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Hypoxia/Reoxygenation of Isolated Rat Heart Mitochondria Causes Cytochrome c Release and Oxidative Stress; Evidence for Involvement of Mitochondrial Nitric Oxide Synthase

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Abstract

The objective of the present study was to delineate the molecular mechanisms for mitochondrial contribution to oxidative stress induced by hypoxia and reoxygenation in the heart. The present study introduces a novel model allowing real-time studying mitochondria under hypoxia and reoxygenation, and describes the significance of intramitochondrial calcium homeostasis and mitochondrial nitric oxide synthase (mtNOS) for oxidative stress. The present study shows that incubating isolated rat heart mitochondria under hypoxia followed by reoxygenation, but not hypoxia *per se*, causes cytochrome *c* release from the mitochondria, oxidative modification of mitochondrial lipids and proteins, and inactivation of mitochondrial enzymes susceptible to inactivation by peroxynitrite. Those alterations were prevented when mtNOS was inhibited or mitochondria were supplemented with antioxidant peroxynitrite scavengers. The present study shows mitochondria independent of other cellular components respond to hypoxia/reoxygenation by elevating intramitochondrial ionized calcium and stimulating mtNOS. The present study proposes a crucial role for heart mitochondrial calcium homeostasis and mtNOS in oxidative stress induced by hypoxia/reoxygenation.

Keywords

mitochondria; calcium; hypoxia; reoxygenation; mtNOS; oxidative stress

1. Introduction

Reoxygenation of the hypoxic cardiac tissue (hypoxia/reoxygenation; H/R) is one of the prime mechanisms underlying cell and tissue damage in pathologic conditions including ischemic heart disease. H/R increases level of oxidative species and attenuation of those species protects cardiomyocytes from H/R-induced damage [1–3]. Mitochondria remain one of the main cellular sources of oxidative species and play a crucial role in oxidative injury during H/R [4]. Mitochondria produce nitric oxide (NO) via mitochondrial NO synthase (mtNOS) that potently produces oxidative species such as peroxynitrite [5]. mtNOS-derived peroxynitrite causes oxidative modification of mitochondrial components and releases the key mitochondrial pro-apoptotic protein, cytochrome *c* [6,7]. During H/R, formation of NO and peroxynitrite are increased that cause cardiac tissue injury [8], and H/R induced cardiac injury is diminished

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when production of peroxynitrite is inhibited [9]. The molecular mechanism by which NO and peroxynitrite contribute to mitochondrial damage during H/R is not fully understood. The present study utilized a novel in vitro model that allows studying isolated mitochondria under hypoxia or H/R. The present study shows that H/R causes mitochondrial cytochrome *c* release, induces oxidative modification of mitochondrial lipids and proteins, and inactivates mitochondrial enzymes susceptible to peroxynitrite. The present study also shows that H/R elevates intramitochondrial Ca^{2+} ($[\text{Ca}^{2+}]_m$) that serve as the mechanism underlying stimulation of mtNOS.

2. Materials and Methods

2.1. Isolation and Purification of Mitochondria and Mitochondrial Subfractions

Intact heart mitochondria were isolated from Sprague-Dawley rats (200–260 g) as described [10] with additional purification steps. All isolation and purification steps were carried out at 4 °C. Euthanasia was performed by decapitation followed by depleting the body of blood to limit exposure of mitochondria to NO reacting molecules such as hemoglobin. The thorax was opened, the heart was excised and placed in 20 ml of ice-chilled solution A, containing MSH buffer pH 7.40, (mannitol, 220 mM; sucrose, 70 mM; HEPES, 10 mM) EDTA (0.2 mM) and fatty acid free bovine serum albumin (0.1%). While in solution A, the heart was gently squeezed to further deplete the organ of blood. The heart was then placed in an ice-chilled Petri dish and dissected along the left descending coronary artery. The aorta, vessels, blood clots, fat, and connective tissues were removed. The heart muscle was washed in solution A and thoroughly minced with a Bio-Homogenizer M133/1821-0 (Biospec Products) with speed adjusted at position one. The minced tissue was transferred into a glass homogenizing tube (Fisher Scientific) and homogenized in 3 ml aliquots using a Teflon pestle (Fisher Scientific) driven at 750 RPM. To avoid heating the homogenate, the tube was placed in a “cold jacket” filled with ice and cold water. The homogenate was centrifuged for 5 min at $600 \times g$, and the supernatant was centrifuged for 10 min at $10,000 \times g$. The supernatant of the second centrifugation was discarded and the enriched mitochondrial pellet was resuspended in 1 ml MSH. The enriched mitochondrial pellet was layered on a Percoll solution (25%) and centrifuged for 30 min at $100,000 \times g$. The middle layer was extracted and washed twice in MSH for 10 min at $10,000 \times g$. The purified mitochondria pellet was resuspended in 0.5 ml MSH. The purity of the mitochondrial preparation was determined by measuring the cytochrome *a* content at 605–630 nm using the extinction coefficient of $12 \text{ mM}^{-1} \text{ cm}^{-1}$ [11]. Only mitochondria with less than 5% impurity were used in this study. Possible contamination of mitochondrial preparation with cytoplasmic proteins was ruled out by the lack of detectable cytoplasmic proteins antibody cross-reactivity in the mitochondrial preparation. The respiratory control ratio of the purified mitochondria was assessed as described [11]. Briefly, purified mitochondria (0.5 mg) were added to a 25 °C thermostated chamber containing 1 ml of buffer (pH 7.40) consisting of HEPES (100 mM), MgCl_2 (5 mM), EDTA (0.5 mM), and potassium phosphate (5 mM). At 45 sec, 105 sec, and 195 sec; rotenone (5 μM), K^+ -succinate (5 mM), and ADP (150 μM) were added, respectively. The ratio of the state 3 to state 4 respiration rates was calculated. Only mitochondria with a ratio of ≥ 8 were used in this study.

2.2. General Incubation procedures

Mitochondrial samples (1 mg protein in a final volume of 100 μL HEPES buffer pH 7.10) were incubated for 30 min on ice with occasional gentle shaking in the presence or absence of L-NMMA (100 μM), glutathione monoethyl ester (GME 100 μM ; [12,13]), or Trolox (100 μM ; Calbiochem).

2.3. In Vitro Hypoxia and H/R

To obtain hypoxia samples, 1 ml of air-saturated buffer (100 μ M HEPES pH 7.10) was added to a 25 °C thermostated tightly sealed chamber equipped with an oxygen-sensitive electrode with a 2.0 mm tip (ISO-OXY-2; 1 μ M O₂ detection limit) that continuously monitored the oxygen concentration ([O₂]; Scheme I). A portion of buffer (100 μ L) was removed and the remaining buffer was purged with N₂. Once the [O₂] reached desired concentration, N₂ was stopped and mitochondria sample (1 mg in 100 μ L) was added to the chamber. After 40 min of stirring, the samples were immediately collected on ice. To obtain H/R samples, the mitochondria were incubated for 30 min under the hypoxic conditions as above, the chamber lid was then gently lifted and the suspension was purged with O₂ for approximately one minute at which time the [O₂] reached the normoxic level. Mitochondria were incubated for 10 additional min at normoxic [O₂] and the samples, referred to as H/R, were immediately collected on ice.

2.4. Apoptosis and Oxidative Stress Markers

Cytochrome c release—A protease inhibitor cocktail consisting of leupeptin (Axxora), phenylmethanesulfonyl fluoride, pepstatin A (Axxora), and aprotinin (Sigma); (10 μ M each), [6,14] was present in the buffer during hypoxia or H/R. After hypoxia or H/R was completed, cytochrome *c* was detected by Western blot as described [6].

Lipid peroxidation (LPO)—Once hypoxia or H/R was completed LPO was determined using thiobarbituric acid assay as described [7].

Protein carbonyl formation—Once hypoxia or H/R was completed protein carbonyl formation was measured by the trichloroacetic acid assay as described [15]. The samples were precipitated by adding 1 ml of trichloroacetic acid (10%). The precipitates were suspended in 1 ml of 2,4-dinitrophenyl hydrazine (0.2%; w/v) and incubated at 37 °C for 60 min. The samples were re-precipitated with trichloroacetic acid and re-centrifuged. The precipitates of the second centrifugation were washed with ethanol: ethyl acetate (50:50) and dissolved in guanidine hydrochloride (6 mM) in phosphate buffer pH 6.50. The protein carbonyls were measured at 370 nm and expressed as pmol per mg mitochondrial protein using $\epsilon_{370\text{nm}} 21 \text{ mM}^{-1} \text{ cm}^{-1}$.

2.5. Determination of the Activity of Mitochondrial Enzymes

Aconitase activity—Once hypoxia or H/R was completed, broken mitochondria were prepared by freeze-thawing the pellets 3 times in liquid nitrogen followed by adding 4 times ice cold water containing protease inhibitor cocktail as above. Membrane rupture was tested by lack of transmembrane potential measured as described [16,17]. The broken mitochondria samples were centrifuged for 20 min at $100,000 \times g$ at 4 °C. The supernatants, referred as to the soluble fraction, were collected. The aconitase activity of soluble fraction (50 μ g) was measured as described [18,19] by following the OD of cis-aconitate (0.2 mM) as the substrate at 240 nm.

MnSOD activity—Onec MnSOD activity of soluble fraction (30 μ g) was measured by the Fridovich method [20] in the presence of KCN (2 mM).

Mitochondrial creatine kinase (mtCK) activity—The mtCK activity of soluble fraction (2.5 μ g) was measured by adding phosphocreatine (10 mM) to the coupled system for adenylate kinase and by subtracting the adenylate kinase activity from the result obtained [21].

Adenylate kinase activity—Adenylate kinase activity of soluble fraction (2 μ g) was measured at 340 nm in a reaction mixture containing ADP (1 mM), glucose (20 mM),

NADP⁺ (0.5 mM), MgCl₂ (2.5 mM), G6PDH (7.2 units), and hexokinase (6.3 units) as described [22].

SCOT activity—SCOT activity was measured as described [7]. Soluble fraction (50–100 µg) was added to Tris HCl (50 mM) pH 8.50 containing succinyl-CoA (0.1 mM), acetoacetate (5 mM), MgCl₂ (5 mM) and iodoacetamide (4 mM) and the formation of acetoacetyl-CoA was followed at 313 nm.

mtNOS Activity—Mitochondrial NOS activity was determined by using the citrulline, fluorometric, and chemiluminescence assays [7,11].

Citrulline assay Mitochondria were incubated in HEPES buffer supplemented with L-[³H] arginine (30,000 – 50,000 cpm) in the absence or presence of Ca²⁺ (20–80 µM), ruthenium red (10 µM), rotenone (5 µM), L-arginine (100 µM), or MgCl₂ (1 mM). After 20 min incubation, the mtNOS activity was terminated by addition of ice-chilled stop solution and L-citrulline was measured as described [7]. To measure heart mtNOS activity in hypoxia or H/R, the hypoxia or H/R chamber was supplemented with L-[³H]arginine and the stop solution was added at the end of hypoxia or reoxygenation.

Fluorometric assay Mitochondria (0.25 mg/ml) were incubated with the membrane-permeable NO-sensitive fluorescent probe, diaminofluorescein diacetate (DAF-2 DA; 5 µM; Calbiochem), excited at 495 nm, and the emission was collected at 515 nm as described [11].

Chemiluminescence assay Mitochondria (100 µg) were injected into the purge vessel containing vanadium chloride (0.8 % in 1M HCl) thermostated at 95 °C, and NO was measured using Chemiluminescence Analyzer (Sievers 280i) as described [7].

2.6. Determination of Intramitochondrial Calcium Concentration

Once hypoxia or H/R was completed, [Ca²⁺]_m was determined in the presence of Arsenazo III using a dual beam spectrophotometer described [7]. The Δψ was collapsed by rotenone (5 µM) and CCCP (1 µM) to allow the [Ca²⁺]_m to equilibrate with extramitochondrial buffer. At the end of the assay, EGTA (5 mM) was added to ensure the test.

A Ca²⁺-sensitive electrode was also used to determine [Ca²⁺]_m for broken mitochondria, as described [23]. In both assays calibration was obtained using known concentrations of Ca²⁺.

To measure [Ca²⁺]_m during H/R in a real-time fashion, the recently described highly sensitive fluorometric assay was used [7]. A fluorescent cuvette was equipped with a gas-exchange system and an O₂ sensor (100 µm; O₂ detection limit 0.1%–100%; response time < 5 sec) that allowed maintaining the [O₂] at the desired concentration (Scheme I). The cuvette containing 2 ml HEPES buffer (100 mM pH 7.10) was purged with N₂ until [O₂] reached the hypoxic concentration. 2 mg mitochondria loaded with 10 µM Fura-2, 15 min were added to the cuvette and incubated at hypoxia for 30 min, followed by reoxygenation for 10 min. The [Ca²⁺]_m was monitored during the entire hypoxia and reoxygenation by exciting mitochondria at dual wavelengths of peak minus isosbestic point (352–362 nm) and recording the fluorescence at 510 nm [7].

2.7. Transmembrane potential (Δψ)

The Δψ was supported by K⁺-succinate (0.8 mM) and determined at 511–533 nm using Safranin (10 µM) as described [17].

2.8. Statistics

Barograms represent Mean $\pm$ SD of $n \geq 6$. Traces are representative of $n \geq 6$. Statistical comparison was performed using ANOVA with significance set at $P < 0.05$.

3. Results and Discussion

3.1. H/R-induces cytochrome c release and oxidative stress

Release of cytochrome *c* from the mitochondria is one of the key events during apoptosis [6, 24,25]. Ischemia-reperfusion induces apoptosis [4] by igniting mitochondrial apoptosis machinery [26] and cytochrome *c* release [22]. Figure 1A shows that hypoxia at different $[O_2]$ did not cause cytochrome *c* release, however, H/R at 50 μM $[O_2]$ caused release of cytochrome *c*. Consistent with a recent report demonstrating release of cytochrome *c* upon ischemia/reperfusion in kidney mitochondria [21], this finding shows that mitochondria independent of other cellular components respond to H/R. H/R of the heart increases peroxynitrite [9] and causes mitochondrial dysfunction [4]. We and several other groups have shown that mitochondria produce peroxynitrite via mtNOS and that mtNOS-derived peroxynitrite causes release of cytochrome *c* from the mitochondria [7,17,21]. Thus we tested possible role of heart mtNOS in cytochrome *c* release and mitochondrial oxidative stress during H/R. Figure 1B shows that cytochrome *c* release induced by H/R was aggravated when mtNOS was stimulated by elevating $[Ca^{2+}]_m$ and inhibited when mtNOS was inhibited by L-NMMA. Trolox, GME, or L-NMMA did not alter cytochrome *c* release from mitochondria not treated under hypoxia or H/R. Since stimulation of mtNOS induces cytochrome *c* release and inhibiting mtNOS prevents the cytochrome *c* release [17], it is plausible that mtNOS activity was increased during H/R. Figure 1B shows that H/R-induced cytochrome *c* release was prevented when mitochondria were supplied with Trolox or GME. Trolox, a hydrophilic alpha-tocopherol analogue and peroxynitrite scavenger, reduced H/R-induced oxidative injury in isolated rat hearts [27], inhibited apoptosis induced by NO [28], and prevented LPO of heart mitochondrial membranes [29]. Likewise, GME, a glutathione derivative peroxynitrite scavenger [27], prevented the harmful effects of peroxynitrite in H/R by converting peroxynitrite to an S-nitrosating species [8]. Thus, result presented in Figure 1B strongly suggests that H/R induces cytochrome *c* release via mtNOS-derived peroxynitrite. LPO [30] and protein carbonylation [31,32] are reliable peroxynitrite biomarkers. Figure 1C shows that while hypoxia did not increase mitochondrial LPO or protein carbonyls, there is a sharp increase in LPO and protein carbonyl levels in mitochondria incubated under H/R. Increases in the LPO and protein carbonyl were fully prevented when mtNOS was inhibited, or mitochondria were supplemented with peroxynitrite scavengers Trolox or GME (Figure 1C).

Results presented in Figure 1 strongly suggest that H/R increases mtNOS activity. Thus, we measured mtNOS activity of mitochondria treated under hypoxia or H/R. As shown in Figure 1, incubation of mitochondria at $[O_2]$ higher than 50 μM followed or not by reoxygenation did not induce cytochrome *c* release or oxidative modification of mitochondrial lipids or proteins. Therefore, 50 μM O_2 is hereafter considered as hypoxia and 50 μM O_2 followed by reoxygenation as H/R. Figure 2A, B show a significant increase in mtNOS activity of H/R samples as compared with the control. This effect of H/R was prevented by L-NMMA. No difference was observed between hypoxia and control samples. These results show that heart mtNOS activity is, indeed, increased during H/R and confirm a recent finding showing that ischemia-reperfusion increases mtNOS activity of kidney mitochondria [21].

In pathologic conditions such as H/R, peroxynitrite inactivates several heart mitochondrial enzymes. To further study possible elevation of mtNOS-derived peroxynitrite during H/R, we tested the activity of mitochondrial enzymes known to be susceptible to peroxynitrite-induced inactivation. Among them are aconitase, a citric cycle enzyme inactivated by reperfusion of

isolated heart mitochondria [32]; mtCK, an enzyme involved in conversion of ADP to ATP that is inactivated by peroxynitrite during reperfusion [33]; MnSOD [34], a key enzyme protecting mitochondria against oxidative injury in H/R [2]; and SCOT, the mitochondrial rate limiting enzyme in ketolysis [35] that is inactivated by mtNOS-derived peroxynitrite [7]. Figures 2C and D show that all tested enzymes were inactivated by H/R but not hypoxia, and that inactivation of those enzymes was prevented when mtNOS was inhibited. Activity of adenylate kinase, a peroxynitrite-insensitive enzyme, was unaltered (0.0151 ± 0.0011 ; 0.0149 ± 0.0009 ; $0.0152 \pm 0.0015 \mu\text{mol} \cdot \text{min}^{-1} \text{mg}^{-1}$ for control, H/R, and hypoxia, respectively). L-NMMA did not alter the activity of neither enzyme in control samples. These results further suggest involvement of mtNOS in H/R-induced oxidative stress via peroxynitrite production.

3.2. Ca^{2+} -dependence of heart mtNOS

Several groups have shown Ca^{2+} sensitivity of mtNOS in various organs, tissues, and cells [19,36–38]. However, an earlier study suggested that mtNOS is not Ca^{2+} sensitive and its activity is stimulated by supplementing mitochondria with L-arginine [39]. Figure 3A shows that rat heart mtNOS activity was stimulated when $[\text{Ca}^{2+}]_{\text{m}}$ was increased in a concentration-dependent manner. This Figure also shows that Ca^{2+} -stimulated mtNOS activity was inhibited by L-NMMA, when mitochondrial Ca^{2+} uptake was blocked by Ruthenium Red or collapsing $\Delta\psi$ by rotenone. The Ca^{2+} stimulated, L-NMMA sensitive and Ruthenium Red sensitive NO production was also confirmed using fluorometric NOS assay (Figure 3B). These results are consistent with a previous report demonstrating Ca^{2+} -dependence of mouse heart mtNOS [38]. Figure 3A also shows that supplementing mitochondria with L-arginine did not increase the mtNOS activity, as suggested in earlier studies [39,40]. We tested whether presence of 1 to 5 mM Mg^{2+} in the buffers used in those studies could affect heart mtNOS activity. Figure 3A shows that 1 mM Mg^{2+} fully prevented Ca^{2+} -induced mtNOS stimulation. Mg^{2+} is a well-known mitochondrial Ca^{2+} uptake blocker [41,42], and blockade of mitochondrial Ca^{2+} uptake significantly decreases mtNOS activity [37,38].

3.3. H/R increases intramitochondrial calcium concentration ($[\text{Ca}^{2+}]_{\text{m}}$)

The results presented thus far provided strong evidence suggesting that H/R stimulates mtNOS activity. Figure 3A and B show that heart mtNOS is Ca^{2+} sensitive and elevation of $[\text{Ca}^{2+}]_{\text{m}}$ stimulates mtNOS activity. Thus, we tested whether H/R increases $[\text{Ca}^{2+}]_{\text{m}}$. Figure 4A shows that mitochondria incubated under H/R contained higher $[\text{Ca}^{2+}]_{\text{m}}$ than mitochondria incubated under hypoxia or untreated mitochondria. In order to exclude possible effects of H/R on intact mitochondrial Ca^{2+} uptake or $[\text{Ca}^{2+}]_{\text{m}}$ efflux, $[\text{Ca}^{2+}]_{\text{m}}$ was tested for broken mitochondria treated under hypoxia or H/R. Broken mitochondria contain all components of the intact mitochondria, except mitochondrial membranes are ruptured to allow detection of $[\text{Ca}^{2+}]_{\text{m}}$ by Ca^{2+} -sensitive probes or electrodes [7]. Figure 4B demonstrates higher $[\text{Ca}^{2+}]_{\text{m}}$ for broken mitochondria treated under H/R, compared to hypoxia or untreated mitochondria. A similar higher $[\text{Ca}^{2+}]_{\text{m}}$ for broken mitochondria was also observed using a Ca^{2+} -sensitive electrode (not shown). Increased $[\text{Ca}^{2+}]_{\text{m}}$ neutralizes negative charge of the inner membrane and decrease the $\Delta\psi$ [7]. Figure 4C shows that mitochondria treated under H/R fail to build and maintain $\Delta\psi$, compared to control or hypoxia samples. This finding supports findings of Figures 4A, B and further suggest H/R increases $[\text{Ca}^{2+}]_{\text{m}}$.

Assays used in Figures 4A and B measured $[\text{Ca}^{2+}]_{\text{m}}$ after H/R was completed. In the present study, we used a set-up shown in Scheme I and performed real-time measurement of $[\text{Ca}^{2+}]_{\text{m}}$ during the course of ischemia and reoxygenation. Figure 4D shows the simultaneous detection of $[\text{O}_2]$ and $[\text{Ca}^{2+}]_{\text{m}}$ during hypoxia and reoxygenation. As shown in this figure, while there is no appreciable alteration of $[\text{Ca}^{2+}]_{\text{m}}$ throughout the hypoxia, a sharp increase in $[\text{Ca}^{2+}]_{\text{m}}$ occurs during the reoxygenation phase. This result supports those presented in Figures 4A and B and clearly demonstrates that $[\text{Ca}^{2+}]_{\text{m}}$ is increased during reoxygenation.

Mitochondria take up large amounts of Ca^{2+} in response to $\Delta\psi$. However, $[\text{Ca}^{2+}]_m$ is maintained very low by several mechanisms including precipitation of the $[\text{Ca}^{2+}]_m$ to electron-dense granules [7,43,44]. Mitochondria maintain intra-organell calcium homeostasis by continuously precipitating $[\text{Ca}^{2+}]_m$ to matrix electron-dense granules and releasing $[\text{Ca}^{2+}]_m$ from the granules [7,43–45]. Hormones, drugs or pathologic conditions alter the balance between precipitation of $[\text{Ca}^{2+}]_m$ to the granules and release of $[\text{Ca}^{2+}]_m$ from the granules. For example, vasopressin decreases the matrix granules in brain mitochondria [46], tamoxifen increases the release of $[\text{Ca}^{2+}]_m$ from the granules in mitochondria of cancer cells [7], and hypoxia increases the number and extent of the granules [43]. The results presented in Figure 4D shows a marked increase in $[\text{Ca}^{2+}]_m$ during reoxygenation, suggesting that reoxygenation shifts ionized/non-ionized equilibrium in the favor of ionized calcium.

Results presented in this study indicate that mitochondria respond to H/R by increasing $[\text{Ca}^{2+}]_m$, that stimulates mtNOS activity leading to elevation of mitochondrial peroxynitrite followed by oxidative stress and apoptosis (Scheme II). This novel mechanism might contribute to oxidative stress of cardiac cells during H/R.

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Abbreviations footnote

H/R	hypoxia/reoxygenation
NO	nitric oxide
mtNOS	mitochondrial nitric oxide synthase
[Ca²⁺]_m	intramitochondrial ionized calcium concentration
MSH	Mannitol-Sucrose-HEPES
Cu	Zn-SOD, copper, zinc superoxide dismutase
L-NMMA	

	L-monomethyl-L-arginine
GME	glutathione monoethyl ester
LPO	lipid peroxidation
TBARS	thiobarbituric acid reacting substances
BM	broken mitochondria
MnSOD	manganese superoxide dismutase
mtCK	mitochondrial creatine kinase
SCOT, succinyl-CoA	3-oxoacid CoA-transferase
DAF-2	diaminofluorescein
$\Delta\psi$	mitochondrial transmembrane potential
[O₂]	oxygen concentration
oxyHb	oxyhemoglobin
COX	cytochrome oxidase
ΔOD	change in optical density

Figure 1. H/R induces cytochrome *c* release, lipid peroxidation and protein carbonylation

A) Cytochrome *c* release from the mitochondria incubated under normoxia (*Norm*) or lowered oxygen concentration (O₂ (μM); 50, 100, 200; *Hypoxia*) or hypoxia/reoxygenation (*H/R*). B) Cytochrome *c* release from mitochondria incubated at 50 μM O₂ hypoxia/reoxygenation (*H/R*) while mtNOS was stimulated by Ca²⁺ (10 μM; Ca²⁺), inhibited by L-NMMA (*L-NMMA*), or mitochondria were supplemented with peroxynitrite scavengers Trolox (*Trolox*) or GME (*GME*). C) Lipid peroxidation (LPO) and protein carbonylation (PC) were measured in mitochondria treated under hypoxia (*Hypoxia*) or hypoxia/reperfusion (*H/R*) in the absence of L-NMMA, Trolox or GME (*LPO*; *PC*) or presence of L-NMMA (*LPO NMMA*; *PC NMMA*), Trolox (*LPO TROLOX*; *PC TROLOX*) or GME (*LPO GME*; *PC GME*). Numbers on the

horizontal axes represent oxygen concentration in μM). *Norm* represents mitochondria sample incubated 40 min under normoxia (air) and *Ctrl* represents freshly isolated mitochondria.

*Significantly different from control.

Figure 2. H/R stimulates mtNOS activity, and inactivates mitochondrial enzymes

In all panels, *Ctrl* represents freshly isolated mitochondria without treatment, *H/R* represents mitochondria treated under H/R, *H/R + NMMA* represents mitochondria treated under H/R while mtNOS was inhibited by L-NMMA, and *hypoxia* represents mitochondria treated under hypoxia. A) mtNOS activity determined by radioassay. B) mtNOS activity of samples as under panel A measured using chemiluminescence assay. C) Aconitase, MnSOD, and creatine kinase (mtCK) activities. D) SCOT activity. *Significantly different from control.

Figure 3. mtNOS activity and $[Ca^{2+}]_m$

A) Heart mtNOS activity measured in the following samples: *Ctrl*: freshly isolated mitochondria; *Ctrl+NMMA*: mtNOS was inhibited by L-NMMA; 20 μ M Ca, 40 μ M Ca, 80 μ M Ca: when $[Ca^{2+}]_m$ was elevated by providing mitochondria with 20, 40, or 80 μ M Ca^{2+} ; *Ca + NMMA*: mitochondria were provided with 40 μ M Ca^{2+} and mtNOS was inhibited by L-NMMA, *Ca + RR*: mitochondria were provided with 40 μ M Ca^{2+} and mitochondrial Ca^{2+} uptake was inhibited by ruthenium red; *Ca + Rot*: mitochondria were provided with 40 μ M Ca^{2+} and mitochondrial Ca^{2+} uptake was prevented by collapsing $\Delta\psi$ with rotenone. mtNOS activity was also measured when mitochondria were supplemented with L-arginine in the absence (*L-arg*) or presence of ruthenium red (*L-arg + RR*). The effect of $MgCl_2$ on mtNOS

activity in the absence (*Mg*) or presence of Ca^{2+} or L-arg (*Mg + Ca*, *Mg + L-arg*) is shown. B) mtNOS activity determined using NO-sensitive fluorescent dye, DAF-2. NO was determined in freshly isolated mitochondria (*Ctrl*) or when mitochondria were supplemented with $40\ \mu\text{M}\ \text{Ca}^{2+}$ (*Ca*), ruthenium red. (*RR*), *RR* plus $40\ \mu\text{M}\ \text{Ca}^{2+}$ (*RR + Ca*) or $40\ \mu\text{M}\ \text{Ca}^{2+}$ and L-NMMA (*Ca + L-NMMA*). *Significantly different from control. #Significantly different from $40\ \mu\text{M}\ \text{Ca}^{2+}$.

Figure 4. H/R, $[Ca^{2+}]_m$ and $\Delta\psi$

A) $[Ca^{2+}]_m$ was measured for untreated mitochondria (Ctrl), mitochondria treated under hypoxia (Hypox) or hypoxia-reoxygenation (H/R). Mitochondria samples (mito) were added to the cuvette, and after 1 minute rotenone and carbonyl cyanide m-chlorophenylhydrazone (rot/cccp) were added to collapse the $\Delta\psi$ to allow $[Ca^{2+}]_m$ equilibrate with buffer. Where indicated, EGTA was added. B) $[Ca^{2+}]_m$ was measured as in panel A for broken mitochondria (BM) untreated (Ctrl), treated under hypoxia (Hypox) or H/R (H/R). Where indicated, 5 μM Ca^{2+} (Ca) or EGTA (EGTA) was added. C) $\Delta\psi$ was measured at 511–533 nm using Safranin. The $\Delta\psi$ was supported by K^+ -succinate (succ) in the presence of rotenone (rot). At the end of the test, carbonyl cyanide m-chlorophenylhydrazone (cccp) was added to ensure the test. D) Real-time and simultaneous detection of oxygen concentration ($[O_2]$) and intramitochondrial Ca^{2+} ($[Ca^{2+}]_m$) during H/R. Where indicated, reoxygenation (Reox) was performed.

Scheme I. Hypoxia and H/R chamber

A) Schematic representation of the *in vitro* hypoxia/reoxygenation system consisting of a tightly sealed thermostated chamber with fine tubes allowing to purge N_2 or air, or adding the mitochondria samples. The chamber is also equipped with an oxygen sensor. B) Schematic $[O_2]$ trace detected by the oxygen sensor during hypoxia and reoxygenation. In a typical experiment, 1 ml of air saturated buffer was added to a chamber, 100 μ l of buffer was removed and the remaining buffer was purged with N_2 (N_2) until the $[O_2]$ measured with the oxygen electrode (*oxygen electrode*) reached the desired concentration. Then mitochondria (*mitochondria*) were added to the chamber and collected after 40 minutes. C) In a fluorescence cuvette buffer was added and purged with N_2 (N_2) until oxygen concentration detected by oxygen sensor ($[O_2]$ *sensor*) was reached the desired concentration. Then Fura-2-loaded

mitochondria were added to a cuvette (*mito*) and $[Ca^{2+}]_m$ was detected throughout the hypoxia and reoxygenation.

Scheme II. Mitochondria: structure, intra-organelle calcium homeostasis, and mtNOS

Mitochondria consist of the inner (IM) and the outer membrane (OM), the matrix, and the intermembrane space (IMS).

The IM carries the respiratory chain. The chain consists of four respiratory complexes (I, II, III, IV) embedded in the IM, coenzyme Q (ubiquinone; Q) and ATP synthase that is often referred to complex V (V). Electrons (e^-) enter the chain through oxidation of NADH at complex I or FADH₂ at complex II and flow down the chain to complex IV to reduce O₂ to H₂O. Coupled to the electron flow protons are extruded from the matrix into the IMS. This proton extrusion establishes a transmembrane potential ($\Delta\psi$). The $\Delta\psi$ is the driving force for mitochondria to take up Ca²⁺. Mitochondria take up relatively large quantities of Ca²⁺, however, the intramitochondrial ionized calcium concentration ([Ca²⁺]_m) is tightly maintained low by precipitating the [Ca²⁺]_m to non-ionized calcium pools, the matrix electron-dense granules. Mitochondria produce NO via mitochondrial NO-synthase (mtNOS) that is Ca²⁺-sensitive, i.e., increased [Ca²⁺]_m stimulates mtNOS activity. Hypoxia/reoxygenation (H/R) alters intramitochondrial calcium homeostasis by shifting the mitochondrial nonionized/ionized calcium equilibrium in the favor of the ionized form. Elevated [Ca²⁺]_m stimulates mtNOS-derived NO synthesis. Mitochondrial NO readily reacts with superoxide anion (O₂⁻) generated by mitochondrial respiratory chain to produce the powerful oxidizing species, peroxynitrite (ONOO⁻). ONOO⁻ releases cytochrome c (cyto c), induces oxidative modification of mitochondrial lipids and proteins including MnSOD, and releases cytochrome c.