

Association of mitochondrial nitric oxide synthase activity with respiratory chain complex I

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Abstract

The present study shows that rat liver and brain mitochondrial nitric oxide synthase (mtNOS) are functionally associated with mitochondrial respiratory chain complex I. When complex I is activated, mtNOS exerts high activity and generates nitric oxide, whereas inactivation of complex I leads mtNOS to abandon its NOS activity. Functional association of mtNOS with complex I is potentially important in regulating mtNOS activity and mitochondrial functions.

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Mitochondrial nitric oxide (NO) synthase (mtNOS) regulates various functions of mitochondria in liver [1–5], heart [6–8], brain [2,9,10], kidney [11,12], as well as cells including endothelial [13], kidney [14], neuronal [15], cardiac myocytes [16], and breast cancer [17]. mtNOS-derived NO competes with oxygen and regulates mitochondrial primary functions including respiration and transmembrane potential ($\Delta\Psi$) [1,5,6,9]. mtNOS-derived NO also produces oxidative nitrogen oxide species that cause oxidative damage to mitochondria and cells and play a key role in apoptosis [1–3,8,12,15–17]. Despite significance of the regulation by mtNOS of mitochondrial functions, it is not fully understood how mitochondria regulate mtNOS activity. The rationale for the present study was to delineate whether mitochondria exert regulatory function over mtNOS activ-

ity. The present study shows that in isolated rat liver and brain mitochondria, mtNOS activity is associated with mitochondrial respiratory chain complex I (RCC-I) and suggests RCC-I regulates mtNOS activity.

Material and methods

Purification of mitochondria

Liver mitochondria were isolated from Sprague–Dawley rats by differential centrifugation and purified by Percoll purification as described [18]. Brain mitochondria were purified as described [19]. Brains were removed, washed, and homogenized in Medium A (sucrose 320 mM, potassium EDTA 1 mM, 0.1% fatty acid-free bovine serum albumin [FAF-BSA], Tris–HCl 10 mM, pH 7.40). The homogenate was centrifuged at 1100g for 10 min, and the supernatant was re-centrifuged at 19,000g for 20 min to obtain crude mitochondrial fraction. The pellet was resuspended in Medium A, laid on 6% Ficoll solution, and centrifuged at 19,000g for 30 min. Supernatant and the fluffy layer above the mitochondria pellet were discarded. Each 10 mg of the brown pellet was resuspended in 70 mg of the Purification Medium (2.56 g of 20% [w/w] dextran T500; 1.28 g of 40% [w/w] polyethylene glycol (PEG) 4000; 2.24 g of 1 M sorbitol; 70 mg of 10 mM potassium EDTA; 700 mg of 1% FAF-BSA; 200 mg of 200 mM potassium phosphate, pH 7.80; H₂O to 7.00 g)

Abbreviations: RCC-I, respiratory chain complex I; RCC-II, respiratory chain complex II; NO, nitric oxide; mtNOS, mitochondrial nitric oxide synthase; L-NMMA, *N*^G-monomethyl-L-arginine; Rot, rotenone; Succ, succinate; Pyr, Pyruvate; Mal, malate.

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and centrifuged at 600g for 2 min in a swing-bucket centrifuge. The upper phase was discarded, 300 μ l of the Washing Medium (96 mg of 20% [w/w] dextran T500; 480 mg of 40% [w/w] PEG 4000; 960 mg of 1 M sorbitol; 75 mg of 200 mM potassium phosphate pH 7.80; 30 mg of 10 mM potassium EDTA; 300 mg 1% FAF-BSA; H₂O to 3.00 g) was added to the lower part, mixed and centrifuged at 600g for 2 min. The upper part was discarded and the lower part was mixed with 3 ml of Medium B (sorbitol 320 mM, potassium EDTA 1 mM, 0.1% FAF-BSA, potassium phosphate 5 mM, pH 7.80). The suspension was centrifuged at 19,000g for 20 min, the supernatant was discarded, and the mitochondrial pellet was collected. All steps were carried out at 4 °C. Purity of the isolated mitochondria was assessed by measuring cytochrome *a* using $\epsilon_{605-630\text{ nm}}$ 12 mM⁻¹ cm⁻¹ as described [18]. Only mitochondria with less than 5% impurity were used. Broken mitochondria were prepared by five times freezing the intact mitochondria in liquid nitrogen followed by thawing as described [1,17]. Submitochondrial particles (SMP) were prepared from mitoplasts as described [20]. Briefly, the outer membrane was removed by treating the intact mitochondria with digitonin (0.12 mg/mg of mitochondrial protein) for 15 min at 4 °C followed by two times wash. Mitoplasts were frozen–thawed as above and the unbroken mitoplasts were removed by two times spinning at 9500g for 10 min. The supernatant of the second centrifugation was centrifuged at 100,000g for 30 min. The pellet referred to as SMP and supernatant referred as to mitochondrial matrix were collected.

Activation or inactivation of RCCs

The following experimental conditions were used in freshly isolated mitochondria: (A) no respiratory substrate (no RS) refers to when respiratory chain was not altered by exogenous substrates; (B) RCC-I activation refers to when RCC-I was activated by pyruvate/malate (10/5 mM); (C) RCC-I inactivation refers to when RCC-I was inactivated by rotenone (5 μ M); (D) RCC-II activation refers to when RCC-I was inactivated by rotenone (5 μ M) and mitochondria were energized from RCC-II by succinate (2.5 mM). In SMP, RCC-I was activated by NADH (100 μ M).

Determination of mtNOS activity

mtNOS activity was determined using the following assays:

Oxyhemoglobin assay. mtNOS activity was detected in broken mitochondria at 401–420 nm in the presence of oxyhemoglobin (4 μ M) using $\epsilon_{401-420\text{ nm}}$ 100 mM⁻¹ cm⁻¹ as described [18].

Citrulline radioassay. Mitochondria were supplemented with L-[³H]arginine (30,000–50,000 cpm). Radio-labeled L-citrulline was separated from L-arginine using Dowex columns and the radioactivity was measured as described [3,17].

Chemiluminescence assay. Mitochondria (50 μ g) were injected into the purge vessel containing vanadium chloride (0.8% in 1 M HCl) thermostated at 93 °C, and NO was measured using chemiluminescence analyzer (Sievers 280i, General Electric, Boulder, CO) as described [17].

Amperometric assay. mtNOS activity in SMP was measured by NO-sensitive electrode (Apollo 4000; World Precision Instruments, FL) using a 7 μ m tip micro-sensor (ISO-NO-007) placed in the chamber containing SMP (200 μ g).

Oxygen consumption

Oxygen consumption was measured using a Clark-type oxygen electrode (Harvard Apparatus, Holliston, MA) as described [18,21].

Intramitochondrial ionized calcium [Ca^{2+}]_m

The [Ca^{2+}]_m was measured by using dual-wavelength excitation fluorometry as described [8,17]. Mitochondria were loaded with fura-2/AM for 20 min at room temperature followed by twice wash. Fura-2/AM loaded mitochondria were excited at 352–362 nm and emission was collected at 510 nm.

Statistical analysis

Barograms are mean \pm SEM of $n = 4$ –6. Differences were tested by ANOVA and considered significant at $p < 0.05$. All computations were performed using PlotIT (Scientific Programming).

Results

mtNOS activity

Fig. 1A–F shows mtNOS activity of liver and brain mitochondria as determined by three NOS assays. As shown, mtNOS activity was significantly higher when mitochondria were respiring on complex I, compared to when mitochondria were not supplied with exogenous respiratory substrates, RCC-I was inactivated, or RCC-II was activated. As shown, mtNOS activity in all samples was inhibited by mtNOS inhibitor, L-N^G-monomethyl arginine (L-NMMA; Ref. [3,18]).

Mitochondrial respiration

Fig. 2A and B shows respiration of liver and brain mitochondria supported by activation of RCC-I, or when RCC-I was inactivated and respiration was supported from RCC-II. As shown, respiration supported by RCC-I was decreased by loading mitochondria with Ca^{2+} , and that Ca^{2+} -induced decrease of respiration was prevented by L-NMMA. However, when RCC-I was inactivated and respiration was supported from RCC-II, Ca^{2+} did not decrease the respiration.

Intramitochondrial calcium ([Ca^{2+}]_m)

mtNOS is Ca^{2+} -dependent [1,7,9,13] and elevation of [Ca^{2+}]_m *per se* is sufficient to increase mtNOS activity [3,8,9,13,16,17]. Thus, the present study determined whether RCC-I activation increases [Ca^{2+}]_m. Fig. 3 shows that [Ca^{2+}]_m was not increased when RCC-I was activated compared to when RCC-I was inactivated and RCC-II was activated.

mtNOS activity of submitochondrial particle (SMP)

Fig. 4A and B shows mtNOS activity of SMP. As shown, activation of SMP RCC-I by NADH stimulates mtNOS in an L-NMMA-sensitive manner. Increased mtNOS activity was inhibited when RCC-I was inactivated by rotenone. Replacing NADH with NADPH did not support mtNOS activity.

Discussion

The present study provides evidence strongly suggesting that mtNOS activity in isolated rat liver and brain mitochondria is associated with RCC-I. Using three well-established NOS detection assays, Fig. 1 shows mtNOS activity

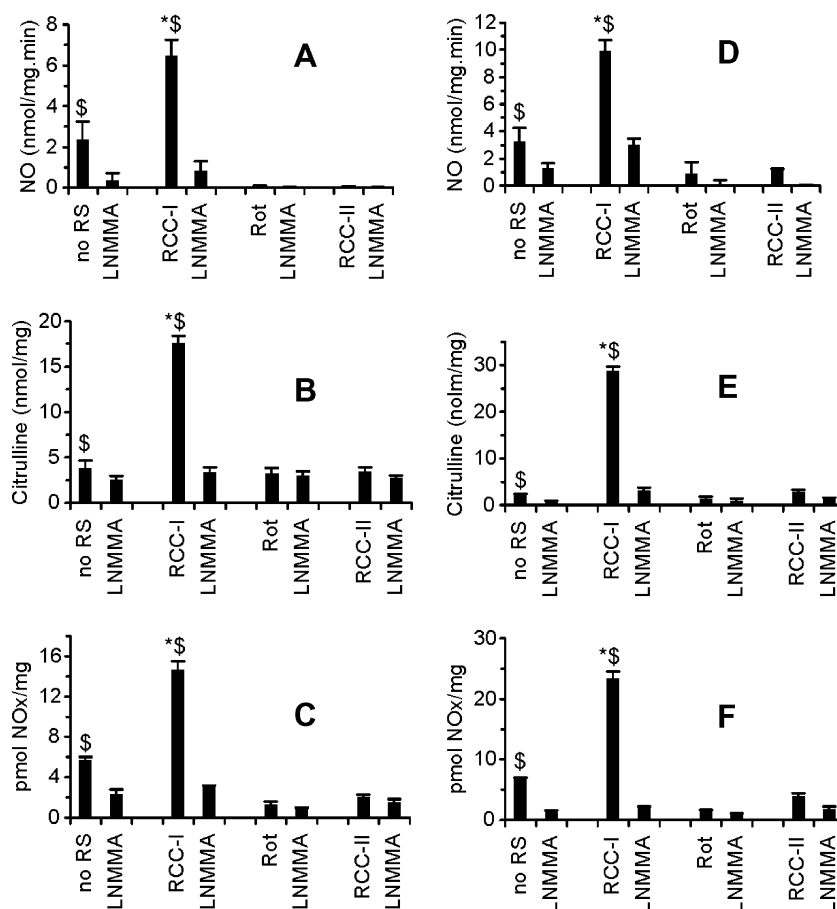


Fig. 1. mtNOS activity and mitochondrial respiratory chain complexes. mtNOS activity in isolated liver (A–C) and brain (D–F) mitochondria were measured using oxyhemoglobin (A, D), citrulline radioassay (B, E), and chemiluminescence assay (C, F). Measurements were performed under the conditions that mitochondrial respiratory chain activity was not altered by exogenous respiratory substrates (no RS), RCC-I was activated (RCC-I), RCC-I was inactivated (Rot), or RCC-I was inactivated and mitochondria were energized from RCC-II (RCC-II). Where indicated mtNOS was inhibited by L-NMMA. Results are expressed as nmol NO per mg mitochondrial protein minute (A, D); nmol citrulline per mg mitochondrial protein (B, E), and pmol NOx per mg mitochondrial protein (C, F). * $P < 0.05$ RCC-I vs. no RS, Rot, RCC-II; $^{\$}P < 0.05$ vs. L-NMMA.

as determined by all NOS assays is significantly higher when RCC-I is activated, compared to when mitochondria were not supplied with exogenous respiratory substrates, RCC-I was inactivated, or when mitochondria were energized from RCC-II. mtNOS is Ca^{2+} -sensitive and increased $[\text{Ca}^{2+}]_{\text{m}}$ stimulates mtNOS activity [1,3,7,8,13,17]. mtNOS-derived NO decreases mitochondrial respiration [22] by competing with O_2 for the binding site of cytochrome oxidase [23]. Fig. 2A and B confirms findings of Fig. 1 by showing that mitochondrial respiration supported from RCC-I was decreased by loading mitochondria with Ca^{2+} and that this effect of Ca^{2+} was prevented when mtNOS was inhibited. However, when RCC-I was inactivated and mitochondrial respiration was supported from RCC-II, Ca^{2+} did not decrease mitochondrial respiration. Findings presented under Figs. 1 and 2 strongly support the hypothesis that mtNOS activity in brain and liver mitochondria is associated with RCC-I activity.

Intramitochondrial calcium homeostasis is governed by continuous precipitation of $[\text{Ca}^{2+}]_{\text{m}}$ to non-ionized calcium stores known as matrix electron-dense granules, and release of $[\text{Ca}^{2+}]_{\text{m}}$ from the granules [8,24]. Since mtNOS is

$[\text{Ca}^{2+}]_{\text{m}}$ -sensitive [8,13,17] and RCC-I activation stimulated mtNOS activity, we tested the effect of RCC-I activation on $[\text{Ca}^{2+}]_{\text{m}}$. As shown in Fig. 3, RCC-I activation did not increase $[\text{Ca}^{2+}]_{\text{m}}$, even $[\text{Ca}^{2+}]_{\text{m}}$ was slightly decreased compared to when RCC-I was inactivated and mitochondria were energized from RCC-II. These findings rule out elevation of $[\text{Ca}^{2+}]_{\text{m}}$ as a mechanism underlying stimulation of mtNOS upon RCC-I activation. To further study mechanisms responsible for association of mtNOS activity with RCC-I, we measured mtNOS activity in SMP. mtNOS is located at the inner mitochondrial membrane [1,3,8,10] and determination of mtNOS in SMP allows excluding potential interference of mitochondrial matrix, intermembrane space, outer membrane, or other components of mitochondria with RCC-I-mtNOS association. Fig. 4A and B shows that NADH that activates RCC-I significantly increased mtNOS activity in an L-NMMA sensitive manner. NOS isozymes utilize L-arginine to produce NO and L-citrulline. For cytoplasmic NOS isozymes, this reaction utilizes electrons donated by NADPH rendering a redox potential of -280 mV [25]. Fig. 4A and B shows that NADPH that serves native electron donor to cytoplasmic

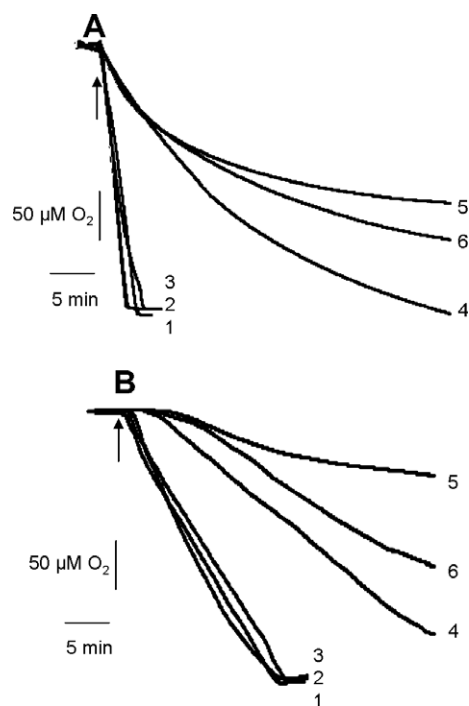


Fig. 2. Oxygen consumption. Oxygen consumption of liver (A) and brain mitochondria (B) are shown. Trace 1 represents respiration supported from RCC-II. Trace 2 is as trace 1 except Ca^{2+} was present in the medium. Trace 3 is as trace 2 except mitochondria were pre-treated with L-NMMA. Trace 4 represents respiration supported from RCC-I. Trace 5 is as trace 4, except Ca^{2+} was present in the medium. Trace 6 is as trace 5 except mitochondria were treated with L-NMMA. Arrows indicates addition of RCC-I or RCC-II substrates to the chamber.

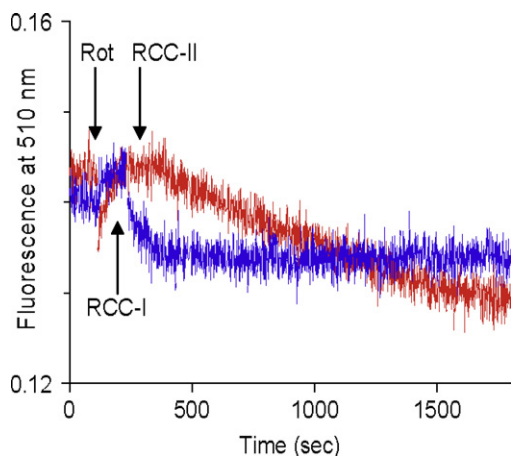


Fig. 3. Mitochondrial calcium ($[\text{Ca}^{2+}]_m$). Intramitochondrial ionized calcium was measured under the conditions that RCC-I was activated (RCC-I) or RCC-I was inactivated by rotenone (Rot) and RCC-II was activated by succinate (RCC-II).

NOS isozymes [26,27] fails to support mtNOS activity of SMP. These figures also show that NADH-increased mtNOS activity was inhibited when RCC-I was inactivated by rotenone. This finding rules out possible direct interaction of NADH with mtNOS. These findings also explain lack of NO formation when SMP were supplied NADPH, as well as inhibition of mtNOS by rotenone.

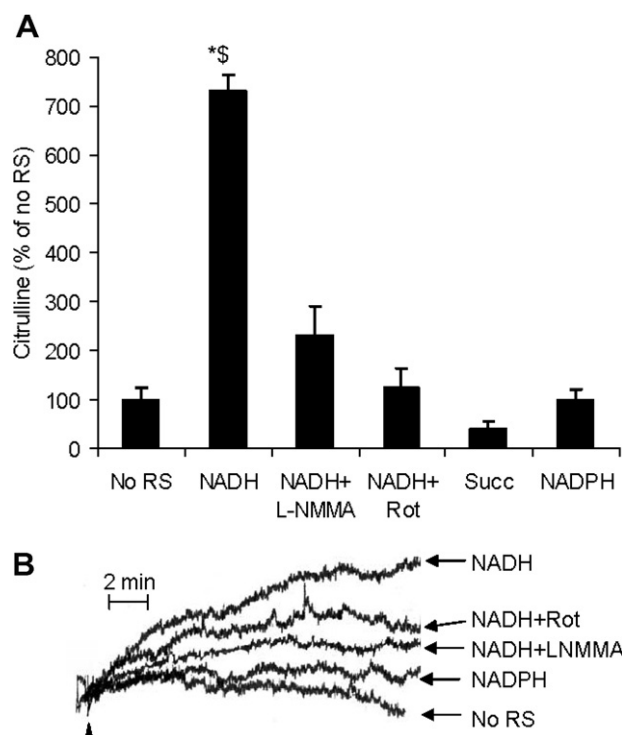


Fig. 4. mtNOS activity of submitochondrial particles. (A) mtNOS activity was measured in submitochondrial particles by using citrulline assay and expressed as percentage of when submitochondrial particles were not supplied with exogenous respiratory substrates (no RS). Bars represent when RCC-I was activated by NADH in the absence (NADH) or presence of L-NMMA (NADH + L-NMMA) or rotenone (NADH + Rot), when RCC-I was inactivated by rotenone and RCC-II was activated by succinate (Succ), or NADPH (NADPH) was present in place of NADH. (B) mtNOS activity was measured by using NO-sensitive electrode under the conditions that submitochondrial particles were not supported with respiratory substrates (no RS), or RCC-I was activated by NADH in the absence (NADH) or presence of L-NMMA (NADH + L-NMMA) or rotenone (NADH + Rot), or NADH was replaced with NADPH (NADPH). * $P < 0.05$ NADH vs. no RS, NADH + Rot, Succ, NADPH; $^{\$}P < 0.05$ vs. L-NMMA.

NOS isozymes are oxidoreductase proteins comprising of an oxygenase and a reductase domain [25]. Each NOS monomer homodimerizes in a configuration that allows the reductase domain of each monomer couples with the oxygenase domain of the coupling monomer. Electrons enter the NADPH-binding site at the reductase domain of a monomer and transverse to the arginine-binding site at the oxygenase domain of the coupling monomer where NO is generated (Diagram 1). Coupling is essential for NOS isozymes to retain their NOS activity and uncoupling NOS isozymes abolishes their NOS activity [25,26]. mtNOS is located at the inner mitochondrial membrane [1,18] where respiratory chain complexes are embedded. Mitochondrial respiratory chain complexes provide a range of redox potentials from -280 mV for RCC-I to $+250$ mV for cytochrome *c* oxidase [28]. Electrons derived from oxidation of NADH enter RCC-I and flow through the chain following the redox hierarchy of the respiratory complexes. Our results show that when RCC-I is active, mtNOS produces NO, while inactivation of RCC-I abolishes mtNOS

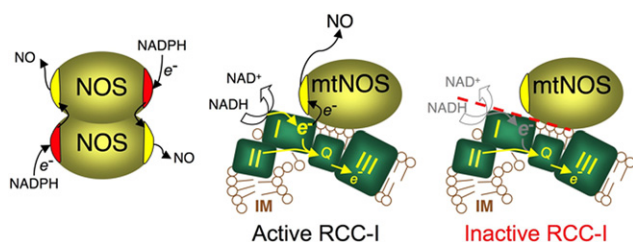


Diagram 1. Functional association of mtNOS with RCC-I. Cytoplasmic NOS isozymes consist of a reductase and an oxygenase domain. Each monomer homodimerizes in a configuration that allows electrons flow from the reductase domain of one monomer to the oxygenase domain of the coupling monomer where NO is produced. mtNOS that is localized at mitochondrial inner membrane (IM) where respiratory chain complexes are located. Electrons enter the chain from respiratory complex I (I) or RCC-II (II) and flow down to ubiquinone (Q) and downstream complexes (only RCC-III is shown). mtNOS functionally associates with RCC-I and utilizes RCC-I as source of electrons. When RCC-I is active, mtNOS receives electrons enabling the enzyme to produce NO. Inactivation of RCC-I ceases mtNOS reducing equivalents and abolishes its NOS activity.

activity. These findings suggest a functional association between mtNOS and RCC-I whereby mtNOS utilizes RCC-I as its source of electrons (Diagram 1), and describe a reciprocal regulation between mtNOS and mitochondrial respiratory chain.

Taken together, the present study demonstrates that liver and brain mtNOS are functionally associated with RCC-I. When RCC-I is activated mtNOS generates NO, whereas RCC-I inactivation leads mtNOS to abandon NO production. Exact molecular mechanism underlying association of mtNOS with RCC-I needs further investigations.

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References

- [1] P. Ghafourifar, C. Richter, Nitric oxide synthase activity in mitochondria, *FEBS Lett.* 418 (1997) 291–296.
- [2] A. Navarro, A. Boveris, Rat brain and liver mitochondria develop oxidative stress and lose enzymatic activities on aging, *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 287 (2004) R1244–R1249.
- [3] 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.
- [4] A. Navarro, M.J. Sanchez-Pino, C. Gomez, M.J. Bandez, E. Cadenas, A. Boveris, Dietary thioproline decreases spontaneous food intake and increases survival and neurological function in mice, *Antioxid. Redox. Signal.* 9 (2007) 131–141.
- [5] L.B. Valdez, T. Zaobornyj, A. Boveris, Mitochondrial metabolic states and membrane potential modulate mtNOS activity, *Biochim. Biophys. Acta* 1757 (2006) 166–172.
- [6] G.F. Gonzales, F.A. Chung, S. Miranda, L.B. Valdez, T. Zaobornyj, J. Bustamante, A. Boveris, Heart mitochondrial nitric oxide synthase is upregulated in male rats exposed to high altitude (4340 m), *Am. J. Physiol. Heart Circ. Physiol.* 288 (2005) H2568–H2573.
- [7] A.J. Kanai, L.L. Pearce, P.R. Clemens, L.A. Birder, M.M. VanBiber, 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. USA* 98 (2001) 14126–14131.
- [8] W.J. Zenebe, R.R. Nazarewicz, M.S. Parihar, P. Ghafourifar, Hypoxia/reoxygenation of isolated rat heart mitochondria causes cytochrome c release and oxidative stress; evidence for involvement of mitochondrial nitric oxide synthase, *J. Mol. Cell Cardiol.* 43 (2007) 411–419.
- [9] S. Lores-Arnaiz, G. D'Amico, A. Czerniczyniec, J. Bustamante, A. Boveris, Brain mitochondrial nitric oxide synthase: in vitro and in vivo inhibition by chlorpromazine, *Arch. Biochem. Biophys.* 430 (2004) 170–177.
- [10] N.A. Riobo, M. Melani, N. Sanjuan, M.L. Fiszman, M.C. Gravielle, M.C. Carreras, E. Cadenas, J.J. Poderoso, The modulation of mitochondrial nitric-oxide synthase activity in rat brain development, *J. Biol. Chem.* 277 (2002) 42447–42455.
- [11] A. Boveris, L.B. Valdez, S. Alvarez, T. Zaobornyj, A.D. Boveris, A. Navarro, Kidney mitochondrial nitric oxide synthase, *Antioxid. Redox. Signal.* 5 (2003) 265–271.
- [12] J.L. Vinas, A. Sola, G. Hotter, Mitochondrial NOS upregulation during renal I/R causes apoptosis in a peroxynitrite-dependent manner, *Kidney Int.* 69 (2006) 1403–1409.
- [13] E.N. Dedkova, X. Ji, S.L. Lipsius, L.A. Blatter, Mitochondrial calcium uptake stimulates nitric oxide production in mitochondria of bovine vascular endothelial cells, *Am. J. Physiol. Cell Physiol.* 286 (2004) C406–C415.
- [14] M.O. Lopez-Figueroa, C. Caamano, R. Marin, B. Guerra, R. Alonso, M.I. Morano, H. Akil, S.J. Watson, Characterization of basal nitric oxide production in living cells, *Biochim. Biophys. Acta* 1540 (2001) 253–264.
- [15] J. Dennis, J.P. Bennett Jr, Interactions among nitric oxide and Bcl-family proteins after MPP+ exposure of SH-SY5Y neural cells I: MPP+ increases mitochondrial NO and Bax protein, *J. Neurosci. Res.* 72 (2003) 76–88.
- [16] R.R. Nazarewicz, W.J. Zenebe, A. Parihar, M.S. Parihar, P. Ghafourifar, 12(S)-Hydroperoxyeicosatetraenoic acid (12-HETE) increases mitochondrial nitric oxide by increasing intramitochondrial calcium, *Arch. Biochem. Biophys.* 468 (2007) 114–120.
- [17] R.R. Nazarewicz, W.J. Zenebe, A. Parihar, S.K. Larson, E. Alidema, J. Choi, P. Ghafourifar, Tamoxifen induces oxidative stress and mitochondrial apoptosis via stimulating mitochondrial nitric oxide synthase, *Cancer Res.* 67 (2007) 1282–1290.
- [18] P. Ghafourifar, M.L. Asbury, S.S. Joshi, E.D. Kincaid, Determination of mitochondrial nitric oxide synthase activity, *Methods Enzymol.* 396 (2005) 424–444.
- [19] M.J. Lopez-Perez, Preparation of synaptosomes and mitochondria from mammalian brain, *Methods Enzymol.* 228 (1994) 403–411.
- [20] P. Ghafourifar, Characterization of mitochondrial nitric oxide synthase, *Methods Enzymol.* 359 (2002) 339–350.
- [21] P. Ghafourifar, S.D. Klein, O. Schucht, U. Schenk, M. Pruschy, S. Rocha, C. Richter, Ceramide induces cytochrome c release from isolated mitochondria. Importance of mitochondrial redox state, *J. Biol. Chem.* 274 (1999) 6080–6084.
- [22] J.G. Peralta, P.V. Finocchietto, D. Converso, F. Schopfer, M.C. Carreras, J.J. Poderoso, Modulation of mitochondrial nitric oxide synthase and energy expenditure in rats during cold acclimation, *Am. J. Physiol. Heart Circ. Physiol.* 284 (2003) H2375–H2383.
- [23] D.L. Boveris, A. Boveris, Oxygen delivery to the tissues and mitochondrial respiration, *Front Biosci.* 12 (2007) 1014–1023.
- [24] M. Ashraf, C.M. Bloor, X-ray microanalysis of mitochondrial deposits in ischemic myocardium, *Virchows Arch. B Cell Pathol.* 22 (1976) 287–297.

- [25] W.K. Alderton, C.E. Cooper, R.G. Knowles, Nitric oxide synthases: structure, function and inhibition, *Biochem J.* 357 (2001) 593–615.
- [26] P.R. Ortiz de Montellano, C. Nishida, I. Rodriguez-Crespo, N. Gerber, Nitric oxide synthase structure and electron transfer, *Drug Metab. Dispos.* 26 (1998) 1185–1189.
- [27] D.K. Ghosh, M.A. Holliday, C. Thomas, J.B. Weinberg, S.M. Smith, J.C. Salerno, Nitric-oxide synthase output state. Design and properties of nitric-oxide synthase oxygenase/FMN domain constructs, *J. Biol. Chem.* 281 (2006) 14173–14183.
- [28] D.D. Tyler, *The Mitochondrion in Health and Disease*, VCH Publishers Inc., New York, 1992.