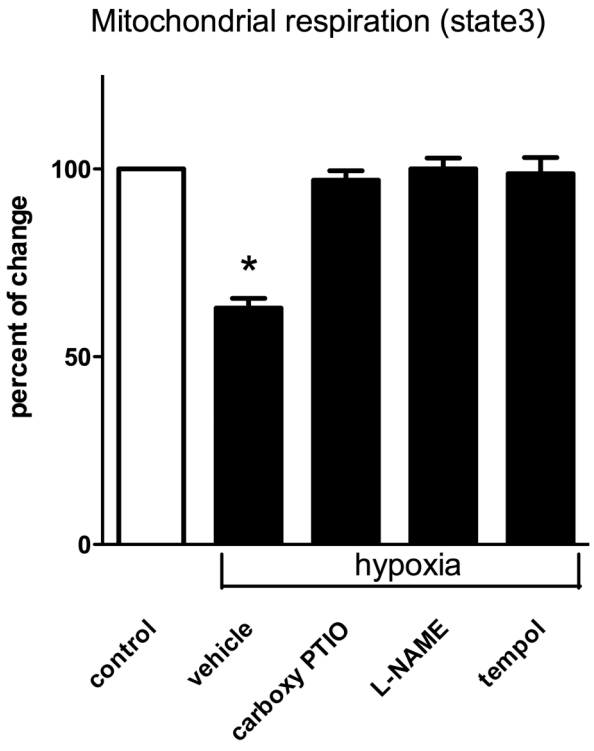

Fig. 1. Changes in mitochondrial respiration (state 3) induced by carboxy-PTIO (10 μM), L-NAME (10 mM) or tempol (2.5 mM) in suspensions of mitochondria exposed to hypoxia and reoxygenation. The results (the mean ± SEM) are expressed as percent of control values. * Indicates $p < 0.05$

ized by the inhibition of respiration, dissipation of membrane potential, cytochrome c release, and major oxidative stress [17, 18, 21, 24]. Indeed, during anoxia, the mitochondrial proton motive force collapses unless ATP is provided to be used by ATPase to generate this force. During hypoxia, isolated mitochondria are able to support the membrane potential until the O_2 supply becomes very low, whereas near anoxia typically fully dissipates the membrane potential ($\Delta\Psi m$). During hypoxia, electrons accumulate in respiratory carriers (e.g., cytochromes) and cofactors (e.g., NADH) to create a reductive stress that promotes the generation of oxygen radicals when molecular oxygen once again becomes available. *In vitro* hypoxia/reoxygenation of isolated mitochondria is a deleterious stimulus mediated by oxidative stress, which is largely prevented during anoxia/reoxygenation. A global sealing to metabolite exchange occurs in mitochondria during anoxia. This condition is characterized by the retention of the mitochondrial proton motive force (pH gradient and membrane potential) despite the absence of energy input from electron flow and ATP hydrolysis [1–3].

Nitric oxide scavenging preserved mitochondrial function in hypoxia/reoxygenation conditions

In contrast to mitochondria exposed to the vehicle control, mitochondrial suspensions incubated with either carboxy-PTIO (10 μM), L-NAME (10 mM) or tempol (2.5 mM) throughout the hypoxic challenge maintained a vigorous respiratory response to ADP (500 μM) after reoxygenation (Fig. 1). Reports of a mitochondrial NO synthase in heart tissues led us to determine the effects of the competitive inhibitor L-NAME on cardiac mitochondrial function in rats. Similarly, increased accumulation of NO within mitochondria led us to use the NO scavenger carboxy-PTIO. As it is possible that mitochondrially generated oxidants under hypoxic conditions include peroxynitrite ($ONOO^-$), we also tested tempol, a superoxide dismutase mimetic and catalytic scavenger of peroxynitrite-derived radicals. Previous studies have shown that these compounds may target the mitochondria [15, 22]. As these compounds prevented the reduction in rate of mitochondrial oxygen consumption (state 3) induced by hypoxia/reoxygenation, we evaluated their effects on mitochondrial membrane potential and calcium retention capacity. Although carboxy-PTIO (10 μM), L-NAME (10 mM), or tem-

Tab. 2. Changes in the mitochondrial membrane potential and Ca^{2+} retention capacity during reoxygenation after a 15-min period of hypoxia

	Membrane potential	Ca^{2+} retention capacity
Vehicle	–195 ± 3	145 ± 30
Carboxy-PTIO	–211 ± 3*	170 ± 15*
L-NAME	–215 ± 2*	175 ± 5*
Tempol	–220 ± 2*	180 ± 15*

Changes in the mitochondrial membrane potential (mV) and Ca^{2+} retention capacity (nmol/mg mitochondrial protein) were measured in the presence of carboxy-PTIO (10 μM), L-NAME (10 mM) or tempol (2.5 mM) in suspensions of mitochondria exposed to hypoxia and reoxygenation. The data are expressed as the mean ± SEM. The results were analyzed with one-way ANOVA and Bonferroni's multiple comparison *post-hoc* adjustment (n = 10 in each group; * indicates $p < 0.05$ compared with the vehicle control)

pol (2.5 mM) had no detectable effects in control mitochondria (data not shown), these drugs supported, at least in part, mitochondrial membrane potential recovery and calcium retention capacity during reoxygenation (Tab. 2). These findings further support the contention that mitochondrial sources of NO modulate mitochondrial functions during hypoxia/reoxygenation.

In conclusion, our study in isolated mitochondria provides new evidence that hypoxia/reoxygenation injury may be largely prevented by nitric oxide scavenging strategies. It should be stated that these results were obtained under conditions of simulated hypoxia/reoxygenation in isolated cardiac mitochondria. To determine the potential consequences of our findings in the prevention of myocardial ischemia reperfusion injury, these pharmacological approaches should be further tested in whole heart preparations.

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