Biochimica et Biophysica Acta 1797 (2010) 1210–1216

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Mitochondria generated nitric oxide protects against permeability transition via formation of membrane protein S-nitrosothiols

Ana Catarina R. Leite [a], Helena C.F. Oliveira [a], Fabiane L. Utino [b], Rafael Garcia [b], Luciane C. Alberici [b], Mariana P. Fernandes [b], Roger F. Castilho [b], Aníbal E. Vercesi [b,*]

[a] Departamento de Fisiologia e Biofísica, Universidade Estadual de Campinas, UNICAMP, Campinas, SP, Brazil
[b] Departamento de Patologia Clínica, Universidade Estadual de Campinas, UNICAMP, Campinas, SP, Brazil

ARTICLE INFO

Article history:
Received 4 November 2009
Received in revised form 27 January 2010
Accepted 27 January 2010
Available online 4 February 2010

Keywords:
Mitochondrial permeability transition
Mitochondrial nitric oxide synthase
S-nitrosothiol
Protein S-nitrosylation
Nitric oxide synthase inhibitor

ABSTRACT

Mitochondria generated nitric oxide ($NO^{\bullet}$) regulates several cell functions including energy metabolism, cell cycling, and cell death. Here we report that the $NO^{\bullet}$ synthase inhibitors (L-NAME, L-NNA and L-NMMA) administered either *in vitro* or *in vivo* induce Ca^{2+}-dependent mitochondrial permeability transition (MPT) in rat liver mitochondria via a mechanism independent on changes in the energy state of the organelle. MPT was determined by the occurrence of cyclosporin A sensitive mitochondrial membrane potential disruption followed by mitochondrial swelling and Ca^{2+} release. In *in vitro* experiments, the effect of NOS inhibitors was dose-dependent (1 to 50 μM). In addition to cyclosporin A, L-NAME-induced MPT was sensitive to Mg^{2+} plus ATP, EGTA, and to a lower degree, to catalase and dithiothreitol. In contrast to L-NAME, its isomer D-NAME did not induce MPT. L-NAME-induced MPT was associated with a significant decrease in both the rate of $NO^{\bullet}$ generation and the content of mitochondrial S-nitrosothiol. Acute and chronic *in vivo* treatment with L-NAME also promoted MPT and decreased the content of mitochondrial S-nitrosothiol. SNAP (a $NO^{\bullet}$ donor) prevented L-NAME mediated MPT and reversed the decrease in the rate of $NO^{\bullet}$ generation and in the content of S-nitrosothiol. We propose that S-nitrosylation of critical membrane protein thiols by $NO^{\bullet}$ protects against MPT.

1. Introduction

Nitric oxide ($NO^{\bullet}$) is an ubiquitous intra and inter-cellular messenger involved in the regulation of diverse physiological processes, such as neurotransmission, vasodilation, immune response and cell cycling and arrest [1,2]. On the other hand, at elevated concentrations, $NO^{\bullet}$ can also be cytotoxic [3,4]. The synthesis of $NO^{\bullet}$ from L-arginine and O_2 is catalyzed by nitric oxide synthases (NOS). Up to the early 90s, three distinct isoforms of NOS had been well characterized, two constitutive (endothelial, eNOS, and neuronal, nNOS) and one inducible (iNOS). They require several co-factors, including FAD, FMN, BH_4, Ca^{2+} and NADPH. All NOS isoforms require Ca^{2+} for their activity, although iNOS is significantly less dependent on Ca^{2+} than the other isoforms [5].

The existence of a mitochondrial $NO^{\bullet}$ synthase (mtNOS) was first suggested by immunohistochemical studies that found that anti-NOS antibodies stained mitochondria [6–8]. Next, biochemical properties of mtNOS were characterized in purified liver mitochondria [9–11]. The activity of mtNOS was also determined in mitochondria purified from different tissues of rats and mice, including brain, heart, kidney, thymus and skeletal muscle (for a review see [12]). The identity of a liver mtNOS was described by Elfering et al. [13]. They sequenced mtNOS, identified the protein as a splice variant of the nNOS, and localized it in the inner mitochondrial membrane. In support for this finding, a previous work described the lack of mtNOS in the heart of nNOS knockout mice [14]. More recent evidences suggest that mtNOS is structurally linked to both complexes I and IV [15,16]. Nonetheless, a considerable debate about the molecular identity of the mtNOS persists [17–20]. Additionally, $NO^{\bullet}$ can be produced inside mitochondria by other NOS independent pathways [21–23].

The mitochondria generated $NO^{\bullet}$ regulates O_2 consumption, energy metabolism, and reactive oxygen species (ROS) formation (for a review see [24]). Mitochondria also generate the anion superoxide ($O_2^{\bullet-}$). Although these two radical species are not toxic at physiological concentrations, an imbalance in their production or

Abbreviations: Alam, alamethicin; CsA, cyclosporin A; DAF-FM, (4-amino-5-methylamino-2′,7′-difluorofluorescein) diacetate; D-NAME, N^G-Nitro-D-arginine methyl ester hydrochloride; DTT, dithiothreitol; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid tetrasodium salt; GSH, glutathione; L-NAME, N^G-nitro-L-arginine methyl ester hydrochloride; L-NMMA, N^G-methyl-L-arginine acetate salt; L-NNA, N^5-(Nitroamidino)-L-2,5-diaminopentanoic acid; MPT, mitochondrial permeability transition; $NO^{\bullet}$, nitric oxide; NOS, nitric oxide synthase; PTP, permeability transition pore; RLM, rat liver mitochondria; RNS, reactive nitrogen species; ROS, reactive oxygen species; SNAP, S-nitroso-N-acetyl-DL-penicillamine; SOD, superoxide dismutase

* Corresponding author. Depto. Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, 13083-970 Campinas, SP, Brazil. Fax: +55 19 3521 9434.
E-mail address: anibal@unicamp.br (A.E. Vercesi).

0005-2728/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2010.01.034

elimination can be responsible for alterations of molecular mechanisms regulating cell life. The reaction of these two species generates peroxynitrite ($ONOO^-$), a potent oxidant that react with most biological molecules causing cell damage. In addition to peroxynitrite, other reactive nitrogen species [6] are derived from $NO^\bullet$, including nitroxyl (HNO), nitrosonium cation (NO^+) and S-nitrosothiols (RSNOs), [25–29].

$NO^\bullet$ reversibly interacts with cytochrome oxidase modulating mitochondria respiration rate [2,30]. In addition, the interaction of $NO^\bullet$ with thiol groups forming S-nitrosothiols in mitochondria may be of physiological importance [31]. The formation of protein S-nitrosothiols may enable the reversible regulation of protein function [32]. The biological role of S-nitrosylation is also related to the protection of protein thiols under oxidative stress conditions, as it will be discussed ahead.

Mitochondrial protein oxidation, lipid peroxidation and DNA damage caused by ROS and RNS have been extensively documented (reviewed in [28,33,34]). The inner membrane proteins are primary targets for oxidative damage, a process that is activated by excessive matrix Ca^{2+}. Oxidation of membrane protein thiols induces the formation of the permeability transition pore (PTP) via protein aggregation due to dithiol formation [35]. We have proposed that these protein aggregates form aqueous channels that confer non-selective permeabilization of the inner mitochondrial membrane [36]. A large body of evidences indicates that mitochondrial permeability transition is involved in cell death mechanisms determining whether the fate of cells will be apoptosis or necrosis [37]. It seems to operate as an ATP switch by establishing the balance between ATP availability and ATP hydrolysis after PTP opening [38].

It is well known that $NO^\bullet$ has pro- and anti-apoptotic effects depending on its concentration range. At high production rates (>2 μM/s), $NO^\bullet$ induces MPT via a Ca^{2+}-dependent mechanism, probably due to the formation of $ONOO^-$. This RNS reacts with thiols by one or two electron oxidations to produce thiyl radicals ($RS^\bullet$) and sulfenic acids (RSOH), respectively, both of which usually react with protein thiols to form disulfides or other forms of higher thiol oxidation [31]. In contrast, at low levels, $NO^\bullet$ may inhibit MPT (IC_{50} of 11 nM $NO^\bullet$/s) [39].

The objective of the present study was to investigate the relationship between mitochondria generated $NO^\bullet$ and MPT using non-selective NOS inhibitors under experimental conditions of fully preserved mitochondrial respiration. We tested the hypothesis that $NO^\bullet$ protects against MPT via S-nitrosylation of critical mitochondrial membrane protein thiol groups.

2. Materials and methods

2.1. Isolation of liver mitochondria

Liver mitochondria from adult rats fasted overnight were isolated by conventional differential centrifugation [40] and partially purified by a discontinuous Percoll gradient. Briefly, livers were homogenized in 250 mM sucrose, 1 mM EGTA, and 10 mM Hepes buffer (pH 7.2), and centrifuged for 10 min at 2500 rpm. The supernatant was centrifuged for 10 min at 8000 rpm. The mitochondrial pellet was purified using a discontinuous Percoll gradient according to [41]. The mitochondrial fraction obtained from the interface between 19 and 52% Percoll layers was washed in medium containing 250 mM sucrose, 10 mM Hepes and 0.1 mM EGTA, for 10 min at 8000 rpm. The final pellet was resuspended in 250 mM sucrose and 10 mM Hepes to a final protein concentration of 80–100 mg/ml.

2.2. Standard incubation procedure

The experiments were carried out at 28 °C, with continuous magnetic stirring, in standard medium containing 125 mM sucrose, 65 mM KCl, 2 mM inorganic phosphate, 1 mM magnesium chloride, 10 mM Hepes buffer (pH 7.2) and a complex I substrate mixture made of malate, pyruvate, ketoglutarate and glutamate (5 mM each). Other additions are indicated in the figure legends.

2.3. Animals and L-NAME treatment

Female Wistar rats (180–250 g) had free access to standard laboratory rodent chow diet (Nuvital CR1, PR, Brazil) and water and were housed at 22 ± 2 °C on a 12 h light–dark cycle. L-NAME (50 mg/kg i.p.) or saline was given once i.p., and after 14 h, rats were killed by cervical dislocation, livers were perfused with cold saline solution and excised for mitochondria isolation. Some rats were also subject to a chronic treatment with L-NAME added to their drinking-water (1 mg kg^{-1} day^{-1}) during 14 days.

2.4. Mitochondrial swelling

Mitochondrial swelling (0.5 mg/ml) was determined as the decrease in the turbidity of the mitochondrial suspension measured at 520 nm using a temperature-controlled Hitachi U-3000 spectrophotometer (28 °C).

2.5. Mitochondrial membrane potential disruption

Mitochondria (0.5 mg/ml) were incubated in the standard reaction medium containing 2 μM tetraphenylphosphonium (TPP^+) at 28 °C. The concentration of TPP^+ in the extramitochondrial medium was continuously monitored with a TPP^+-selective electrode prepared in our laboratory according to [42]. The membrane potential was then calculated by assuming that the TPP^+ distribution between mitochondria and the medium follows the Nernst equation. Corrections due to the binding of TPP^+ to the mitochondrial membrane were made according to Jensen et al. [43]. No corrections were made to compensate $\Delta\psi$ values for the continuous alteration in mitochondrial volume that occurs during the experiments.

2.6. Mitochondrial calcium transport

Calcium uptake and release by isolated liver mitochondria (0.5 mg/ml) was determined following the fluorescence of 0.1 μM Calcium Green-5N hexapotassium salt (Molecular Probes) using a temperature-controlled Hitachi F4500 spectrofluorometer (28 °C) at excitation and emission wavelengths of 506 and 531 nm, respectively, and slit widths of 5.0 nm [44].

2.7. Mitochondrial nitric oxide production

The probe DAF-FM (10 μM) was used to monitor $NO^\bullet$ release from rat liver mitochondria (0.5 mg/ml) using a temperature-controlled Hitachi F4500 spectrofluorometer (28 °C) at excitation and emission wavelengths of 495 and 515 nm, respectively, and slit widths of 2.5 nm. Superoxide dismutase (1 μM) and catalase (1 μM) were added to prevent interference of $O_2^{\bullet-}$ and H_2O_2. To test DAF specificity to $NO^\bullet$ under our experimental conditions we added the $NO^\bullet$ donor SNAP at different concentrations, and observed a rapid and proportional increase in the probe fluorescence. The calibration was made using DAF-2T the product of the reaction between DAF-FM and $NO^\bullet$.

2.8. Mitochondrial S-nitrosothiol determination

The samples were prepared according to [3]. Mitochondria were disrupted by shaking with 0.5% Triton X-100 for 60 min at 4 °C and insoluble proteins were removed by centrifugation at 20,000 *g* for 60 min. The supernatant was used to determine S-nitrosothiol

content using a modified Saville assay [45]. Briefly, the samples were incubated with 300 µM 2,3-diaminonaphthalene (DAN) diluted in HCl (0.5 M). $HgCl_2$ was used to release NO• during a 10 min incubation in the dark. After this, samples were alkalinized with NaOH (1 M) and incubated for an additional 10 min period in the dark. Then, fluorescence was followed in a temperature-controlled Hitachi F4500 spectrofluorometer (28 °C) at excitation and emission wavelengths of 381 and 407 nm, respectively, and slit widths of 2.5 nm (excitation) and 5.0 nm (emission). Nitrosothiol content was calculated using a standard curve generated with $NaNO_2$.

2.9. Statistical analyses

Data from the experiments were analyzed by one-way ANOVA, followed by a post hoc Tukey test, using OriginLab 7.5 software (OriginLab Corp.). Student *t*-test was used for two mean comparisons. The data are presented as averages ± SD of at least four experiments conducted with different preparations.

2.10. Chemicals

L-NAME, D-NAME, L-NNA, L-NMMA, SNAP, Cyclosporin A, catalase, SOD, EGTA, FCCP, NEM, $HgCl_2$, 2,3-Diaminonaphthalene (DAN), and $NaNO_2$ were purchased from Sigma (St. Louis, USA). Calcium green-5N and DAF-FM were purchased from Molecular Probes. DAF-2T was obtained from Alexis Biochemicals. All other reagents were products of the highest purity grade available.

3. Results

The effects of NOS inhibitors on mitochondrial swelling and membrane potential are shown in Fig. 1. The experiments depicted in Fig. 1A show that L-NAME, L-NNA and L-NMMA (50 µM each), in contrast to the L-NAME isomer D-NAME, induced extensive swelling of mitochondria suspended in the incubation medium containing 30 µM Ca^{2+}. The effect of L-NAME was dose-dependent (1–50 µM) both on mitochondrial swelling (Fig. 1B) and on the disruption of membrane potential (Fig. 1C).

The mechanisms by which L-NAME induced the loss of mitochondrial integrity *in vitro* were additionally investigated. Fig. 2 shows that the effects of L-NAME on mitochondrial swelling (panel A) and membrane potential disruption (panel B) were inhibited, totally or partially, by the MPT inhibitors CsA, Mg^{2+} plus ATP, catalase, and DTT, a disulfide reductant.

Since MPT is stimulated by Ca^{2+} accumulation and the inhibitory effects of NO• on MPT could be secondary to inhibition of Ca^{2+} uptake by the organelle [39], we examined the effect of L-NAME on mitochondrial Ca^{2+} handling, under our experimental conditions (Fig. 2C). The data show that initially the mitochondrial Ca^{2+} influx was unaffected by the presence of L-NAME. However, after a Ca^{2+} pulse, the capacity to retain Ca^{2+} was decreased by L-NAME, via a mechanism sensitive to cyclosporin A. The results indicate that L-NAME, as well as the other NOS inhibitors, the arginine analogues L-NNA and L-NMMA, has the ability to act as a MPT inducer independently of changes in the capacity of mitochondrial Ca^{2+} accumulation and respiration (data not shown).

To investigate whether a NO• donor could prevent the effect of L-NAME on MPT, we performed experiments in the presence of SNAP (Fig. 3). Interestingly, Fig. 3A shows that SNAP, at the concentration of 10 nM, retards both the swelling induced by Ca^{2+} alone and that induced by Ca^{2+} plus L-NAME. The presence of 10 nM SNAP did not affect Ca^{2+} influx to mitochondria but, in agreement with the experiment of Fig. 3A, retards Ca^{2+} release in the presence or absence of L-NAME (Fig. 3B). These results suggest that neither L-NAME nor 10 nM SNAP have a direct effect on mitochondrial permeabilization and that the observed effects are certainly mediated by NO•, in a concentration range that does not affect either Ca^{2+} influx or respiration (not shown).

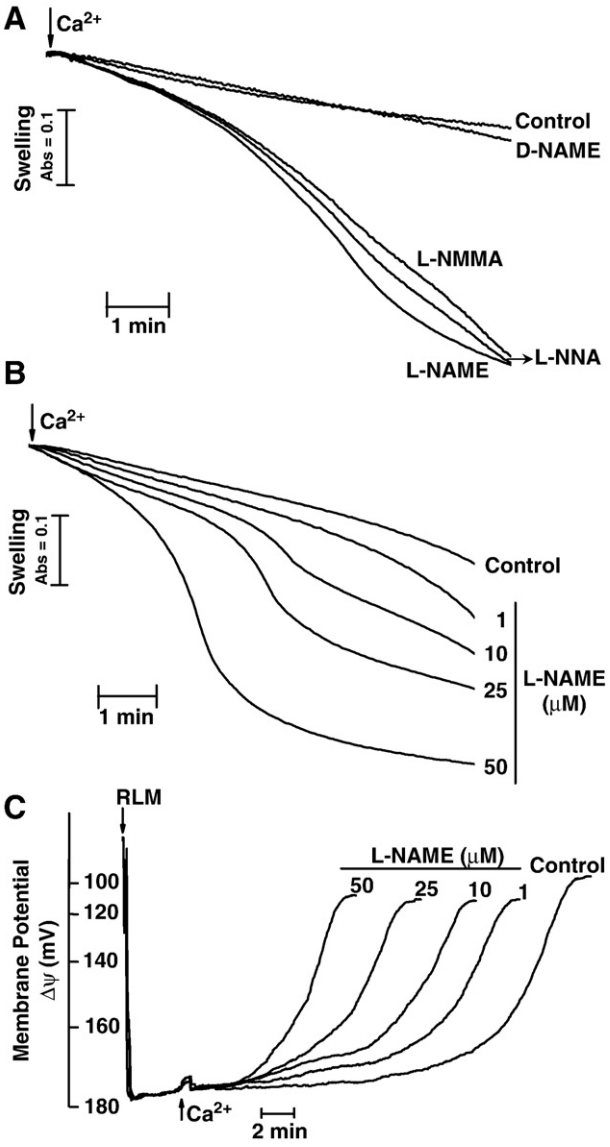

Fig. 1. Mitochondrial swelling induced by NOS inhibitors *in vitro* (A) and dose-dependent effect of L-NAME on mitochondrial swelling (B) and on mitochondrial membrane potential ($\Delta\psi$) disruption (C). Panel A: Rat liver mitochondria (RLM, 0.5 mg/ml) were incubated in the standard medium containing 30 µM Ca^{2+} in the presence of 50 µM of L-NAME, L-NMMA, L-NNA, or D-NAME. Absorbances at 7 min were: 1.270 ± 0.062 (Control), 1.253 ± 0.093 (D-NAME), 0.955 ± 0.075 (L-NAME), 0.958 ± 0.037 (L-NNA), and 0.966 ± 0.186 (L-NMMA), $p<0.001$ for control and D-NAME *vs.* all NOS inhibitors. Panel B: L-NAME was present at indicated concentrations. Absorbances at 7 min were: 1.465 ± 0.104 (Control), 1.368 ± 0.196 (1 µM L-NAME), 1.175 ± 0.172 (10 µM L-NAME), 1.145 ± 0.167 (25 µM L-NAME), and 0.948 ± 0.023 (50 µM L-NAME), $p<0.05$ for control *vs.* 10, 25 and 50 µM L-NAME. Panel C: Standard medium containing 2 µM TPP^+, 25 µM Ca^{2+} and L-NAME at indicated concentrations. Average time of $\Delta\psi$ maintenance was: 24.9 ± 2.8 (Control), 18.6 ± 1.5 (1 µM L-NAME), 16.3 ± 7.3 (10 µM L-NAME), 14.8 ± 3.0 (25 µM L-NAME) and 12.7 ± 1.9 (50 µM L-NAME), $p<0.05$ for control *vs.* 10, 25 and 50 µM L-NAME. Lines are representative of five independent experiments.

In order to further characterize the role of endogenous NO• on MPT, we performed experiments analyzing the effect of L-NAME on NO• generation by mitochondria (Fig. 4). The experiments started by the addition of mitochondria to the standard medium containing 10 µM of the NO• probe, DAF-AM (Fig. 4A). A biphasic increase in DAF fluorescence was observed: an initial phase with higher slope was followed by a second phase with lower slope that begun 3–4 min after

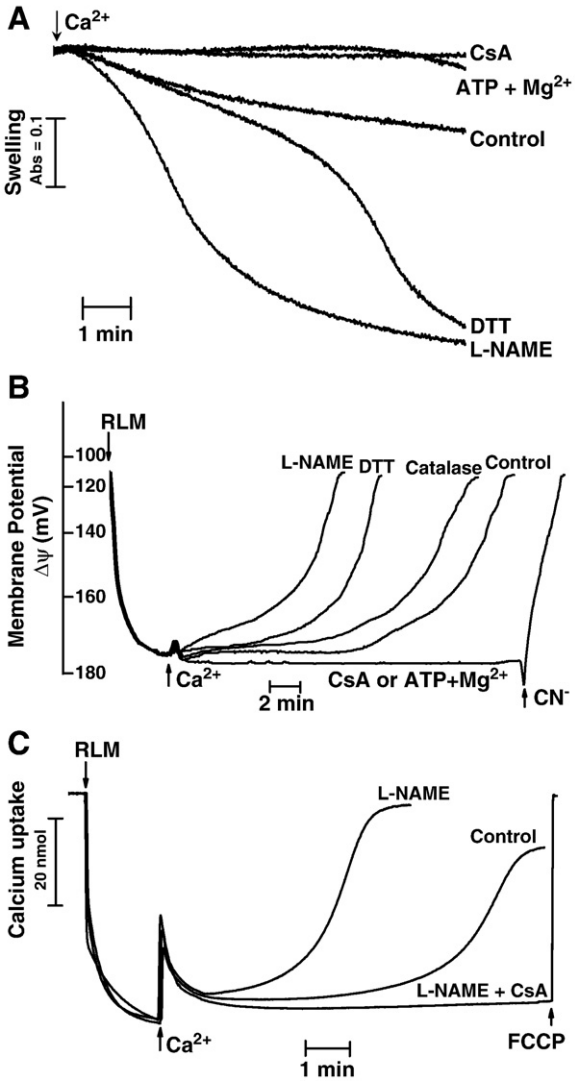

Fig. 2. Inhibition of L-NAME-induced mitochondrial swelling (A), membrane potential disruption (B) and calcium release (C) by MPT inhibitors. Rat liver mitochondria (RLM, 0.5 mg/ml) were incubated in the standard medium with 50 μM L-NAME and 30 μM Ca^{2+} in the presence of the MPT inhibitors: cyclosporin A (CsA, 1 μM), ATP (200 μM) plus Mg^{2+} (5 mM), catalase (2 μM), or dithiothreitol (DTT, 500 μM). Panel A: Absorbances at 7 min were: 1.332 ± 0.138 (Control), 1.395 ± 0.172 (CsA), 1.387 ± 0.137 (ATP + Mg^{2+}), 1.020 ± 0.047 (DTT), and 0.980 + 0.043 (L-NAME), $p<0.05$ for L-NAME *vs.* control, CsA, and ATP + Mg^{2+}. Panel B: Average time of $\Delta\psi$ retention (min) was: 24.0 ± 5.4 (Control), 18.8 ± 3.5 (DTT), 20.8 ± 1.8 (Catalase) and 14.9 ± 3.6 (L-NAME), $p<0.05$ for L-NAME *vs.* control, CsA, and ATP + Mg^{2+}. Panel C: Average time of calcium release (min) was: 7.1 ± 0.6 (Control) and 2.8 ± 1.0 (L-NAME), $p<0.05$. Lines are representative of five independent experiments.

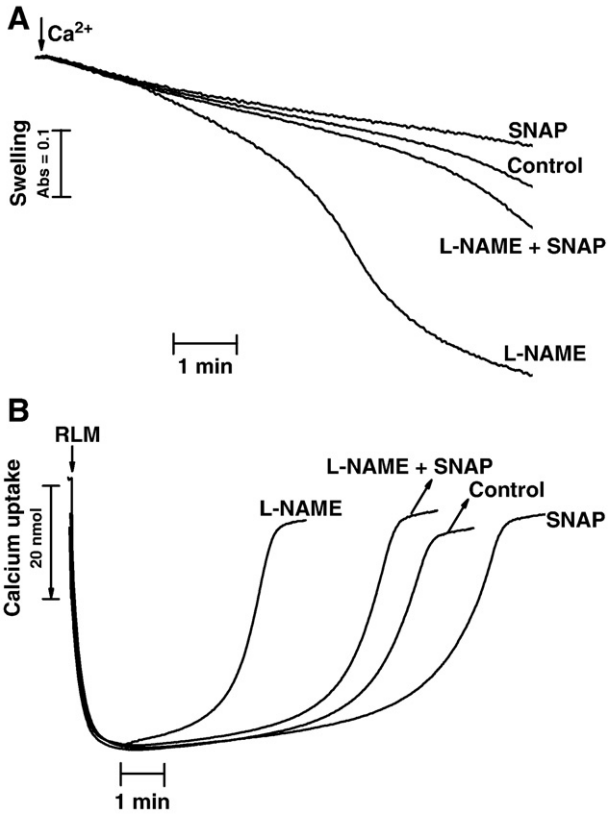

Fig. 3. SNAP protects against L-NAME-induced mitochondrial swelling (A) and calcium release (B). Rat liver mitochondria (RLM, 0.5 mg/ml) were incubated in standard medium containing 30 μM Ca^{2+} in the presence or absence of SNAP (10 nM) and L-NAME (50 μM) as indicated. Panel A: Absorbances at 7 min were: 1.327 ± 0.066 (SNAP), 1.295 ± 0.118 (Control), 1.250 ± 0.144 (L-NAME + SNAP) and 1.040 ± 0.121 (L-NAME), $p<0.05$ for L-NAME *vs.* all others. Panel B: Average calcium retention time (min) was: 8.7 ± 0.9 (SNAP), 7.2 ± 0.5 (Control), 6.1 ± 2.0 (L-NAME + SNAP), and 2.9 ± 0.9 (L-NAME), $p<0.05$ for L-NAME *vs.* all others. Lines are representative of five independent experiments.

mitochondria addition. The first faster phase of fluorescence increase may reflect the reaction of DAF with a pre-formed mitochondrial pool of $NO^{\bullet}$ and the second slower phase of fluorescence increase probably represents the steady state rate of $NO^{\bullet}$ generation by mitochondria. This second phase was considered for the estimations of mitochondrial $NO^{\bullet}$ generation rates. According to our standard curve built using DAF-2T, the rates of $NO^{\bullet}$ generation were 0.35 pmol $NO^{\bullet}$/mg under the control conditions and 0.18 pmol $NO^{\bullet}$/mg in the presence of L-NAME (Fig. 4B). Therefore, 50 μM L-NAME caused an inhibition of 47% in the mitochondria generation rate of $NO^{\bullet}$. Furthermore, an experiment using D-NAME, instead of L-NAME, did not show any difference in the rate of DAF fluorescence as compared to the control (Fig. 4B). Additions of increasing concentrations of the $NO^{\bullet}$ donor SNAP were done to the preparations containing L-NAME and significantly increased the slope of DAF fluorescence (Fig. 4A and B). No mitochondrial swelling was detected under these experimental conditions (low Ca^{2+} concentration) (data not shown).

Since $NO^{\bullet}$ promotes S-nitrosylation of cysteine residues in mitochondrial membrane proteins [4] we analyzed the influence of L-NAME on the content of mitochondrial S-nitrosothiol. Fig. 5 shows that L-NAME decreases S-nitrosothiol content by 36% when compared to the control. On the other hand, 10 nM SNAP totally abrogates the effect of L-NAME. In order to eliminate the possible interference of mitochondrial swelling in S-nitrosothiol estimation, we performed a control experiment in the presence of alamethicin, a non-selective inducer of mitochondrial membrane permeabilization. This compound did not cause any change in S-nitrosothiol content when compared to the control (Fig. 5), despite the extensive swelling it induces. The higher content of S-nitrosothiol found in our determinations in relation to literature data [30,46,47] is probably due to the fact that our assay was done in whole mitochondria, which presents a high content of matrix S-nitrosylated glutathione content [48].

The effect of L-NAME was also tested *in vivo*, using two treatment protocols that inhibit all isoforms of NOS in animal tissues. The experiments presented in Fig. 6A show that liver mitochondria isolated from rats 14 h after an acute dose of L-NAME (50 mg/kg i.p.) are more susceptible to MPT than those isolated from control animals. Under these experimental conditions, mitochondrial swelling was also prevented by the MPT inhibitors, CsA, ATP plus Mg^{2+} or catalase (Fig. 6B). More important, *in vivo* L-NAME treatment decreased mitochondrial

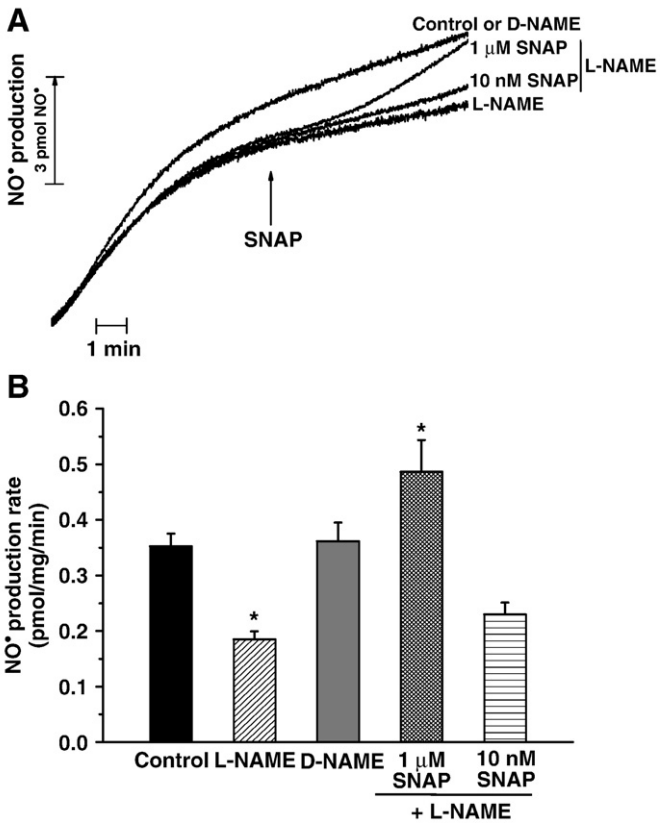

Fig. 4. Inhibition of mitochondrial NO• generation by L-NAME. NO• production by mitochondria (0.5 mg/ml) was measured using 10 μM DAF-FM in the presence of 1 μM SOD and 1 μM catalase. Standard medium contained 10 μM Ca^{2+}. Panel A: Representative experiment of mitochondrial NO• production in the presence of D-NAME (50 μM) or L-NAME (50 μM), with addition of SNAP (10 nM or 1 μM) as indicated. Panel B: Quantification of total NO• generated (pmol/mg mitochondrial protein/min). *$p<0.05$ for L-NAME and SNAP (1 μM) *vs.* control. Results represent six independent experiments.

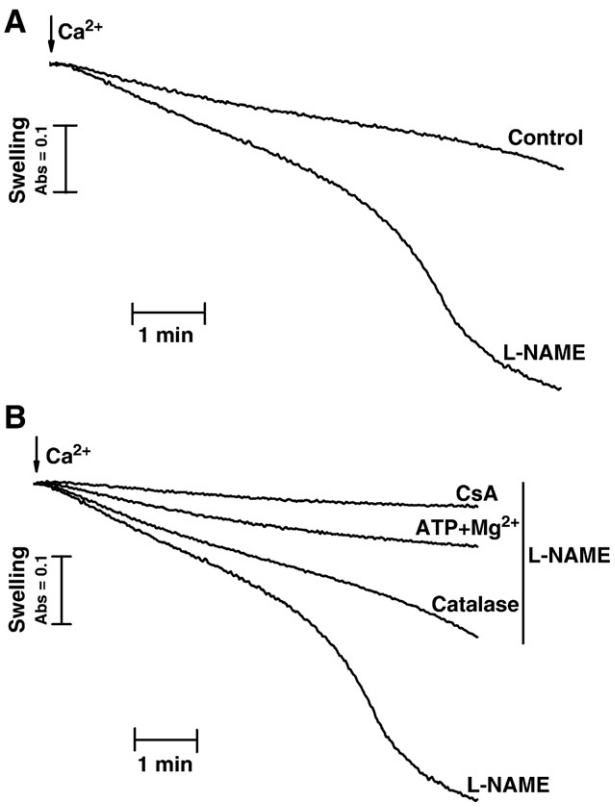

Fig. 6. *In vivo* treatment with L-NAME promotes Ca^{2+} induced mitochondrial swelling (A) sensitive to MPT inhibitors (B). Rat liver mitochondria (RLM, 0.5 mg/ml) from control and L-NAME treated rats were incubated in the presence of 30 μM Ca^{2+}. Panel A: Absorbances for swelling at 7 min were: 1.300 ± 0.105 (Control) *vs.* 1.042 ± 0.187 (L-NAME), $p<0.05$. Panel B: RLM from L-NAME treated rats were incubated in the presence of MPT inhibitors cyclosporin A (CsA, 1 μM), ATP (200 μM) + Mg^{2+} (5 mM) and catalase (2 μM). Absorbances at 7 min were: 1.396 ± 0.029 (CsA), 1.324 ± 0.024 (ATP + Mg^{2+}), 1.156 ± 0.104 (Catalase), and 1.062 ± 0.173 (L-NAME), $p<0.05$ for L-NAME *vs.* MPT inhibitors. Lines are representative of five independent experiments.

S-nitrosothiol content by 45% when compared to the control (Fig. 7). Similar results were obtained when the rats were chronically treated with L-NAME added to their drinking-water (1 mg kg^{-1} day^{-1} during 14 days; results not shown).

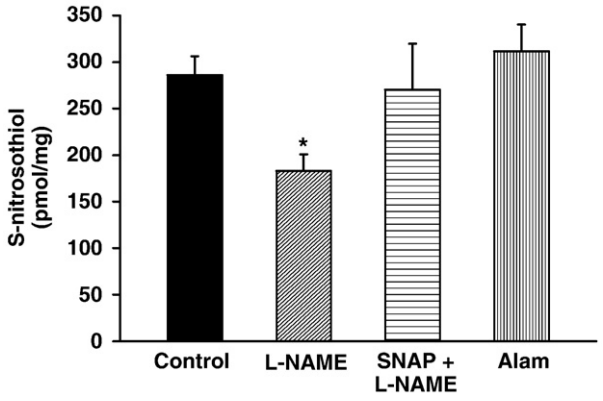

Fig. 5. SNAP protects against L-NAME-induced decrease in the content of mitochondrial S-nitrosothiol. Assay was performed in mitochondria (0.5 mg/ml) samples after a 10 min incubation in the presence of 30 μM Ca^{2+} and 50 μM L-NAME, 10 nM SNAP or L-NAME plus SNAP as indicated. Alamethicin (Alam, 5 μg/ml) plus EGTA (200 μM), a calcium independent mitochondria swelling inducer, was used to show no interference of mitochondrial swelling on the nitrosothiol quantification. *$p<0.05$ for L-NAME *vs.* all others. Data are averages ± SD ($n=8$). Lines are representative of eight independent experiments.

4. Discussion

The first evidences that mitochondria generate NO• and possess their own mtNOS were published more than 10 years ago [9–11]. Due to the enormous physiological relevance of NO•, the issue attracted great interest among researchers in the area and generated a large controversy. This subject has been critically analyzed in several recent

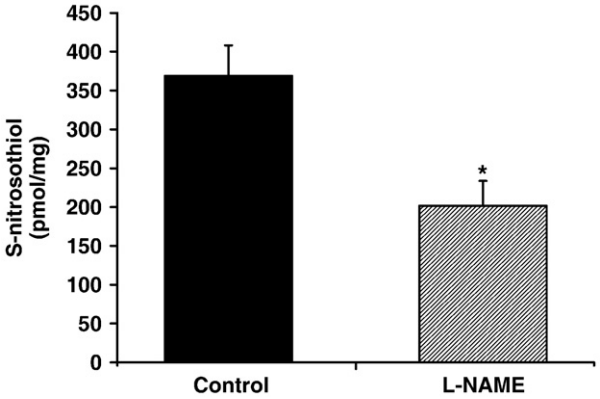

Fig. 7. *In vivo* treatment with L-NAME decreased the content of mitochondrial S-nitrosothiol. Assay was performed in mitochondria (0.5 mg/ml) samples after a 10 min incubation in the presence of 30 μM Ca^{2+}, data are averages ± SD ($n=5$). *$p<0.05$.

reviews [19,29,34,49]. Although the results reported in this work are not a final proof, they support the existence of such a mtNOS since they provide evidence that the lack of mitochondrially generated $NO^{\bullet}$ impairs the mitochondrial resistance to Ca^{2+}-dependent MPT. The previous demonstrations that Ca^{2+} induced MPT is sensitive to catalase and thiol reagents (mainly DTT) indicated that MPT is favored by accumulation of H_2O_2 and triggered by oxidation of membrane protein thiols [35,36,50,51]. The stimulation of MPT by NOS inhibitors, *in vitro* and *in vivo*, and conversely the protection against MPT conferred by the $NO^{\bullet}$ donor SNAP in fully functioning mitochondria, are strong evidences of a new relevant physiological role of mtNOS, as opposed to others' claims [19].

In this study, the role of $NO^{\bullet}$ on the MPT susceptibility was approached by using low concentrations of either NOS inhibitors or the $NO^{\bullet}$ donor SNAP, which effectively modified the rates of $NO^{\bullet}$ generation in the organelle. All non-selective NOS inhibitors used here, named L-NAME, L-NNA and L-NMMA showed the ability to induce Ca^{2+}-dependent MPT by a dose-dependent mechanism antagonized by the $NO^{\bullet}$ donor SNAP, in a process independent on changes of mitochondrial respiration rates and Ca^{2+} uptake. The effect of SNAP and lack of effect by D-NAME support the idea that these NOS inhibitors had no direct effect on MPT *per se*, but rather acted via changes in $NO^{\bullet}$ availability in the organelle. Indeed, 50 μM L-NAME inhibited the rate of $NO^{\bullet}$ generation by about 50% and this was totally reversed by 1 μM SNAP. The ability of these NOS inhibitors to promote MPT was also documented in mitochondria isolated from perfused livers of rats treated with L-NAME, either acutely or chronically. In addition, the lack of effect by D-NAME confirms the concept that all types of NOS are enantiomer specific for their amino acid substrate [52].

It is well established that $NO^{\bullet}$ can have dual effects on mitochondrial functions. At physiological concentrations, it regulates some pathways such as metabolic rate [30,53], mitochondrial biogenesis [54,55], resistance to MPT [31,38,56] and cell death [57]. Recent work on mitochondria-target $NO^{\bullet}$ donors shows protection during ischemia/reperfusion injury in the heart [30]. In contrast, high concentrations of $NO^{\bullet}$ generate RNS and lead to pore opening [58], modify protein function, mainly through nitration of tyrosine residues [59,60] and impair lipid function also via nitration [59].

Concerning the mechanism of protection against MPT it is worth to consider that: first, many biological effects of $NO^{\bullet}$ are mediated through S-nitrosylation of protein cysteine thiol and that mitochondria have been identified as organelles with high activity of reversible S-nitrosylation reactions [46]; second, PTP opening is mediated by membrane protein polymerization via dithiol formation [35,36]. In this regard, we observed that NOS inhibitors, under the experimental conditions that induced MPT, significantly decreased both the rate of $NO^{\bullet}$ generation and the content of mitochondrial S-nitrosothiol. In contrast, SNAP that protected against MPT, recovered the rate of $NO^{\bullet}$ generation and the content of S-nitrosothiols. Therefore, it is conceivable to propose that S-nitrosylation protects against MPT by competing for critical protein thiols and blocking the formation of dithiols. This putative role for $NO^{\bullet}$ and S-nitrosothiols in the protection against MPT is in agreement with data showing that protein S-nitrosothiols can be generated by pre-formed S-nitrosothiols that persist mainly when the mitochondrial glutathione pool is oxidized [31,47], a condition that favors MPT [36]. This suggests that the reversible S-nitrosylation in mitochondria is related to the protection of protein thiols against the attack of reactive oxygen when the antioxidant mitochondrial system, mainly represented by NADPH and GSH, is deficient, a new biological role of mitochondrially generated $NO^{\bullet}$.

In summary, we have identified that NOS inhibitors induce MPT through the decrease of both mitochondrial generation of $NO^{\bullet}$ and S-nitrosylation of membrane protein thiols. These changes were shown to be independent on changes in respiration rates, membrane potential and Ca^{2+} content in the organelle. It is proposed that S-nitrosylation of critical membrane protein thiols protects against MPT and thus against mitochondrial damage.

Acknowledgements

This work was supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional para o Desenvolvimento Científico e Tecnológico (CNPq/INCT: National Institute for Science and Technology in Diabetes and Obesity). A.C.R.L. and M.P.F. were supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) fellowships and F.L.U., R.G. and L.C.A. were supported by FAPESP fellowships.

References

[1] L.J. Ignarro, Nitric oxide: a unique endogenous signaling molecule in vascular biology, Biosci. Rep. 19 (1999) 51–71.
[2] S. Moncada, J.P. Bolanos, Nitric oxide, cell bioenergetics and neurodegeneration, J. Neurochem. 97 (2006) 1676–1689.
[3] C.A. Piantadosi, L.G. Tatro, A.R. Whorton, Nitric oxide and differential effects of ATP on mitochondrial permeability transition, Nitric Oxide 6 (2002) 45–60.
[4] G.C. Brown, Nitric oxide and mitochondria, Front. Biosci. 12 (2007) 1024–1033.
[5] W.K. Alderton, C.E. Cooper, R.G. Knowles, Nitric oxide synthases: structure, function and inhibition, Biochem. J. 357 (2001) 593–615.
[6] T.E. Bates, A. Loesch, G. Burnstock, J.B. Clark, Immunocytochemical evidence for a mitochondrially located nitric oxide synthase in brain and liver, Biochem. Biophys. Res. Commun. 213 (1995) 896–900.
[7] U. Frandsen, M. Lopez-Figueroa, Y. Hellsten, Localization of nitric oxide synthase in human skeletal muscle, Biochem. Biophys. Res. Commun. 227 (1996) 88–93.
[8] L. Kobzik, B. Stringer, J.L. Balligand, M.B. Reid, J.S. Stamler, Endothelial type nitric oxide synthase in skeletal muscle fibers: mitochondrial relationships, Biochem. Biophys. Res. Commun. 211 (1995) 375–381.
[9] P. Ghafourifar, C. Richter, Nitric oxide synthase activity in mitochondria, FEBS Lett. 418 (1997) 291–296.
[10] C. Giulivi, J.J. Poderoso, A. Boveris, Production of nitric oxide by mitochondria, J. Biol. Chem. 273 (1998) 11038–11043.
[11] A. Tatoyan, C. Giulivi, Purification and characterization of a nitric-oxide synthase from rat liver mitochondria, J. Biol. Chem. 273 (1998) 11044–11048.
[12] A. Navarro, A. Boveris, Mitochondrial nitric oxide synthase, mitochondrial brain dysfunction in aging, and mitochondria-targeted antioxidants, Adv. Drug Deliv. Rev. 60 (2008) 1534–1544.
[13] S.L. Elfering, T.M. Sarkela, C. Giulivi, Biochemistry of mitochondrial nitric-oxide synthase, J. Biol. Chem. 277 (2002) 38079–38086.
[14] A.J. Kanai, L.L. Pearce, P.R. Clemens, L.A. Birder, M.M. VanBibber, S.Y. Choi, W.C. de Groat, J. Peterson, Identification of a neuronal nitric oxide synthase in isolated cardiac mitochondria using electrochemical detection, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 14126–14131.
[15] T. Persichini, V. Mazzone, F. Polticelli, S. Moreno, G. Venturini, E. Clementi, M. Colasanti, Mitochondrial type I nitric oxide synthase physically interacts with cytochrome *c* oxidase, Neurosci. Lett. 384 (2005) 254–259.
[16] M.S. Parihar, R.R. Nazarewicz, E. Kincaid, U. Bringold, P. Ghafourifar, Association of mitochondrial nitric oxide synthase activity with respiratory chain complex I, Biochem. Biophys. Res. Commun. 366 (2008) 23–28.
[17] P.S. Brookes, Mitochondrial nitric oxide synthase, Mitochondrion 3 (2004) 187–204.
[18] P. Ghafourifar, E. Cadenas, Mitochondrial nitric oxide synthase, Trends Pharmacol. Sci. 26 (2005) 190–195.
[19] Z. Lacza, E. Pankotai, D.W. Busija, Mitochondrial nitric oxide synthase: current concepts and controversies, Front. Biosci. 14 (2009) 4436–4443.
[20] P. Venkatakrishnan, E.S. Nakayasu, I.C. Almeida, R.T. Miller, Absence of nitric-oxide synthase in sequentially purified rat liver mitochondria, J. Biol. Chem. 284 (2009) 19843–19855.
[21] A.V. Kozlov, K. Staniek, H. Nohl, Nitrite reductase activity is a novel function of mammalian mitochondria, FEBS Lett. 454 (1999) 127–130.
[22] S. Basu, N.A. Azarova, M.D. Font, S.B. King, N. Hogg, M.T. Gladwin, S. Shiva, D.B. Kim-Shapiro, Nitrite reductase activity of cytochrome *c*, J. Biol. Chem. 283 (2008) 32590–32597.
[23] P.R. Castello, P.S. David, T. McClure, Z. Crook, R.O. Poyton, Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: implications for oxygen sensing and hypoxic signaling in eukaryotes, Cell Metab. 3 (2006) 277–287.
[24] C. Giulivi, K. Kato, C.E. Cooper, Nitric oxide regulation of mitochondrial oxygen consumption I: cellular physiology, Am. J. Physiol. 291 (2006) C1225–C1231.
[25] A. Boveris, S. Alvarez, A. Navarro, The role of mitochondrial nitric oxide synthase in inflammation and septic shock, Free Radic. Biol. Med. 33 (2002) 1186–1193.
[26] P. Ghafourifar, U. Schenk, S.D. Klein, C. Richter, Mitochondrial nitric-oxide synthase stimulation causes cytochrome *c* release from isolated mitochondria. Evidence for intramitochondrial peroxynitrite formation, J. Biol. Chem. 274 (1999) 31185–31188.
[27] J.J. Poderoso, C. Lisdero, F. Schopfer, N. Riobo, M.C. Carreras, E. Cadenas, A. Boveris, The regulation of mitochondrial oxygen uptake by redox reactions involving nitric oxide and ubiquinol, J. Biol. Chem. 274 (1999) 37709–37716.

[28] E. Cadenas, K.J. Davies, Mitochondrial free radical generation, oxidative stress, and aging, Free Radic. Biol. Med. 29 (2000) 222–230.
[29] R. Radi, A. Cassina, R. Hodara, C. Quijano, L. Castro, Peroxynitrite reactions and formation in mitochondria, Free Radic. Biol. Med. 33 (2002) 1451–1464.
[30] T.A. Prime, F.H. Blaikie, C. Evans, S.M. Nadtochiy, A.M. James, C.C. Dahm, D.A. Vitturi, R.P. Patel, C.R. Hiley, I. Abakumova, R. Requejo, E.T. Chouchani, T.R. Hurd, J.F. Garvey, C.T. Taylor, P.S. Brookes, R.A. Smith, M.P. Murphy, A mitochondria-targeted S-nitrosothiol modulates respiration, nitrosates thiols, and protects against ischemia–reperfusion injury, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 10764–10769.
[31] M. Whiteman, Y.L. Chua, D. Zhang, W. Duan, Y.C. Liou, J.S. Armstrong, Nitric oxide protects against mitochondrial permeabilization induced by glutathione depletion: role of S-nitrosylation? Biochem. Biophys. Res. Commun. 339 (2006) 255–262.
[32] D.T. Hess, A. Matsumoto, S.O. Kim, H.E. Marshall, J.S. Stamler, Protein S-nitrosylation: purview and parameters, Nat. Rev. Mol. Cell. Biol. 6 (2005) 150–166.
[33] A. Bindoli, Lipid peroxidation in mitochondria, Free Radic. Biol. Med. 5 (1988) 247–261.
[34] A.J. Kowaltowski, N.C. de Souza-Pinto, R.F. Castilho, A.E. Vercesi, Mitochondria and reactive oxygen species, Free Radic. Biol. Med. 47 (2009) 333–343.
[35] M.M. Fagian, L. Pereira-da-Silva, I.S. Martins, A.E. Vercesi, Membrane protein thiol cross-linking associated with the permeabilization of the inner mitochondrial membrane by Ca2+ plus prooxidants, J. Biol. Chem. 265 (1990) 19955–19960.
[36] A.J. Kowaltowski, R.F. Castilho, A.E. Vercesi, Mitochondrial permeability transition and oxidative stress, FEBS Lett. 495 (2001) 12–15.
[37] M. Crompton, The mitochondrial permeability transition pore and its role in cell death, Biochem. J. 341 (Pt 2) (1999) 233–249.
[38] J.S. Kim, T. Qian, J.J. Lemasters, Mitochondrial permeability transition in the switch from necrotic to apoptotic cell death in ischemic rat hepatocytes, Gastroenterology 124 (2003) 494–503.
[39] P.S. Brookes, E.P. Salinas, K. Darley-Usmar, J.P. Eiserich, B.A. Freeman, V.M. Darley-Usmar, P.G. Anderson, Concentration-dependent effects of nitric oxide on mitochondrial permeability transition and cytochrome *c* release, J. Biol. Chem. 275 (2000) 20474–20479.
[40] R.S. Kaplan, P.L. Pedersen, Characterization of phosphate efflux pathways in rat liver mitochondria, Biochem. J. 212 (1983) 279–288.
[41] C. Lopez-Mediavilla, A. Orfao, M. Gonzalez, J.M. Medina, Identification by flow cytometry of two distinct rhodamine-123-stained mitochondrial populations in rat liver, FEBS Lett. 254 (1989) 115–120.
[42] N. Kamo, M. Muratsugu, R. Hongoh, Y. Kobatake, Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state, J. Membr. Biol. 49 (1979) 105–121.
[43] B.D. Jensen, K.K. Gunter, T.E. Gunter, The efficiencies of the component steps of oxidative phosphorylation. II. Experimental determination of the efficiencies in mitochondria and examination of the equivalence of membrane potential and pH gradient in phosphorylation, Arch. Biochem. Biophys. 248 (1986) 305–323.
[44] A.N. Murphy, D.E. Bredesen, G. Cortopassi, E. Wang, G. Fiskum, Bcl-2 potentiates the maximal calcium uptake capacity of neural cell mitochondria, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 9893–9898.
[45] J.K. Park, P. Kostka, Fluorometric detection of biological S-nitrosothiols, Anal. Biochem. 249 (1997) 61–66.
[46] M.W. Foster, J.S. Stamler, New insights into protein S-nitrosylation. Mitochondria as a model system, J. Biol. Chem. 279 (2004) 25891–25897.
[47] C.C. Dahm, K. Moore, M.P. Murphy, Persistent S-nitrosation of complex I and other mitochondrial membrane proteins by S-nitrosothiols but not nitric oxide or peroxynitrite: implications for the interaction of nitric oxide with mitochondria, J. Biol. Chem. 281 (2006) 10056–10065.
[48] M. Steffen, T.M. Sarkela, A.A. Gybina, T.W. Steele, N.J. Trasseth, D. Kuehl, C. Giulivi, Metabolism of S-nitrosoglutathione in intact mitochondria, Biochem. J. 356 (2001) 395–402.
[49] A. Boveris, A. Navarro, Systemic and mitochondrial adaptive responses to moderate exercise in rodents, Free Radic. Biol. Med. 44 (2008) 224–229.
[50] V.G. Valle, M.M. Fagian, L.S. Parentoni, A.R. Meinicke, A.E. Vercesi, The participation of reactive oxygen species and protein thiols in the mechanism of mitochondrial inner membrane permeabilization by calcium plus prooxidants, Arch. Biochem. Biophys. 307 (1993) 1–7.
[51] R.F. Castilho, A.J. Kowaltowski, A.R. Meinicke, E.J. Bechara, A.E. Vercesi, Permeabilization of the inner mitochondrial membrane by Ca^{2+} ions is stimulated by t-butyl hydroperoxide and mediated by reactive oxygen species generated by mitochondria, Free Radic. Biol. Med. 18 (1995) 479–486.
[52] O.W. Griffith, R.G. Kilbourn, Nitric oxide synthase inhibitors: amino acids, Meth. Enzymol. 268 (1996) 375–392.
[53] J.J. Poderoso, The formation of peroxynitrite in the applied physiology of mitochondrial nitric oxide, Arch. Biochem. Biophys. 484 (2009) 214–220.
[54] E. Clementi, E. Nisoli, Nitric oxide and mitochondrial biogenesis: a key to long-term regulation of cellular metabolism, Comp. Biochem. Physiol. 142 (2005) 102–110.
[55] E. Nisoli, E. Clementi, M.O. Carruba, S. Moncada, Defective mitochondrial biogenesis: a hallmark of the high cardiovascular risk in the metabolic syndrome? Circ. Res. 100 (2007) 795–806.
[56] E.N. Dedkova, L.A. Blatter, Characteristics and function of cardiac mitochondrial nitric oxide synthase, J. Physiol. 587 (2009) 851–872.
[57] A. Tripathi, S. Khatun, A.N. Pandey, S.K. Mishra, R. Chaube, T.G. Shrivastav, S.K. Chaube, Intracellular levels of hydrogen peroxide and nitric oxide in oocytes at various stages of meiotic cell cycle and apoptosis, Free Radic. Res. 43 (2009) 287–294.
[58] M. Balakirev, V.V. Khramtsov, G. Zimmer, Modulation of the mitochondrial permeability transition by nitric oxide, Eur. J. Biochem. 246 (1997) 710–718.
[59] H. Rubbo, R. Radi, Protein and lipid nitration: role in redox signaling and injury, Biochim. Biophys. Acta 1780 (2008) 1318–1324.
[60] C. Quijano, N. Romero, R. Radi, Tyrosine nitration by superoxide and nitric oxide fluxes in biological systems: modeling the impact of superoxide dismutase and nitric oxide diffusion, Free Radic. Biol. Med. 39 (2005) 728–741.