

NO-synthase activity in mitochondria of uterus smooth muscle: identification and biochemical properties

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Abstract. Information about the catalytic and kinetic properties of mitochondria NO-synthase from uterus smooth muscle is missing currently. According to the data on MitoTracker Orange CM-H2TMRos and 4-amino-5-methylamino-2',7'-difluorescein, diaminofluorescein-FM (DAF-FM) dye co-localization in uterine smooth muscle cells, presented in this paper, NO can be synthesized in their mitochondria. High activity of NO synthase requires the presence of substrates of respiration, L-arginine, Ca^{2+} and NADPH. It is established that the dependence of NO production on the concentration of L-arginine has a bell-shaped character with a maximum of 75 μM , and the apparent affinity constant for L-arginine is $28.9 \pm 9.1 \mu\text{M}$. The dependence of NO production on Ca^{2+} concentration has a maximum at 100-250 μM ; the activation constant for Ca^{2+} is $44.4 \pm 14.5 \mu\text{M}$. The inhibitor of Ca^{2+} transport in mitochondria ruthenium red (RuR), as well as the inhibitor of NO-synthase N^G -nitro-L-arginine (NA), reduces NO production. The biosynthesis of NO by mitochondria depends on its energized level: it is stimulated by the addition of respiration substrates, suppressed with specific inhibitors of the electron transport chain (rotenone and antimycin A) and carbonyl-cyanide 3-chlorophenylhydrazone (CCCP) protonophore.

Key words: Nitric oxide — Mitochondria — Calcium — Smooth muscle — Myometrium

Introduction

Nitric oxide (NO) is a universal signaling and regulatory molecule in a cell. The control of the contractile function of smooth muscles, including myometrium, is an important example of its biological activity (Buxton 2004).

To date, it has been found that NO modulates energy, metabolic and transport processes in mitochondria. It is a well-known point of view that mitochondria are the primary cell targets of NO (Giulivi 2007). The membrane of mitochondria is an effective target of nitrocompounds, since there is a high content of thiol residues, iron-sulfur centers, hemic groups in the localized enzymes, and the membrane itself is the place of superoxide anion formation (Boveris 1977; Korshunov et al. 1997; Ghimire 2017). Nitric oxide is capable of regulating the activity of the mitochondrial electron transport chain, reversibly suppressing

cytochrome c-oxidase, and controlling mitochondrial pH (Giulivi 2007; Piantadosi and Suliman 2012; Ghimire 2017). At low nanomolar concentrations, NO limits the intensity of respiration and oxidative phosphorylation, which is considered as an adaptive physiological response (Brown 2007; Brown and Borutaite 2007; Levine et al. 2012; Zaobornyj and Ghafourifar 2012). NO regulates the homeostasis of Ca^{2+} in mitochondria and, accordingly, Ca^{2+} -dependent processes, primarily the activity of dehydrogenases (Traaseth et al. 2004; Giulivi et al. 2006; Gellerich et al. 2010). NO/cGMP-dependent decrease in the electrical potential on the internal membrane of mitochondria, which affects the intensity of electrophoretic accumulation of Ca^{2+} , is described (Levine et al. 2012). The synthesis of ATP by mitochondria is regulated by NO in a GMP-dependent manner (Forstermann and Sessa 2012; Levine et al. 2012). Nitric oxide stimulates mitochondria biogenesis in various types of tissues and, in particular, increases the expression of sirtuin-1, which together with 5'-AMP protein kinase regulates peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α), which is a modulator of mitochondrial biogenesis (Nisoli and

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Carruba 2006; Levine et al. 2012; Piantadosi and Suliman 2012; Tengan et al. 2012). There is no doubt that biochemistry and molecular physiology of mitochondria, which combines the processes of oxygen consumption, oxidative phosphorylation, lipolytic catabolism, heme biosynthesis, Ca^{2+} homeostasis maintenance, oxygen active form production and apoptosis, are largely controlled by NO (Giulivi et al. 2006).

Considering the central role of mitochondria in cellular energy supply, and also taking into account modern notions of mitochondria as a key element in biochemical processes of endothelial dysfunction in cardiovascular diseases, type II diabetes, neurodegenerative pathologies etc. ("mitochondrial diseases"), considerable attention in modern literature is given to the problem of the relationship: NO – mitochondria (Davidson and Duchon 2006; Levine et al. 2012; Litvinova et al. 2015).

In 1997 P. Ghafourifar and C. Richter demonstrated the existence of functionally active mitochondrial NOS (common abbreviation – mtNOS) in rat liver mitochondria. The enzyme formed NO and L-citrulline in the presence of L-arginine, and the process was Ca^{2+} -dependent. Such analogues of L-arginine as N^G -monomethyl-L-arginine (L-NMMA) and N^G -nitro-L-arginine (NA) inhibited the enzyme. The activity of mtNOS was associated with the internal mitochondrial membrane. The enzyme was constitutively active, dependent on the intensity of mitochondrial respiration and was under the control of membrane potential in intact energized by succinate addition mitochondria (Ghafourifar and Richter 1997). In 1998 C. Giulivi and A. Boveris with co-authors (Giulivi et al. 1998) showed NO production in rat liver mitochondria by direct, reliable, precise independent methods: electron-paramagnetic resonance using spin traps (derivatives of dithiocarbamates) and two-wavelength spectrophotometry of oxidized hemoglobin. Studies were conducted on purified preparations of mitochondria (intact, permeabilized organelles, sub-mitochondrial particles) and crude fraction of mtNOS. The apparent K_m for L-arginine was also determined, which was 5–7 μM , being close to that for neuronal NO-synthase (nNOS) (Haynes et al. 2004; Valdez et al. 2006).

The majority of experimental data suggests that in mitochondria a special isoform of NOS exists – mtNOS, with a molecular weight of 144 kDa, which is a splice variant of nNOS- α different from those already identified. It has two post-translational modifications: N-terminal myristoylation on Thr or Ser residues and C-terminal phosphorylation on Ser-1412 (Elfering et al. 2002; Haynes et al. 2004; Traaseth et al. 2004; Ghafourifar and Cadenas 2005). Immune co-localization of mtNOS with mitochondrial markers (succinate dehydrogenase, cytochrome c-oxidase) further proves its placement precisely in the internal mitochondrial membrane. mtNOS myristoylation is necessary to direct the enzyme to a mitochondria or to anchor it in the mem-

brane. Phosphorylation of C-terminal Ser residue regulates the activity of mtNOS (Elfering et al. 2002; Zaobornyj and Ghafourifar 2012; Tengan et al. 2012).

Immune-electron microscopy and immune-precipitation techniques show the interaction of mtNOS with the subunit V_a of cytochrome c-oxidase *via* the PDZ domain (acronym from words: post, Drosophila, Zonula). Other studies have found that the proteins of complex I of the electron transport chain are immune-precipitated with intra-mitochondrial NOS (Ghafourifar and Cadenas 2005; Persichini et al. 2005; Franco et al. 2006; Carreras and Poderoso 2007; Zaobornyj and Ghafourifar 2012).

mtNOS-generated NO provides reciprocal regulation between the biosynthesis of NO in this compartment and the concentration of intra mitochondrial Ca^{2+} , L-arginine, pH, O_2 partial pressure and oxidation-reduction state of mitochondria (Nisoli and Carruba 2006; Dedkova and Blatter 2009; Santos et al. 2011; Zaobornyj and Ghafourifar 2012). Localization of NOS in mitochondria is today reliably shown in various organs and tissues of mammals: liver (Giulivi 1998; Tatoyan and Giulivi 1998), heart (Davidson and Duchon 2006; Dedkova and Blatter 2009; Litvinova et al. 2015), brain (Dolińska and Albrecht 1998), diaphragm (Haynes et al. 2003; Alvarez and Boveris 2004), kidneys (Boveris 2003), thymus (Bustamante et al. 2002). The biosynthesis of NO by mitochondria is a powerful process: heart and diaphragm organelles are capable to produce 67% and 24% of the total cell content of NO, respectively.

However, the possibility and the biochemical properties of NO formation in smooth muscle mitochondria remain to be clarified, and the problem of NO biosynthesis in myometrium mitochondria is not considered. The presented work is dedicated to the solution of these issues.

Materials and Methods

Isolation of myocytes from the uterine smooth muscle (myometrium) of non-pregnant rats

Rats were anesthetized by diethyl ether inhalation and decapitated. Myocytes were isolated from the uteruses of white nonlinear nonpregnant rats, by applying Mollard method using collagenase and soybean trypsin inhibitor (Mollard et al. 1986).

Isolation of mitochondria from the uterine smooth muscle

The mitochondrial fraction was isolated from the myometrium of non-pregnant rats using differential centrifugation, as described by Kosterin and coworkers (Kolomiets et al. 1985). Rats were anesthetized by diethyl ether inhalation and then decapitated. The isolated mitochondrial fraction was kept on ice for the duration of the experiment. The protein

content in the mitochondrial fraction was determined by a standard procedure by Bradford (1976). The total protein content in the mitochondrial fraction was 2 mg/ml.

Animal experiments were conducted in accordance with the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (International Convention, Strasbourg, 1986).

Laser scanning confocal microscopy

Spatial distribution of fluorescent dyes in cells was investigated with the confocal laser scanning microscope LSM 510 META («Carl Zeiss», Germany). Myocytes were immobilized on poly-L-lysine. All experimental procedures were conducted in physiological Henks's solution (mM): NaCl, 136.9; KCl, 5.36; KH_2PO_4 , 0.44; NaHCO_3 , 0.26; Na_2HPO_4 , 0.26; CaCl_2 , 0.03; MgCl_2 , 0.4; MgSO_4 , 0.4; glucose, 5.5; Hepes (pH 7.4; 37°C), 10. For the visualization of mitochondria and cell nucleus the fluorescent dyes MitoTracker Orange CMH₂TMRos (200 nM) and Hoechst 33342 (50 nM) were used, respectively (Buckman et al. 2001). The loading of immobilized myocytes with NO-sensitive fluorescent probe 4-amino-5-methylamino-2',7'-difluorescein, diaminofluorescein-FM (DAF-FM) in concentration of 10 μM was carried out for 15 min at 24°C. The experiments with confocal microscopy were performed in MultiTrack mode. The fluorescence of Hoechst 33342 was excited using excitation laser at wavelength 405 nm, and registered with the BP 420–480 filter. The excitation laser at the wavelength of 543 nm was used for MitoTracker Orange CM-H2TMRos, and the fluorescence was registered in the spectral range 560–615 nm; DAF-FM fluorescence was excited at the wavelength 488 nm, and the emission was registered in the spectral range 505–530 nm.

Study of NO biosynthesis by isolated mitochondria using DAF-FM and flow cytometry

The measurements were performed using COULTER EPICS XLTM (Beckman Coulter, USA) cytometer with an argon laser ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{fl}} = 515 \text{ nm}$ (Fl1 channel)). The NO-sensitive DAF-FM fluorescence probe was added to the mitochondria in the working medium, the experimentally-selected concentration was 0.5 μM . In experiments DAF-FM was used at concentrations of 0.1–10 μM to find optimal conditions for the determination of NO.

The dependence of mtNOS activity on the presence of respiratory substrates (5 mM pyruvate and succinate), L-arginine (1–100 μM), Ca^{2+} (10–500 μM , in some cases 1 mM), cofactors (10 μM tetrahydrobiopterin (BH_4) and FAD), NADPH (10 μM), as well as 10 μM ruthenium red (RuR), 7.5 $\mu\text{g/ml}$ alamethicin and N^{G} -nitro-L-arginine

(NA), was studied. ADP (0.5 mM), inhibitors of the electron transport chain (5 μM rotenone, 1 $\mu\text{g/ml}$ antimycin A, 2.5 μM oligomycin) and 10 μM protonophore carbonyl-cyanide 3-chlorophenylhydrazone (CCCP) were used in the investigation of mitochondria NO production depending on their energy status.

The working medium in all cases, except for the concentration dependences on L-arginine and Ca^{2+} , was composed of (mM): 20 Hepes (pH 7.4; 37°C), 2 K^+ -phosphate buffer (pH 7.4, 37°C), 120 KCl, 5 pyruvate, 5 succinate, 0.05 L-arginine, 0.1 Ca^{2+} , 0.01 NADPH, 0.01 BH_4 , aliquot of the mitochondria fraction contained 20 μg of protein.

The activation constant of NO-synthase reaction in mitochondria for Ca^{2+} and the apparent affinity constant for L-arginine were evaluated by Heinz method (Keleti 1986). These constants were calculated in Heinz coordinates $\{(S)/V : (S)\}$, where (S), L-arginine or Ca^{2+} concentration; V, mtNOS activity ($n = 5$). Correlation coefficient formulas are used to find how strong a relationship between the data is. The formulas return a value between –1 and 1. Curves with the correlation coefficient $R^2 > 0.9$ were taken into account. The data are presented as mean \pm SE.

In the work the following reagents were used: Hepes, glucose, saccharose, sodium succinate, sodium pyruvate, bovine serum albumin, poly-L-lysine, collagenase type IA, ATP, ADP, Pluronic F-27, DAF-FM ($\text{C}_{21}\text{H}_{14}\text{F}_2\text{N}_2\text{O}_5$, 4-amino-5-methylamino-2',7'-difluorescein, diaminofluorescein-FM), EGTA, CaCl_2 , N^{G} -nitro-L-arginine, oligomycin, rotenone, antimycin A, CCCP, ruthenium red, L-arginine, NADPH, BH_4 , FAD (Sigma, USA); Hoechst 33342, soybean trypsin inhibitor (Fluka, Switzerland); MitoTracker Orange CM-H2TMRos (Invitrogen, USA). Any other reagents are produced in Ukraine.

Results

We used NO-sensitive fluorescent probe DAF-FM, an active form of dye that enters the cell through simple diffusion, in order to prove the presence of NO in mitochondria. DAF-FM reacts with NO in the presence of O_2 , resulting in its triazolofluorescein derivative, which has a higher fluorescence efficiency in the green region (Li and Wan 2015). While using MitoTracker Orange and DAF-FM fluorescence dyes, their co-localization was observed (Fig. 1), proving the similar distribution of fluorescence probes in the cytoplasm. This indicates the presence of NO in mitochondria, which is consistent with the notion of the presence of mitochondrial NO-synthase in these organelles.

Further research was aimed at the selection of optimal conditions for the determination of NO-synthase activity in isolated mitochondria of rat myometrium using DAF-FM and flow cytometry. The analysis of the fluorescence

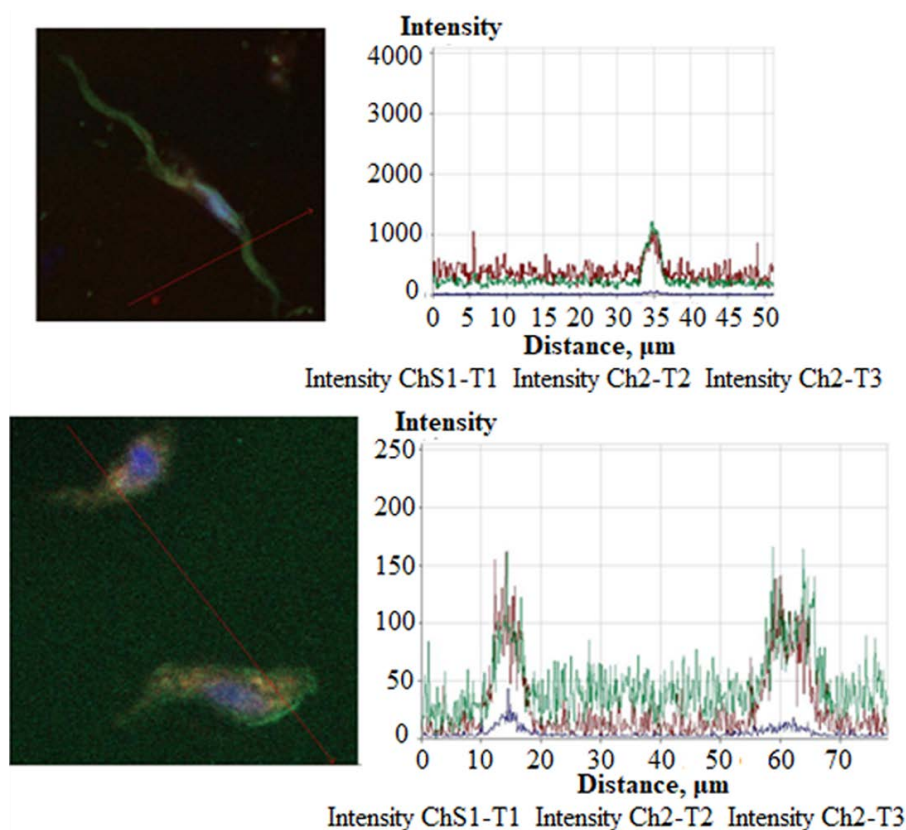


Figure 1. Co-localization of DAF-FM and MitoTracker Orange CM-H₂TMRos fluorescent probes in a myocyte, immobilized and spread on a treated with poly-L-lysine glass surface: red color, MitoTracker Orange CM-H₂TMRos (200 nM); green, DAF-FM (10 μM); blue, specific to the nucleus dye Hoechst 33342 (50 nM). The computer analysis of the fluorescent dyes distribution profiles (right panels). Data of two different experiments (top and bottom images). (See online version for color figure.)

response of the probe in a wide range of concentrations from 0.1 to 10 μM has found that 0.5 μM is the optimal dye concentration to test the formation of NO in the presence of Ca²⁺ in the reaction medium. The fluorescence dependence on the concentration has a bell-shaped form, and 10 μM DAF-FM fluorescence response showed a decrease in the signal below the control values (Fig. 2A). The time dependence of fluorescence has the appearance of a curve with the plateau (Fig. 2B), and the signal was significantly higher in the presence of non-mitochondrial Ca²⁺ (1 mM) in all cases, (Fig. 2), except for experiments with 10 μM DAF-FM.

Data on the study of the isolated mitochondria NO-synthase activity depending on the presence of exogenous substrate and cofactors is presented in Fig. 3. The mitochondria were able to endogenous NO production in the working solution containing the substrates of respiration, namely 5 mM pyruvate and succinate, and the balanced ionic composition. The biosynthesis of NO was found to be lower by 40% in the absence of respiration substrates. Sequential addition of 1 μM L-arginine and 100 μM Ca²⁺ into the incubation medium resulted in a significant ($p < 0.05$) increase in NO biosynthesis regarding to the control values. The addition

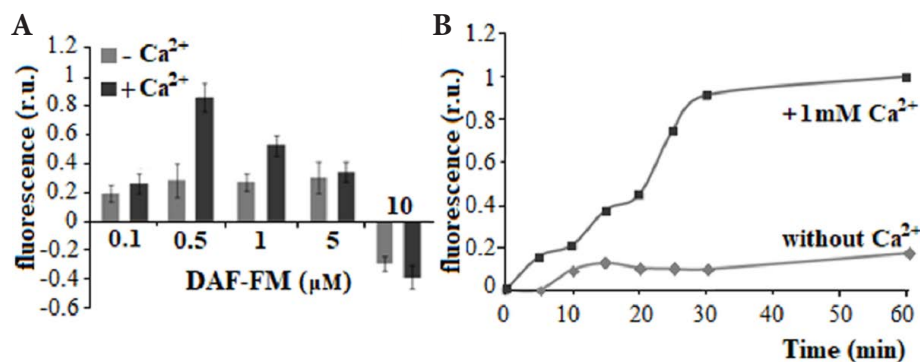


Figure 2. Dependency of DAF-FM fluorescence in isolated mitochondria on probe concentration (A) and on incubation time (B). Data are means \pm SE, $n = 5$. B. Typical experiment of changes in fluorescent intensity of DAF-FM in time. r.u., relative units.

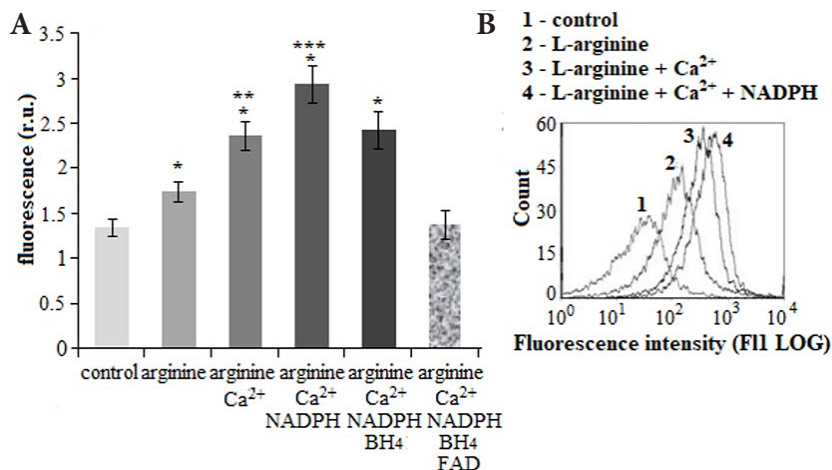


Figure 3. A. Dependency of mtNOS activity on the presence of substrate (1 μM L-arginine), 10 μM NADPH and cofactors (100 μM Ca²⁺, 10 μM BH₄, 10 μM FAD) in mitochondria. Data are means \pm SE, $n = 7$. * $p < 0.05$ vs. control, ** $p < 0.05$ vs. arginine, *** $p < 0.05$ vs. arginine+Ca²⁺. B. Typical experiment of the displacement of the fluorescence intensity peaks in conditions of different medium composition. The time of the mitochondria incubation with substances was 30 minutes. r.u., relative units.

of exogenous Ca²⁺ revealed a significant increase ($p < 0.05$) of NO formation in relation to the experiments with its absence (Fig. 3). Adding 10 μM of NADPH has also resulted in further growth of NO-synthase activity. At the same time, the introduction of BH₄ into the reaction medium did not stimulated NO-synthase activity of mitochondria (Fig. 3). The presence of 10 μM FAD resulted in a significant reduction in NO formation in the incubation medium containing all of the above-mentioned components. The concentration of the substances was used considering the results of such experiments on other objects.

The perforation of the internal mitochondrial membrane with a channel forming antibiotic alamethicin (7.5 $\mu\text{g}/\text{ml}$) did not significantly affected NO-synthase activity of the mitochondria in the presence of the substrate and cofactors in the reaction medium (graphic data is not given).

The dependence of NO-synthase activity of isolated mitochondria on Ca²⁺ and L-arginine concentrations in the incubation medium has been studied. As the concentration of exogenous Ca²⁺ increased, NO production by mitochondria augmented. The maximum of NO-synthase activity was

observed in the range from 100 to 250 μM Ca²⁺ (Fig. 4). Further growth of Ca²⁺ concentration slightly reduced NO production. Concentration-dependent curve on Fig. 4 allows to calculate the activation constant of NO-synthase reaction for Ca²⁺, which is 44.4 ± 14.5 μM ($n = 5$) at 1 μM L-arginine in the incubation medium.

It has also been established that the growth of NO biosynthesis with a tendency to enter the plateau level (the concentration of exogenous Ca²⁺ is 100 μM) is observed with increasing concentration of L-arginine from 1 to 75 μM , which allows us to calculate the apparent affinity constant for the substrate. NO formation is significantly reduced by further increase in the concentration of amino acid (up to 100 μM in these experiments) (Fig. 5). The apparent affinity constant for L-arginine is 28.9 ± 9.1 μM being calculated in Heinz coordinates ($n = 5$).

A widespread inhibitor of the constitutive NO-synthase NA in micromolar concentrations inhibited NO synthesis in mitochondria of myometrium (Fig. 6). A known blocker of Ca²⁺-transport in the internal membrane of mitochondria RuR (10 μM) significantly inhibited NO production by mi-

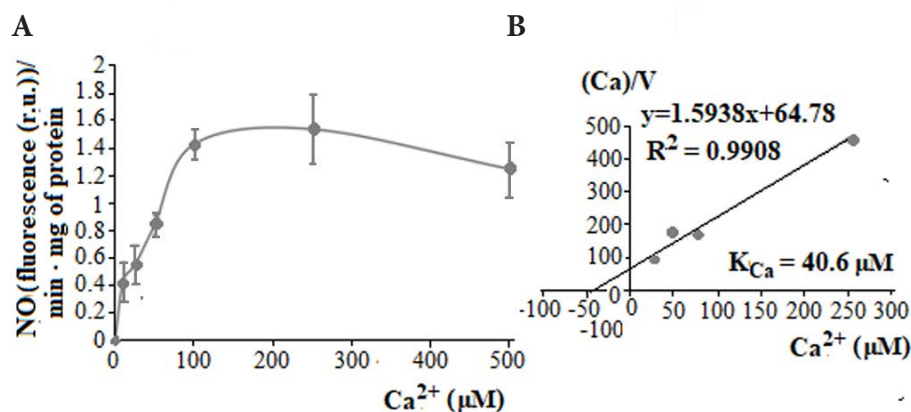


Figure 4. A. Dependency of mtNOS activity on exogenous Ca²⁺ concentration, data are means \pm SE, $n = 5$. B. Example of activation constant calculation by Heinz method. r.u., relative units; V, mtNOS activity.

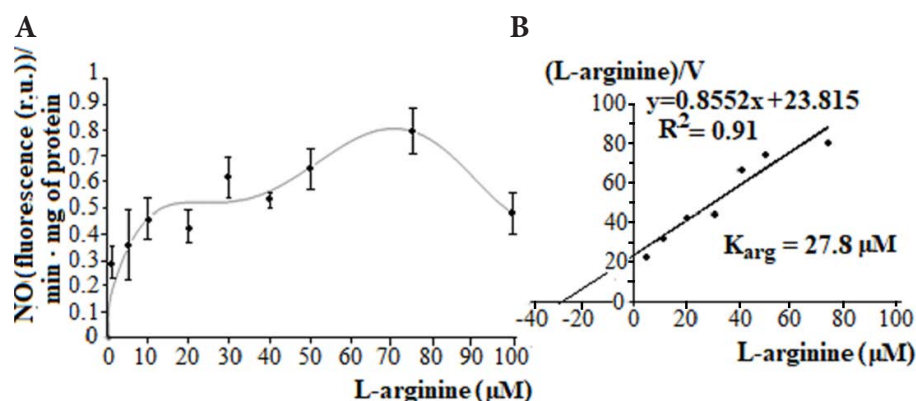


Figure 5. A. Dependency of mtNOS activity on exogenous L-arginine concentration. Data are means \pm SE, $n = 5$. B. Example of apparent affinity constant for L-arginine calculation by Heinz method. r.u., relative units; V, mtNOS activity.

tochondria in the presence of $100 \mu\text{M Ca}^{2+}$ in the incubation medium (Fig. 6).

The dependence of NO-synthase activity of mitochondria on their energy status has been also studied. Isolated mitochondria synthesized NO in the absence of exogenously added substrates (Fig. 7), although with insignificant efficiency. The addition of 5 mM pyruvate and succinate in the incubation medium caused the stimulation of their oxidation and the functioning of the electron transport chain was enhanced. Mitochondria were energized and the NO synthesis was increased. The similar effect on NO-synthase activity was carried out with the widespread inhibitor of H^+ -ATPase H^+ -channel (F_0 complex) oligomycin ($2.5 \mu\text{M}$). The addition of 0.5 mM ADP (inorganic phosphate and Mg^{2+} were present in the incubation medium) to the energized mitochondria

resulted in the creation of conditions for ATP synthesis, and weakening of NO-synthase activity in mitochondria (Fig. 7). The blocking of the electron transport chain complex I with rotenone ($5 \mu\text{M}$) or complex III with antimycin A ($1 \mu\text{g/ml}$) resulted in a significant decrease in NO biosynthesis in energized mitochondria (Fig. 7). The similar effect was caused by CCCP, added into the incubation medium, which rapidly destroyed the electrochemical gradient of protons on the internal mitochondrial membrane (Fig. 7).

Discussion

The presence of NO in uterine smooth muscle cells, in particular in mitochondria, has been visualized, involving

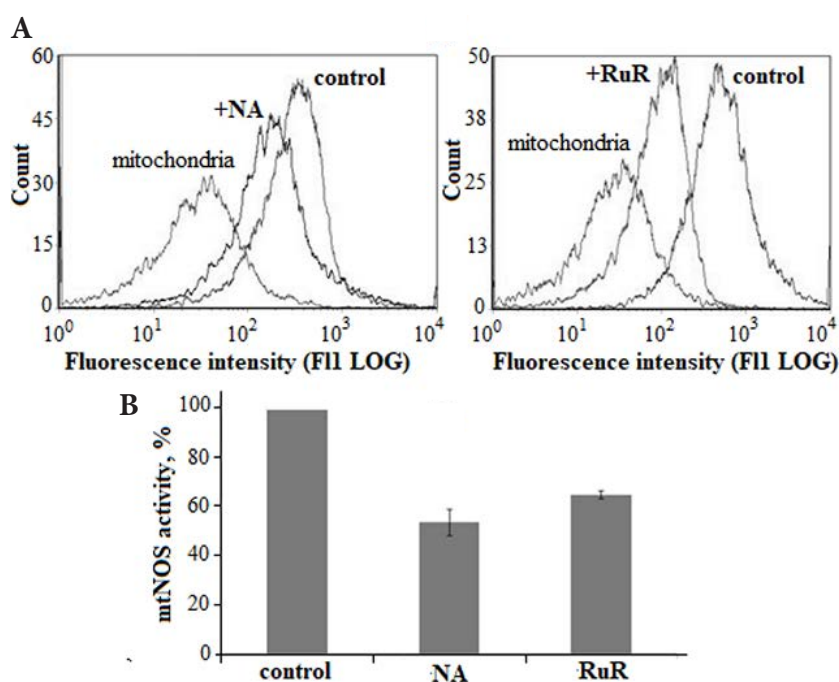


Figure 6. A. Typical experiment of the displacement of the fluorescence intensity peaks in condition of N^{G} -nitro-L-arginine (NA) and ruthenium red (RuR) action. Mitochondria, peak position at “0” time; control, peak position after 30 min. B. Changes in fluorescent intensity of DAF-FM in mitochondria after adding $25 \mu\text{M}$ NA and $10 \mu\text{M}$ RuR. Data are means \pm SE, $n = 5$.

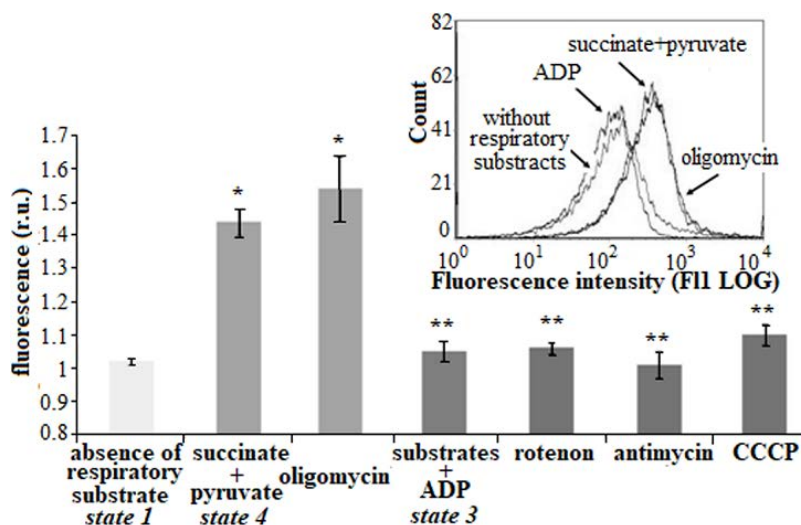


Figure 7. Dependency of mtNOS activity on the presence of respiratory substrates and electron transport chain inhibitors in mitochondria: 5 mM pyruvate, 5 mM succinate, 2.5 μ M oligomycin, 0.5 mM ADP, 5 μ M of rotenone, 1 μ g/ml of antimycin A, 10 μ M protonophore CCCP. States 1, 3, 4, mitochondria respiration states by Chance and Williams (1955). * $p < 0.05$ vs. state 1, ** $p < 0.05$ vs. state 4. Data are means \pm SE, $n = 5$. Insert: the fluorescence intensity peaks displacement in condition of different medium composition; the result of a typical experiment. r.u., relative units.

fluorescence confocal microscopy, NO-sensitive DAF-FM probe and the specific marker of functionally active (energized) mitochondria MitoTracker Orange CM-H2TMRos. The use of DAF-FM fluorescent dye in these experiments is logical, since this probe is less sensitive to the action of laser and does not photo-bleach with time (Li and Wan 2015). Computer analysis of cell slices, that was chosen outside the cell nucleus (red lines on Fig. 1), showed identical profile of distribution of both fluorescent dyes. The co-localization of these dyes in freshly-isolated rat uterus myocytes has been observed, which indicates the possibility of NO formation in mitochondria of the myometrium (Fig. 1).

Subsequent studies have been conducted on isolated mitochondria of the rat myometrium by using DAF-FM and the flow cytometry method. According to the preliminary results, isolated mitochondria are oval particles with a size of 500–700 nm and cristae (own data of electron microscopy and photon correlation spectroscopy) (Kolomiets et al. 2013). These sub-cell structures had an intact inner membrane, their hydrodynamic diameter increased with adding a channel forming antibiotic alamethicin. Freshly-isolated mitochondria were able to accumulate Ca ions from the incubation medium in the presence of respiratory substrates and Mg-ATP²⁻ (Kolomiets et al. 2013). Consequently, they have certain morphological and functional characteristics of intact organelles.

We have selected optimal concentrations of fluorescent dye to determine changes in NO content in the mitochondrial suspension using flow cytometry. This method has significant advantages over conventional spectrofluorimetry. They include a high signal/noise ratio in the fluorescence channel, an estimate of the fluorescence change of mitochondria precisely rather than the working medium and non-mitochondrial subcellular fragments, as well as the

possibility of using a relatively small number of membrane preparations in the samples. It has been found that the optimal concentration of DAF-FM is 0.5 μ M in the presence of exogenous Ca²⁺. Both lower and higher concentrations of the probe turned out to be less effective. There was even a decrease in fluorescence referring to control values for 10 μ M DAF-FM, which is probably due to the interaction between the probe molecules (Fig. 2A). In the study of time dependence of enzymatic activity it has been established that the steady state is reached up to 30 minutes (Fig. 2B), which we have used for further study of NO biosynthesis by mitochondria.

mtNOS, like non-mitochondrial NOS isoforms, requires L-arginine, O₂, Ca²⁺ as well as NADPH and cofactors (calmodulin, BH₄) for the synthesis of NO. It is predicted, that mtNOS is co-localized together with the complex I of the respiratory chain at least in the case of heart mitochondria (Parihar et al. 2008; Zaobornyj and Ghafourifar 2012). Such specific compartmentalization involves the possibility of regulation of NOS activity by the local microenvironment of the organelles and the components of the matrix, as well as the dependence of NO production on the activity of the electron transport chain and the electrochemical gradient of hydrogen ions on the internal mitochondrial membrane. We tried to find out the necessity of certain components for constitutive NO-synthase reactions in the case of isolated myometrium mitochondria mtNOS. It should be noted that isolated mitochondria possess basal NO-synthase activity in the absence of cofactors and substrate, indicating the presence of necessary reaction components in the matrix in sufficient quantities and the energetic state of mitochondria in the medium with pyruvate and succinate (Fig. 3). The synthesis of NO was significantly lower in non-energized mitochondria without exogenous respiratory substrates

(see comments on Fig. 7). Addition of 1 μM L-arginine to the mitochondria significantly increased NO production regarding the control values. The effect of L-arginine in these concentrations can be surprising. It's the fact that the concentration of this amino acid in the matrix of liver mitochondria reaches a level of 150–300 μM , according to the experimental data (it is supposed that the level in cardiomyocytes is lower) (Giulivi et al. 1998). It is difficult to assume that NOS is in short supply, since its affinity for L-arginine is in the micromolar range. However, the well-known phenomenon is the so-called “arginine paradox”, which is based on the fact that the introduction of L-arginine into the body is accompanied by an increase in the biosynthesis of NO (the concentration of this amino acid in liquids and tissues reaches millimoles) (Elms et al. 2013). It is believed that the presence in cells of asymmetric dimethylarginine, which competes with L-arginine, plays an essential role in this phenomenon (Palm et al. 2007; Peng et al. 2016). It should also be taken into account that the presence and high activity of arginase II in mitochondria cleaves the amino acid (Morris 2009).

Addition of 100 μM Ca^{2+} to the working medium resulted in further significant growth of NO production by mitochondria (Fig. 3) in relation to the L-arginine values. It is known that energized mitochondria of the heart synthesize NO even in the absence of exogenous Ca^{2+} due to the sufficient concentration of Ca^{2+} in the matrix (Elfering et al. 2002; Zaobornyj and Ghafourifar 2012). NO biosynthesis, which is independent from exogenous Ca^{2+} too, is observed in our experiments. However, Ca^{2+} addition to mouse and rat heart mitochondria leads to an increase in NO production, and extra-mitochondrial Mg^{2+} , as well as RuR, suppresses it (Zaobornyj and Ghafourifar 2012). Thus, the biosynthesis of NO by mitochondria of myometrium also depends on the concentration of exogenous Ca^{2+} .

The introduction of 10 μM NADPH as the main source of electrons for non-mitochondrial NOS into the incubation medium in the presence of 100 μM Ca^{2+} and 1 μM L-arginine resulted in further growth of NO production (Fig. 3). On the other hand, addition of BH_4 (10 μM), a cofactor and a dissociation carrier of electrons, did not cause any probable difference in the biosynthesis of NO concerning the results in the absence of the cofactor (Fig. 3). Perhaps these results can be explained by the sufficient mitochondria energization in the conditions of our experiments (presence of succinate and pyruvate as respiratory substrates in the medium), functional coupling of mtNOS with the respiratory chain, as well as the possibility of reverse transport of electrons from II to I complex. In the case of L-arginine or BH_4 lack and stimulation of Ca^{2+} accumulation by mitochondria, NO formation was significantly lower, but at the same time there was an increase in the production of active forms of oxygen (Dedkova and Blatter 2009). Thus, according to cited data, the presence of BH_4 in the incubation medium is a prereq-

uisite for the correct testing of NO-synthase activity. This was taken into account for further experiments.

Addition of 10 μM FAD as an essential element of electron transport from NADPH to the heme prosthetic group of non-mitochondrial NOS has a significant inhibitory effect on NO production by mitochondria (Fig. 3). We explain this effect by the specific location and, accordingly, the functioning of mtNOS as a part of complex I or IV of the respiratory chain. Due to the presence of a sufficient amount of calmodulin in mitochondria, this cofactor was absent in the medium of incubation. It should be noted that calmodulin antagonists, namely, trifluoperazine and calmidazolium, significantly inhibited NO-synthase activity in myometrium mitochondria (our unpublished data).

In all separate variants of the above-described experiments we used alamethicin (7.5 $\mu\text{g}/\text{ml}$) as an agent that forms nonspecific permeability of the internal mitochondrial membrane to cofactors and substrates (Gostimskaya et al. 2003). After alamethicin insertion, the value of the membrane potential decreases to the Donnan potential in isolated mitochondria (Nicholls and Ferguson 2002). Although alamethicin may increase the availability of exogenous cofactors and substrates and mtNOS activity may increase, mitochondrial depolarization will have the opposite effect. We have not registered any significant effect of alamethicin on NO products.

There is evidence that energized mitochondria are capable to produce NO in the absence of NADPH, L-arginine and exogenous Ca^{2+} in the incubation medium (Elfering et al. 2002; Valdez et al. 2006; Zaobornyj and Ghafourifar 2012). The intra-mitochondrial concentration of NADPH is 1.04–1.78 mM, which significantly exceeds the corresponding value of K_{NADPH} for mtNOS (0.1–1 μM) (Valdez et al. 2006). The concentration of L-arginine in the mitochondrial matrix corresponds to 150–310 μM and is substantially higher than the K_m for L-arginine (5 μM for liver mitochondria (Giulivi et al. 1998), 36 μM for cardiomyocytes (Alvarez and Boveris 2004)). Thus, the concentrations of L-arginine, Ca^{2+} and NADPH are not rate-limiting NO synthesis factors in mitochondria (Giulivi 1998; Zaobornyj and Ghafourifar 2012; Porcelli et al. 2014). Lack of L-arginine in the matrix and the corresponding decrease in mtNOS activity may be due to the increased activity of arginase II in mitochondria, which competes with the NOS for the substrate (Morris 2009).

Consequently, NO-synthase activity in isolated myometrium mitochondria has been identified in the presence of appropriate cofactors and substrates in the reaction medium using DAF-FM fluorescence probe and the flow cytometry method. The analysis of the results suggests that the optimal work of mtNOS in myometrium mitochondria requires the presence of respiration substrates, L-arginine, Ca^{2+} and NADPH. These data will be used for further studies of kinetic and catalytic properties of NO biosynthesis in mitochondria.

Dependence of enzymatic activity on the concentration of exogenous Ca^{2+} in the range of 10–500 μM has been investigated (Fig. 4). It has been found that the optimal range of concentrations of Ca^{2+} is 100–250 μM , further increase in the content of Ca^{2+} decreased enzymatic activity. The activation constant for Ca^{2+} is about 45 μM . This concentration of Ca^{2+} is close to the physiological value near mitochondria, where Ca^{2+} /inositol-1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release from the sarco(endo)plasmic reticulum creates significant local Ca^{2+} concentrations (Graier et al. 2007). For comparison, the activation constant for energy-dependent accumulation of Ca^{2+} by mitochondria reaches 54 μM (according to our preliminary calculations) (Kolomiets et al. 2013). E. Dedkova and L. Blatter showed a dose-dependent increase in NO production in mitochondria in the presence of L-arginine and an increase in the concentration of Ca^{2+} in myocytes permeabilized by digitonin (Dedkova and Blatter 2009). In this work NO-sensitive fluorescence probe 4,5-diaminofluorescein diacetate (DAF-2) and MitoTracker Red CMXRos mitochondrial specific dye were used.

The production of NO was analyzed according to the changes in L-arginine concentration in the range of 1–100 μM (Fig. 5). It has been established that the dependence of enzymatic activity on the concentration of the substrate has a bell-shaped character, and the apparent affinity constant for L-arginine is about 30 μM . For comparison, the corresponding constants for mitochondria of the heart make up 35–36 μM , the skeletal muscle (diaphragm) – 37 μM , and the liver – 5 μM (Tatoyan and Giulivi 1998; Alvares and Boveris 2004). The authors who have carried out these calculations emphasize that the apparent affinity constants are several times lower than the calculated concentration of L-arginine in the matrix.

A nonspecific inhibitor of the constitutive NOS NA at a concentration of 25 μM inhibited NO production in mitochondria almost by 50% (Fig. 6). This fact suggests that mtNOS is one of the variants of constitutive enzymes, possibly neuronal NOS. These isoforms are Ca^{2+} -dependent, therefore the biosynthesis of NO is suppressed by ruthenium red, a known blocker of Ca^{2+} transport in mitochondria (Fig. 6). However, there is evidence that the outer membrane of mitochondria of endothelial cells contains endothelial NOS, and 85% of the mitochondria of the heart are positive in relation to this isoform. Solubilized and purified by affinity chromatography, liver mtNOS synthesized 250–350 nmol NO/min/mg protein, the enzyme was inhibited by L-NMMA. A detailed analysis of the amino acid composition of mtNOS found that the protein structure corresponds to the initial sequence of nNOS (Tatoyan and Giulivi 1998).

Thus, important kinetic parameters of NO-synthase activity in mitochondria are calculated: the apparent affinity constant for L-arginine and the activation constant for

Ca^{2+} , which have a physiological meaning. NO synthesis was inhibited by a known inhibitor of constitutive NOS NA and was dependent on Ca^{2+} concentration.

The current conception of mitochondria metabolic states and respiratory control is based on the rate of O_2 uptake by isolated organelles. The high rate of respiration and ATP production have been observed in metabolic state 3 in case of addition of respiratory substrates and ADP. The absence of ADP causes low rate of O_2 uptake, ATP is not synthesized, and the internal mitochondrial membrane is hyperpolarized (metabolic state 4) (Chance and Williams 1955; Valdez et al. 2006). We obtained the following results, using the panel of the electron transport chain modifiers. Isolated mitochondria produced NO in the absence of exogenously added substrates of respiration at the internal reserves. This process in conditions of mitochondria energization and the hyperpolarization of their internal membrane is significantly enhanced by addition of respiratory substrates or oligomycin (Fig. 7). Instead, when effective oxidative phosphorylation occurs (state 3), the synthesis of NO decreases. The inhibition of certain complexes of the respiratory chain by rotenone and antimycin, as well as the dissipation of the electrochemical gradient by protonophore CCCP, suppresses mtNOS (Fig. 7). The transition from relative rest (state 4) to active respiration in case of fully coupled mitochondria (available substrates of respiration, sufficient oxygen concentration and present ADP, state 3) is accompanied by an decrease in NO production in heart mitochondria. NO release exponentially depends on the value of the inner membrane electric potential (Valdez 2006). Formation of NO is observed even without exogenous NADPH under the condition of electron transport from II to I complex in the presence of ATP and succinate. mtNOS activity is maximal during optimal functionality of the electron transport chain, in particular, complex I, which is the source of electrons for the functioning of the enzyme. Inactivation of complex I reduces mtNOS activity (Parihar et al. 2008).

Our results are consistent with those obtained on permeabilized cardiomyocytes using the method of confocal microscopy. In particular, collapse of the membrane potential in the presence of protonophore FCCP, blockage of the Ca^{2+} -uniporter of the internal membrane of mitochondria with the compound Ru360, the use of the electron transport chain inhibitors rotenone or antimycin A in combination with oligomycin resulted in inhibition of NO synthesis in mitochondria (Dedkova and Blatter 2009).

It should be noted that the decrease in NO production during the transition from the state 4 to 3 is the opposite of what one could expect from the slight matrix acidification and pH dependence of mtNOS activity. Dependence of NO products on mitochondrial pH in the range of 5.5–8.5 has a bell-shaped character with a maximum at pH 7.4 (Valdez et al. 2006). Taking into account the pH value of the matrix

at about 7.5 in the state 3 and about 7.8 in the state 4, mtNOS activity should be higher in the first case. These results indicate that a larger synthesis of NO in the state 4 is not associated with the changes in pH. There is a view that mtNOS activity is regulated by an electrical, not a proton component of the membrane potential on the internal mitochondrial membrane (Valdez et al. 2006).

Thus, our results convincingly show and are in line with the notion that the biosynthesis of NO by mitochondria significantly depends on the level of their energy and the magnitude of hydrogen ion electrochemical gradient on the inner membrane.

Conclusions

The formation of NO in mitochondria of uterine smooth muscle cells has been firstly demonstrated in the presented work using laser confocal microscopy and the methodology of the specific fluorescent dyes co-localization. The conditions for determining NO-synthase activity in isolated mitochondria of rat myometrium have been selected employing DAF-FM fluorescence probe and flow cytometry. The optimal work of mtNOS in isolated myometrium mitochondria requires the presence of respiration substrates, Ca^{2+} , NADPH and L-arginine. The key kinetic parameters of this enzymatic reaction are calculated: the apparent affinity constant for L-arginine is $28.9 \pm 9.1 \mu\text{M}$, and the activation constant for Ca^{2+} is $44.4 \pm 14.5 \mu\text{M}$, which has a physiological value. It is proved that the biosynthesis of NO by mitochondria significantly depends on the level of their energization, the intensity of exogenous Ca^{2+} entry to the matrix and suppression by N^{G} -nitro-L-arginine.

Conflict of interest. Authors declare no conflict of interest.

Acknowledgement and financial support. Authors received no grant for this study. Authors thank Dr. Sergiy O. Karakhim and Dr. Alexander Y. Chunikhin for their technical assistance.

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Received: May 29, 2018

Final version accepted: September 10, 2018

First published online: January 18, 2019