Original Paper

The Effect Of NO Donor on Calcium Uptake and Reactive Nitrogen Species Production in Mitochondria

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Key Words

Mitochondria • Nitric oxide • Nitroglycerine • Calcium • Mitochondrial NO synthase • Reactive nitrogen and oxygen species • Permeability transition pore

Abstract

Background/Aims: NO and reactive nitrogen species (RNS) are thought to be physiologically important effectors of mitochondrial calcium transport, but this issue was not studied in a living organism. According to literature, the modulation of Ca²⁺ uptake could influence RNS production *via* the action on mitochondrial NO synthase (mtNOS). The aim of this work was to study the effect of *in vivo* administration of NO donor nitroglycerine (NG) on matrix Ca²⁺ accumulation, RNS production and mtNOS activity. **Methods:** Ca²⁺ uptake was studied spectrophotometrically with arsenazo-III. The amounts of stable RNS (nitrite, nitrate and nitrosothiols) and L-citrulline, the product of enzymatic NOS activity, were determined analytically. **Results:** NG administration resulted in dose-dependent short-term increase in Ca²⁺-uptake accompanied by essential rise in L-citrulline and RNS content in mitochondria. In parallel, dose-dependent elevation of hydroperoxide production was detected. Ca²⁺-uniporter activity was not affected, but mitochondrial permeability transition pore (MPTP) was effectively blocked by NO. **Conclusion:** Our results indicate that MPTP blockage by NO was the primary cause for the increase in calcium uptake which eventually resulted in the activation of mtNOS and RNS production. Improved Ca²⁺ accumulation in mitochondria, together with MPTP blockage, may contribute to well-known cardioprotective effects of pharmacological donors of nitric oxide.

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Introduction

Wide spectrum of cardioprotective actions of nitric oxide (NO) and pharmacological NO donors includes the activation of soluble guanylate cyclase and cGMP-dependent modulation of ion channel activity in plasma membrane and sarcoplasmic reticulum which eventually

reduce the level of cytosolic calcium and serve cardioprotective effects [1, 2]. In agreement with contemporary knowledge, mitochondria play important role in modulation of cytosolic calcium dynamics [3-5]. Several diseases are accompanied by the impairments of both cellular and mitochondrial Ca^{2+} homeostasis [3-6]. However, thus far little is known of the NO action on mitochondrial handling calcium.

Based on the studies *in vitro* [7-9], NO is thought to be physiologically important effector of calcium accumulation *via* potential-dependent channel of inner membrane, mitochondrial Ca^{2+} uniporter [4]. The data obtained with different respiratory substrates showed the release of Ca^{2+} after the loss of membrane potential ($\Delta\Psi_m$) in mitochondria exposed to NO [7, 8]. Besides, it was shown that prevention of Ca^{2+} uptake, because of the inhibition of respiratory chain by NO and membrane depolarization, blocked the opening of mitochondrial permeability transition pore (MPTP) [9], the megachannel involved in triggering cellular death.

MPTP is composed of mitochondrial membrane proteins and opened by Ca^{2+} , phosphate and oxidants; its role in pathogenesis of several diseases was well documented in the literature [10, 11]. However, MPTP structure since its discovery was debated. Initially, membrane proteins – adenine nucleotide translocase (ANT), voltage dependent anion channel, and matrix protein – cyclophilin D, were considered to be the main MPTP components [10]. But in the light of very recent findings, it was proposed that MPTP is formed by dimerization of F_0F_1 ATP synthase, c-subunit of which being the main pore-forming component [11], and cyclophilin D playing minor regulatory role [4, 11].

Based on the above reports, it is reasonable to suppose that NO would affect Ca^{2+} uptake in mitochondria of a living organism, but thus far little is known about the action of NO on mitochondrial handling calcium *in vivo*. One of the most important consequences of NO targeting the Ca^{2+} transport system of mitochondria could be the modulation of cytosolic and matrix levels of calcium [4, 5], which is intimately coupled to reactive nitrogen and oxygen species (RNS and ROS) production.

There is plenty of evidence of the crosstalk between nitric oxide, calcium and ROS in mitochondria (reviewed in [12-14]). According to literary data, mitochondria are the potent source of cellular ROS and RNS [15, 16]. The main sources of ROS are the respiratory chain and several oxidoreductases localized into matrix and intermembrane space [16]. mtNOS is considered to be the main source of RNS [14, 15], but devoid of the substrate L-arginine and the cofactor tetrahydrobiopterine, it would produce superoxide [17]. Calcium, which enters the matrix *via* Ca^{2+} -uniporter [18], is the effector of several mitochondrial enzymes, such as ATP-synthase, some dehydrogenases of tricarboxylic acid cycle, mitochondrial NO synthase (mtNOS) [13, 15, 19], many of which are capable of producing ROS and RNS. Accordingly, studies *in vitro* have shown that mitochondrial nitric oxide synthase (mtNOS) activity was dependent on Ca^{2+} accumulation [20]. This allows an assumption that modulation of Ca^{2+} uptake *in vivo* likewise might result in the modulation of mitochondrial reactive nitrogen species (RNS) production.

Although already studied by many authors on different cell models and isolated mitochondria, molecular mechanisms of the crosstalk between nitric oxide and calcium uptake in mitochondria and its physiological role are far from being clear. Successful applications of NO donors in clinic need a careful study of these processes in a living organism, but this topic was not addressed under physiological conditions. Thus the aim of this work was to estimate the effect of NO on mitochondrial calcium uptake and RNS production *in vivo* under administration of the widely used NO donor nitroglycerine.

Materials and Methods

Animals

All procedures performed in studies were in accordance with EU directive 86/609/EEC and the ethical standards approved by the Ethics Committee at A.A. Bogomoletz Institute of Physiology, NAS Ukraine. Adult

Wistar rats with 180-200 g body weight were used. Nitroglycerine (NG) was administered intraperitoneally at the doses 0.25, 0.5, 1.0 and 1.5 mg/kg weight. Control animals were administered physiological solution.

Isolation of mitochondria

Mitochondria were isolated by the standard procedure. Hearts and liver removed 5 and 30 min after NG administration were thoroughly washed with 0.9% KCl (2°C) minced and homogenized in standard isolation medium: 250 mM sucrose, 20 mM Tris-HCl buffer, 1 mM EDTA, pH 7.4. Homogenate was centrifuged 7 min at 700 g and, after removal of the pellet, 15 min at 11,000 g. The sediment was resuspended in a small volume of EDTA-free medium and stored on ice. The protein content was determined by the method of Lowry.

Calcium transport study in isolated mitochondria

Ca²⁺ transport was studied in the incubation medium: 120 mM KCl, 5 mM sodium succinate, 1 mM KH₂PO₄, 5 mM Tris-HCl buffer, pH 7.4 in the presence of 50 μM of Ca²⁺ indicator arsenazo-III. CaCl₂ was added into incubation medium. 10⁻⁶ M of cyclosporine A and 10⁻⁵ M of ruthenium red were added dependent on conditions. Starting from the addition of mitochondria (0.5 mg protein), Ca²⁺-uptake was monitored by standard dual-wavelength technique [9], and the absorption of mitochondrial samples at wavelengths pair 654 and 700 nm was registered.

Membrane potential measurement

Membrane potential ($\Delta\Psi_m$) was monitored spectrophotometrically [21] with the use of 10 μM safranin as membrane potential probe in the same incubation medium. Dual-wavelength technique was used [21], and absorption of mitochondrial samples at wavelengths pair 510 and 525 nm was registered. Complete membrane depolarization was verified by the addition of 10⁻⁶ M CCCP. Difference between the absorbance of deenergized and fully energized mitochondria was taken for $\Delta\Psi_m$; $\Delta\Psi_m$ registered in mitochondria of control animals was taken for 100%.

The study of RNS production and mtNOS activity

RNS production was estimated by the determination of stable metabolites content: nitrite, nitrate and S-nitrosothiols. Mitochondrial NOS activity was estimated by measuring L-citrulline content in mitochondrial extractions. For the determination of metabolite content in mitochondrial preparations aliquots of mitochondrial suspensions were sampled and protein was removed by the addition of 0.5 M HClO₄ with consequent precipitation 10 min at 10,000 g. After the pellet was discarded, supernatant was neutralized by the additions of 5 M KOH and centrifuged repeatedly 5 min at 10,000 g. Protein-free extracts were used for the metabolite analysis.

Nitrite content

Nitrite content was determined by the method of Green with the Griess reagent [22]. Griess reagent was prepared by mixing equal volumes of 0.1% water solution of N-(1-naphthyl)ethylenediamine dihydrochloride with 1% sulfanilamide in 5% H₃PO₄ just before use. 0.5 ml aliquots of mitochondrial protein-free extractions were sampled and mixed with Griess reagent in a proportion 1:1 by volume. 5 min after absorbance at 546 nm was measured; nitrite concentration was determined from calibration curves.

The determination of nitrate

Nitrate was determined by the method based on the reduction of NO₃⁻ to NO₂⁻ by zinc [23]. For the determination of the summary content of NO₂⁻ + NO₃⁻ 1.0 ml aliquots of the protein-free preparations at pH 7.4 were mixed with 50 μl of zinc suspension (100 mg·ml⁻¹ in water). After 5 min centrifugation 0.5 ml of the supernatants were sampled and mixed with equal volume of the Griess reagent; after that absorbance at 546 nm was measured. For the determination of nitrate content, that of nitrite, established in parallel missing the reduction procedure, was subtracted.

S-nitrosothiols content

Total S-nitrosothiol content was determined by the method of Saville [24], using Griess reagent with HgCl₂ for S-nitrosothiol decomposition. Briefly, HgCl₂/sulfanilamide solution was prepared by

mixing 1 volume of 1% HgCl_2 in water with 4 volumes of 3.4% sulfanilamide in 0.4 M HCl. 0.5 ml aliquots of mitochondrial suspensions, were added to 0.7 ml HgCl_2 /sulfanilamide solution and 5 min after mixed with 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 0.4 M HCl in a proportion 2:1 by volume. Control probes were treated as above but without HgCl_2 . After protein removal by centrifugation and the neutralization of the probes, absorbance at 546 nm was measured. *S*-nitrosothiol content was determined from the increment in nitrite concentration respective to controls using calibration curves.

The determination of L-citrulline

The determination of L-citrulline was carried out according to the method described by Buga et al. [25]. Because the urea intervenes in L-citrulline determination with diacetyl monoxime [25], the samples were previously freed of it by incubating the probes with the urease. For this purpose 170 μl aliquots of protein-free samples were added to 30 μl 50 mM triethanolamine/HCl buffer containing urease at 1 activity unit and incubated 15 min at 25°C [25]. After that 1 ml of the solution made of 1 part of 3% diacetyl monoxime in ethanol and 29 parts of the mixture of sulfuric and phosphoric acids in a proportion: 1 part of H_2SO_4 , 3 parts of H_3PO_4 , 7 parts H_2O was added to the probes. Reaction mixture was held for 30 min on a water bath (100°C) and cooled. Absorbance at 492 nm was recorded. The concentration of L-citrulline was found from the calibration curves.

H_2O_2 production

The method for H_2O_2 determination was based on the indirect registration of H_2O_2 consumption in the course of iodide (I^-) oxidation to iodine (I_3^-) in the presence of excess lactoperoxidase, the stoichiometric ratio between H_2O_2 consumption and I_3^- formation being 1:1 [26]. Aliquots of mitochondrial protein-free extractions were incubated in 2 ml of incubation medium which contained 0.1 M KI in the presence of the excess lactoperoxidase (0.5 $\mu\text{g}\cdot\text{ml}^{-1}$) in 0.05 M Tris buffer, pH 7.33 (25°C). I_3^- formation was followed spectrophotometrically at 353 nm and the amount of hydroperoxide was determined using molar absorption coefficient 26,000 $\text{mol}^{-1}\cdot\text{cm}^{-1}$.

Materials

Cyclosporine A, ruthenium red, arsenazo-III were of "Fluka" (Switzerland); nitroglycerine, sodium nitroprusside, CCCP, safranine, *N*-(1-naphthyl)ethylenediamine dihydrochloride, sulfanilamide, lactoperoxidase, urease, diacetyl monoxime, and other reagents were of "Sigma" (USA).

Statistical analysis

The data were expressed as mean $\pm$ S.E. of 4-6 independent experiments. Statistical analysis was performed using paired Student's *t*-test; $P < 0.05$ was taken as the level of significance.

Results

Short-term effect of nitroglycerine on Ca^{2+} uptake and NO_3^- formation in mitochondria

According to the data obtained, NG administration resulted in remarkable modulation of mitochondrial Ca^{2+} -uptake. The ability of mitochondria to take up calcium (Ca^{2+} accumulation capacity) was estimated as the highest amount of Ca^{2+} taken up by mitochondria after the addition of increasing amounts of CaCl_2 . The dose- and time-dependent effects of NG on Ca^{2+} accumulation capacity of mitochondria were observed (Fig. 1A, B). Rapid increase in Ca^{2+} uptake in rat heart and liver mitochondria took place at once after NG administration with peak effect at 0.5-1.0 $\text{mg}\cdot\text{kg}^{-1}$ body weight (Fig. 1A, B, bars 1). The effect weakened 30 min later (Fig. 1A, B, bars 2).

The rise in calcium uptake was accompanied by the activation of RNS production. The amount of nitrate, the end product of NO oxidation, exhibited similar dose- and time-dependent effects of NG in heart and liver mitochondria (Fig. 1C, D). Thus, nitrate content in heart mitochondria rose from 0.04 ± 0.01 to $1.4 \pm 0.015 \mu\text{mol}\cdot\text{mg}^{-1}$, with subsequent decrease to $0.13 \pm 0.01 \mu\text{mol}\cdot\text{mg}^{-1}$ 30 min after the drug administration (Fig. 1C 1, 2). Similar data were obtained in liver mitochondria (Fig. 1D) where the nitrate level at first rose from 0.035

Fig. 1. The effect of NG on Ca^{2+} uptake (A, B) and NO_3^- formation (C, D) in rat heart and liver mitochondria. Ca^{2+} uptake in $\text{nmol}\cdot\text{mg}^{-1}$ and NO_3^- content in $\mu\text{mol}\cdot\text{mg}^{-1}$ in mitochondria was determined 5 (1) and 30 (2) min after NG administration. Ca^{2+} uptake was determined as the peak amount of Ca^{2+} taken up by mitochondria. The data were expressed as a mean $\pm$ SE ($M \pm m$, $n = 6$); $p < 0.05$ was considered to be statistically significant relative to control (*); relative to data collected at 5 min (#).

± 0.01 to $0.90 \pm 0.01 \mu\text{mol}\cdot\text{mg}^{-1}$ and then declined to $0.17 \pm 0.015 \mu\text{mol}\cdot\text{mg}^{-1}$. Dramatic rise in NO_3^- content in mitochondria and its decline with time indicated fast short-term activation of RNS metabolism under the action of pharmacological donor of NO.

The effect of nitroglycerine on RNS and ROS production in mitochondria

The above assumption was verified further by determining the amount of other NO metabolites – nitrite and *S*-nitrosothiols. Thus, 5 min after NG administration, in heart mitochondria nitrite content rose from 8.0 ± 0.7 to $20.0 \pm 2.0 \text{ pmol}\cdot\text{mg}^{-1}$, whereas total *S*-nitrosothiol content simultaneously rose from 0.70 ± 0.03 (the basal level) to $1.50 \pm 0.09 \text{ nmol}\cdot\text{mg}^{-1}$ (Fig. 2A, B, 1). The data obtained in liver mitochondria resembled those in heart mitochondria (Fig. 2A, B, 2). In parallel, it was observed that the rise in RNS production in mitochondria was accompanied by the dose-dependent increase in hydroperoxide formation (Fig. 2C).

Unlike this, only moderate increase in the amount of metabolites was detected in the whole (intact) tissue. So, in the liver NO_3^- content rose from 0.120 ± 0.008 to only $0.180 \pm 0.02 \mu\text{mol}\cdot\text{mg}^{-1}$ relative to the increase from 0.035 ± 0.01 to $0.90 \pm 0.01 \mu\text{mol}\cdot\text{mg}^{-1}$ obtained in liver mitochondria. In the same way, the rise in *S*-nitrosothiols content from 1.20 ± 0.04 to $2.0 \pm 0.03 \text{ nmol}\cdot\text{mg}^{-1}$ in liver relative to that from 0.80 ± 0.08 to $2.50 \pm 0.1 \text{ nmol}\cdot\text{mg}^{-1}$ in liver mitochondria was found. The change in NO_2^- content in liver after NG administration was statistically insignificant, contrary to threefold increase of it in liver mitochondria (Fig. 2A, 2). In the case of H_2O_2 , the effect of NG again was specifically localized to mitochondria. So, the increase in H_2O_2 content from 0.090 ± 0.007 to 0.30 ± 0.01 and from 0.050 ± 0.006 to $0.80 \pm 0.008 \text{ nmol}\cdot\text{mg}^{-1}$ in heart and liver mitochondria was detected 5 min after NG injection, against statistically insignificant increase of hydroperoxide production in liver tissue, which remained close to basal level. This proved that effect of NG was much more specific for mitochondrial than whole cell RNS metabolism and showed that NG administration *in vivo* resulted in the instant dose-dependent activation of mitochondrial RNS and ROS production.

mtNOS activity is increased under the action of NG

The primary source of RNS is NO synthase (NOS) activity. Mitochondria are known to possess its own NOS (mtNOS) which is Ca^{2+} -dependent isoform of the enzyme [15]. Thus, it was of especial interest to assess mtNOS activity under the action of NG in the living organism. With this aim, the amount of L-citrulline, the stable product of enzymatic NOS activity, was determined.

As previously, the effect observed in heart mitochondria resembled that obtained in liver mitochondria. Thus, rapid dose-dependent rise in L-citrulline content from $0.45 \pm 0.05 \text{ nmol} \cdot \text{mg}^{-1}$ to $6.3 \pm 0.1 \text{ nmol} \cdot \text{mg}^{-1}$ in heart mitochondria (Fig. 3A, 1) and from $0.45 \pm 0.06 \text{ nmol} \cdot \text{mg}^{-1}$ to $2.5 \pm 0.08 \text{ nmol} \cdot \text{mg}^{-1}$ in liver mitochondria (Fig. 3A, 2) was found. Such an increment in L-citrulline formation (5.6 times in liver mitochondria and 14.0 times in heart mitochondria) gave convincing evidence of mtNOS activation just starting from NG injection.

In the literature, it was shown that mitochondrial NO production was dependent on Ca^{2+} accumulation in mitochondria of vascular endothelium cells [20]. In our experiments, the increase in RNS production in heart and liver mitochondria also took place along with the increase in Ca^{2+} accumulation capacity (Fig. 1-2). For this reason, the activation of mtNOS by Ca^{2+} accumulation in the matrix appears to be a plausible explanation for the activation of mitochondrial RNS metabolism. The reliability of this assumption was supported by the correlation established between the dose-dependent increase in Ca^{2+} -uptake and RNS production under the action of moderate ($\leq 1.0 \text{ mg}\cdot\text{kg}^{-1}$) doses of NG (Fig. 3B). Based on the data, linear dependences of NO_2^- and S-nitrosothiols content on Ca^{2+} uptake in heart mitochondria were obtained (Fig. 3B, 1, 2). Similar to RNS, H_2O_2 formation too showed the dependence on matrix Ca^{2+} accumulation (Fig. 3B, 3).

Thus, the activation of metabolic processes in mitochondria was in a good agreement with short-term dose-dependent increase of Ca^{2+} uptake (Fig. 1A, B). This allows hypothesize that the rise in matrix calcium might be the direct cause for the activation of mtNOS and RNS metabolism in mitochondria of the living organism. In support of this assumption, the decrease of NO_3^- formation was observed along with the reduced uptake of Ca^{2+} 30 min after NG administration (Fig. 1A-D, 2).

NO enhances Ca^{2+} uptake in mitochondria in vivo due to the blockage of mitochondrial permeability transition pore

In agreement with the published data [7, 9], nitric oxide *in vitro* prevented uptake of calcium because of mitochondrial depolarization. Unlike this, NG administration *in vivo* potently activated Ca^{2+} accumulation in rat heart and liver mitochondria (Fig. 1A, B). Membrane potential ($\Delta\Psi_m$) *in vivo* was unaffected by moderate doses of NG, but exceeding $1 \text{ mg}\cdot\text{kg}^{-1}$, slight depolarization (by $\sim 20\%$) was observed. Nevertheless, this coincided with the peak effects of NO on Ca^{2+} uptake and RNS production at $\sim 1 \text{ mg}\cdot\text{kg}^{-1}$ NG (Fig. 1A, B).

Calcium accumulation capacity of mitochondria is considered to be dependent on the equilibrium between uptake and the efflux of the cation [4]. The main component of Ca^{2+} -uptake system is mitochondrial Ca^{2+} -uniporter, whereas $\text{Na}^+/\text{Ca}^{2+}$ and $\text{H}^+/\text{Ca}^{2+}$ exchangers serve for Ca^{2+} -efflux [4, 18]. Convincing data support the notion that in sub-conductance states MPTP too could function as a Ca^{2+} -releasing channel to prevent Ca^{2+} -overload of mitochondria in a living organism [3, 27-29]. The ability of mitochondria to take up Ca^{2+} was shown to be essentially dependent on MPTP activity. So, MPTP blockage by cyclosporine A increased Ca^{2+} uptake in rat heart mitochondria under reperfusion conditions [30], and, on the contrary, mitochondrial uptake of Ca^{2+} greatly diminished along with the increase of MPTP activity with ageing [29], which show MPTP to be physiologically relevant Ca^{2+} -efflux pathway. In our experiments, NO-donor treatment *in vivo* resulted in twofold increase of Ca^{2+} uptake in liver mitochondria, from ~ 90 to $\sim 180\text{-}200 \text{ nmol Ca}^{2+}\cdot\text{mg}^{-1}$, the same as it was observed under the action of MPTP blocker cyclosporine A *in vitro* (from ~ 100 to $\sim 200 \text{ nmol Ca}^{2+}\cdot\text{mg}^{-1}$). So, for better understanding the effect of NO on calcium accumulation, a direct study of NO action on Ca^{2+} -uniporter and MPTP activity as most essential for matrix Ca^{2+} accumulation was conducted. With this purpose NO donors, sodium nitroprusside (SNP) and NG, were used.

To test whether the direct action of NO on mitochondrial Ca^{2+} -uptake system could contribute to the overall increase in Ca^{2+} uptake, we studied the effect of NO donors on Ca^{2+} -uniporter activity. With the notion that NO could depolarize mitochondria because of the blockage of respiratory chain [7-9], the effect of NO donors on mitochondrial Ca^{2+} -uniporter activity was assessed from its dependence on membrane potential [18], in the presence of MPTP blocker cyclosporine A. With this aim, the initial rates of Ca^{2+} uptake (V_0) were determined under membrane depolarization induced by increasing concentrations of SNP, NG and malonate in mitochondria respiring on succinate. Obtained values were plotted against membrane potential, measured in parallel under the same conditions (Fig. 4).

According to our experiments, the initial rate of Ca^{2+} uptake in energized liver mitochondria taken for 100% in the absence of NO donors and malonate was 280 ± 12

Fig. 4. The assessment of the effect of NO donors on Ca^{2+} -uniporter activity. The initial rate (V_0) of Ca^{2+} -uptake in rat liver mitochondria under the action of SNP (1), NG (2), and malonate (3) was determined in parallel with membrane potential ($\Delta\Psi_m$). On the ordinate axis: initial rate of Ca^{2+} -uptake (V_0); on the abscissa axis: $\Delta\Psi_m$ at different concentrations of SNP, NG and malonate in mitochondria respiring on succinate. V_0 of Ca^{2+} -uptake and $\Delta\Psi_m$ in the absence of NO donors and malonate was taken for 100%. Ca^{2+} was added into standard incubation medium at 50 $\text{nmol}\cdot\text{mg}^{-1}$. The data represent mean $\pm$ SD.

Fig. 5. The effect of NO donors on MPTP activity: representative time traces (A) and peak Ca^{2+} uptake (B) in mitochondria of control animals (A, 1, 2) and treated by NG, 1 $\text{mg}\cdot\text{kg}^{-1}$ (A, 3, 4, grey color) in the absence (A, B, 1, 3) and the presence (A, B, 2, 4) of cyclosporine A (CsA); C – RR-sensitive (1, 3) and RR-insensitive (2, 4) Ca^{2+} transport after the addition of CCCP (1, 3), RR (2-4) and 10^{-6} M CsA (4) as shown by the arrows; D – the effect of NO on RR-insensitive Ca^{2+} -efflux; the additions of 10^{-5} M RR (1-3), 10^{-4} M NG (1), $5\cdot 10^{-4}$ M SNP (2, 3), 10^{-6} M of Ca^{2+} -ionophore A-23187 (1-3), and 10^{-6} M CCCP (3) were as shown. CaCl_2 was added into standard incubation medium at 30 μM ; mitochondria were added at 0.5 $\text{mg}\cdot\text{ml}^{-1}$; CsA was added at 10^{-6} M (A, B, 2, 4; C, 1, 3); peak Ca^{2+} uptake in $\text{nmol}\cdot\text{mg}^{-1}$ (B) is shown as mean $\pm$ SE (* - $P < 0.05$). On the ordinate axis: Ca^{2+} concentration in the medium, $[\text{Ca}^{2+}]_o$, μM .

$\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$. It declined exponentially with the decrease of $\Delta\Psi_m$ which agreed with the literature [18]. The differences between the dependences of Ca^{2+} -uniporter activity on $\Delta\Psi_m$ in all cases were statistically insignificant (Fig. 4, 1-3), thus we came to the conclusion that Ca^{2+} -uniporter was not affected by nitric oxide and was not responsible for the increase in Ca^{2+} accumulation capacity.

To explain the improvement of Ca^{2+} accumulation capacity under NG treatment, we supposed further that MPTP activity could be affected by NO. Analogous time traces, representative of Ca^{2+} transport in isolated mitochondria, show that properties of Ca^{2+} transport were changed in NO treated animals (at 1 $\text{mg}\cdot\text{kg}^{-1}$ NG). So, in control animals Ca^{2+} uptake typically was followed by Ca^{2+} efflux sensitive to cyclosporine A, which diminished related peak Ca^{2+} accumulation (Fig. 5A, B, 1, 2). Unlike this, after NO-treatment cyclosporine

A-sensitive Ca^{2+} efflux was considerably suppressed, while peak Ca^{2+} uptake increased almost to control level (Fig. 5A, B, 3, 4), which directly indicated MPTP inhibition by NO *in vivo*. To prove this observation more, we studied direct effect of NO donors on MPTP activity.

As showed kinetic curves, RR-sensitive and RR-insensitive Ca^{2+} transport were the main pathways of Ca^{2+} efflux from mitochondria under experimental conditions (Fig. 5C, 1, 2). Mitochondrial depolarization by CCCP in the presence of cyclosporine A delivered all Ca^{2+} taken up by mitochondria *via* Ca^{2+} uniporter in RR-sensitive way (Fig. 5C, 1, 3). In the presence of cyclosporine A CCCP-induced Ca^{2+} efflux was instantly blocked any time since the addition of RR to the suspension (*not shown*). Ca^{2+} release from energized mitochondria required the blockage of Ca^{2+} uniporter by RR (Fig. 5C, 2). Most part of RR-insensitive Ca^{2+} transport was blocked by cyclosporine A (Fig. 5C, 2, 4), which allowed to assess MPTP activity from RR-insensitive Ca^{2+} efflux. With this aim RR was added after Ca^{2+} accumulation, and the effects of NO donors on MPTP activity were studied (Fig. 5D). To exclude the contribution of $\text{Na}^+/\text{Ca}^{2+}$ and $\text{H}^+/\text{Ca}^{2+}$ exchangers to RR-insensitive Ca^{2+} efflux, only cyclosporine A-sensitive component of Ca^{2+} transport was taken into account. Such an approach allowed avoiding possible effects of membrane depolarization caused by NO on Ca^{2+} uptake and thus MPTP activity [9].

Unlike the case with Ca^{2+} uniporter, nitric oxide proved to be effective MPTP blocker (Fig. 5D). Both NO donors, SNP and NG almost completely inhibited MPTP activity at concentrations $\geq 5 \cdot 10^{-4}$ M (Fig. 5D, 1, 2). MPTP blockage occurred at once since the addition of NO donor into incubation medium (Fig. 5D, 3), and the blockage of Ca^{2+} transport in all cases was bypassed by the addition of Ca^{2+} -ionophore, A-23187 (Fig. 5D). The use of chemically different compounds as NO donors ensured that observed effects may be ascribed to the action of nitric oxide.

Unlike MPTP, other Ca^{2+} efflux pathways were little effective in releasing calcium from mitochondria, which was seen from the low rate of RR-insensitive Ca^{2+} efflux in the presence of cyclosporine A (Fig. 5C, 4). Thus we came to the conclusion that MPTP blockage by nitric oxide was the major cause for the increase in Ca^{2+} uptake under NG administration *in vivo*. This, in turn, resulted in a strong activation of mtNOS and mitochondrial RNS metabolism accompanied by the increase in ROS production.

Discussion

The effect of nitric oxide on mitochondrial calcium accumulation

In this work, it was shown that administration of NO donor nitroglycerine resulted in remarkable increase in calcium accumulation capacity of rat heart and liver mitochondria because of MPTP blockage by NO. Increased mitochondrial ability to take up calcium correlated with the activation of mtNOS and the rise in mitochondrial RNS production, shown to be preferably localized in mitochondria. The activation of RNS metabolism resulted in high level of protein nitrosylation supposed to contribute to MPTP inhibition under NO-donor administration.

The pattern of calcium accumulation in mitochondria isolated from NO-treated animals exhibited suppressed MPTP activity (Fig. 5A, 3, 4). Study *in vitro* confirmed that MPTP could be effectively blocked by NO (Fig. 5D), while uniporter activity was not affected (Fig. 4). In our experiments, mitochondrial Ca^{2+} buffering properties for the most part were dependent on Ca^{2+} -uniporter and MPTP activity, the contribution of other Ca^{2+} efflux pathways (such as $\text{Na}^+/\text{Ca}^{2+}$ and $\text{H}^+/\text{Ca}^{2+}$ exchangers [18] or non-specific Ca^{2+} efflux due to the presence of fatty acids in mitochondrial membrane [31]) being quite negligible. Unlike indirect MPTP inhibition by nitric oxide caused by membrane depolarization and prevention of Ca^{2+} uptake [9], our data gave the evidence for the instant MPTP blockage since the addition of NO donor to the medium (Fig. 5D). This implies the direct mechanisms of MPTP blockage by NO. But thus far, molecular mechanisms of NO interaction with MPTP remain obscure.

MPTP activity is known to be strongly dependent on the redox state of pyridine nucleotides and functionally active thiol groups [32]. NO can react with SH-groups of

several proteins and enzymes with the formation of derivatives such as mixed disulfides, sulphonic and sulphenic acids, and *S*-nitrosothiols resulting in the loss of the enzyme activity [24, 33]. Recently it was shown that nitrosylation of Cys-203 of cyclophilin D inhibited MPTP opening [34]. Thus, critical thiol modification of MPTP or regulatory protein(s) may serve a plausible explanation for observed blockage of MPTP activity both *in vitro* and *in vivo*. Accordingly, high level of chemically modified (nitrosylated) proteins and other NO derivatives in mitochondria of NG-treated animals (Fig. 2B) allowed us to assume that that direct action of NO/RNS on the pore or adjacent protein moiety could contribute to its inhibition in the living organism.

Nitroglycerine becomes physiologically active as NO donor after the hydrolysis of NO_3^- group with its following reduction to NO [35], and the rise in NO_3^- formation starting from NG injection (Fig. 1C, D, 1) largely may be caused by intracellular biotransformation of this drug. Thus, elevated matrix calcium could not be solely responsible for the increase in RNS production. On the other hand, instant dose-dependent activation of mtNOS, and mitochondrial localization of metabolic processes definitely indicated mitochondria as the primary source of RNS under the action of NG. In the literature, the activation of mtNOS was shown to occur due to Ca^{2+} accumulation *in vitro* [20]. Because mtNOS is known to be the main source of mitochondrial RNS [13-15], mtNOS activation along with the increase in Ca^{2+} uptake might reliably explain the rise in RNS production under NG administration (Fig. 2, 3).

Most part of hydroperoxide production under the action of NG too was of mitochondrial origin (Fig. 2C); however, at present it is rather difficult to indicate precisely the sources of H_2O_2 . Besides the respiratory chain, mitochondria possess too many sources of ROS, and several Ca^{2+} -dependent enzymes, including mtNOS, could contribute to ROS production [12-17]. The one plausible cause for the increase in hydroperoxide formation is high level of *S*-nitrosothiols (Fig. 2B), capable to inhibit the respiratory chain and promote ROS production [33]. Thus, observed rise in *S*-nitrosothiol formation after NG administration was coincident with partial (~20%) depolarization of mitochondria at the doses $\geq 1 \text{ mg}\cdot\text{kg}^{-1}$ NG. Increase in Ca^{2+} uptake too could be one of the major causes for the enhancement of hydroperoxide production. Earlier we have shown that Ca^{2+} uptake critically increased ROS production in isolated brain mitochondria [36]. So it was reasonable to suppose close effect of calcium in heart and liver mitochondria under the action of NG *in vivo*. This assumption was confirmed by the correlation established between Ca^{2+} accumulation and the activation of ROS metabolism (Fig. 3B); however, it needs to be verified by more detailed studies.

Physiological implications

Mitochondria are central in pathogenesis of cardiac and neurodegenerative diseases because of ROS overproduction, MPTP opening and impaired Ca^{2+} -buffering capacity [10, 30, 37]. MPTP exhibits several conductance states [11, 38, 39] with different permeability to solutes. Convincing data support the notion that in sub-conductance states MPTP could function as Ca^{2+} -releasing channel [11, 27, 28], and MPTP flickering between the open and closed state is supposed to be relevant under physiologically normal conditions [38-40]. This is in agreement with its function as cyclosporine A-sensitive pathway of Ca^{2+} efflux (Fig. 5D). Because at moderate doses *in vivo* NG did not impair mitochondrial energy state, physiologically, MPTP blockage by NO means the improvement of Ca^{2+} buffering capacity of mitochondria (Fig. 1 B; 5 B, 1, 3). Thus, physiological meaning of MPTP blockage by NO *in vivo* is: 1) suppression of MPTP activity as Ca^{2+} -releasing channel; 2) increase in Ca^{2+} buffering capacity of mitochondria, which help the removal of Ca^{2+} from cytosol; 3) prevention of MPTP transition to high conductance states and suppression of apoptosis induction. It is tempting to speculate that rise in mtNOS activity and RNS production caused by elevated matrix Ca^{2+} could serve a sort of feedback mechanism aimed to keep MPTP in a closed state and contribute to beneficial therapeutic action of nitric oxide.

The crosstalk between nitric oxide, calcium and ROS in mitochondria has many both physiological and pathophysiological aspects, which are not very well understood yet. In our work too, not all effects of NO donor in mitochondria were purely beneficial. So, the

reverse of MPTP blockage was calcium overload of mitochondria, simultaneous with ROS overproduction, seen in our experiments. Possibly, it may be overcome by mitochondrial antioxidant system, but this issue needs further studies.

Based on the data obtained, we came to the conclusion that improved matrix Ca^{2+} accumulation, together with MPTP blockage, are important contributions to vasodilatory and anti-apoptotic action of NO under pathophysiological conditions. So we consider that results of our work could serve better understanding of the mechanisms underlying cardioprotective effects of NO donors in clinics.

Disclosure Statement

The authors declare no conflict of interest.

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