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Calix[4]arenes Modulate Ca^{2+}-Dependent Processes in Smooth Muscle Cells Mitochondria

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Abstract Calix[4]arenes are macrocyclic polyphenolic nanocompounds that can selectively influence the transport of ions in subcellular membrane structures and do not have pronounced toxicity. The purpose of the work was to demonstrate the possibility of the selected calix[4]arene C-956 penetration into uterus smooth muscle cells and study out some mechanisms of its influence on the functional activity of mitochondria. The study was conducted on freshly isolated myocytes and isolated mitochondria from rats utera. The methods of laser confocal microscopy, flow cytometry and spectrofluorimetry were used. It was found that calix[4]arene C-956 has its own fluorescence in the blue-violet region of the spectrum. It is shown that it interacts with the plasmalemma of myocytes, penetrates into the cells and colocalizes with mitochondria. When interacting with mitochondria, C-956 (50, 100 μM) suppresses H^+-Ca^{2+}-exchanger of an internal membrane and does not affect the energy-dependent accumulation of Ca^{2+} by organelles. Along with this, 50 μM C-956 significantly stimulates NO-synthase activity in isolated mitochondria and inhibits the functioning of the electron transport chain by inhibiting the oxidation of NADH and $FADH_2$. No significant effect of the test compound on the DCF-fluorescence of the sub-cellular fraction was detected.

Keywords calix[4]arenes, mitochondria, smooth muscle, calcium, nitric oxide

Introduction

Calixarenes - macrocyclic compounds of cup-shaped structure obtained by cyclo-condensation of formaldehyde-substituted para-phenols are widely used as molecular platforms for the design of specific receptors capable of recognizing closely similar in size and properties of unique molecules. The ability to recognize substrates and to form supramolecular complexes with them is the basis for the use of calixarenes in chemistry, biology and nanotechnology [1].

Today, calix[4]arenes, supramolecular macrocyclic polyphenol nanocompounds, have being intensively studied, since they are high affinity modifiers of ATP hydrolases in the smooth muscles, including the uterus, which draws attention to their importance as potential regulators of calcium homeostasis of the myocytes [2-4].

Mitochondria play a central role in many fundamental biological processes, such as energy supply of cellular functions, oxidative metabolism, steroidogenesis, programmed cell death, etc. [5]. It has been established that mitochondria are the key stage in the intracellular Ca^{2+} signaling in smooth muscle cells owing to their ability to

accumulate and release significant amounts of Ca ions [6-8]. Changes in the Ca^{2+} concentration in mitochondria are major factors in the regulation of biochemical processes in the matrix and the functioning of the electron transport chain. The vital task of modern membranology is the search for exogenous non-toxic compounds that could efficiently and selectively regulate Ca^{2+} transport in the inner mitochondrial membrane and, thus, modulate Ca^{2+} homeostasis.

Nitric oxide (NO) is a universal signaling and regulatory molecule in a cell. Currently, mitochondrial localization of NO-synthase (mtNOS) is reliably shown in various mammalian organs and tissues [9-11]. In mitochondria, nitric oxide can perform several key functions: inhibition or induction of biogenesis, respiration control and oxidative phosphorylation, etc. [5, 12-13].

The ratio of oxidized and recovered forms of NADH (nicotinamide adenine dinucleotide) and $FADH_2$ (flavin adenine dinucleotide) serves an indicator of mitochondrial bioenergetics. NADH and FAD have own fluorescence and act as a marker for functional activity of mitochondria [14]. Consequently, changes in the Ca^{2+}/NO concentrations in mitochondria under the action of both endogenous and exogenous modifiers are a significant factor affecting the functioning of the electron transport chain.

The ability of calix[4]arenes to change the functional activity of mitochondria raises the question of studying their permeability through the plasma membrane of myocytes and interaction with mitochondria. This, in turn, will be a prerequisite for the search for calix[4]arenes that would have been able to correct mitochondrial dysfunction. However, systematic studies on the interaction between selected calix[4]arenes with uterine myocytes and the possibility of penetration into the cytoplasmic compartments were not carried out.

We have shown that calix[4]arene C-956 has autofluorescence in the blue-violet spectrum range. This fact allows us to apply methods of optical spectroscopy (spectrofluorimetry, laser confocal microscopy) to analyze the interaction and clarify the possibility of C-956 penetration in the smooth muscle cells.

Therefore, the purpose of the work was to study the interaction of calix[4]arene C-956 with myocytes of the rats uterus and the possibility of its penetration into mitochondria, to investigate the effect of C-956 on Ca^{2+}-transport, nitric oxide biosynthesis, changes in autofluorescence of adenine dinucleotides and DCF-fluorescence in isolated mitochondria of smooth muscle of the uterus.

Materials and methods

Experiments were performed on white wild-type nonpregnant rats weighing 150-180 g. All manipulations with animals were carried out according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, and the Law of Ukraine "On protection of animals from cruelty". Rats were anesthetized by chloroform inhalation and decapitated.

Isolation of myocytes from the uterine smooth muscle (myometrium). Myocytes were isolated from the uteruses by applying Mollard method using collagenase and soybean trypsin inhibitor [15].

Isolation of mitochondria from the uterine smooth muscle. The mitochondrial fraction was isolated from the myometrium using differential centrifugation, as described earlier [16]. 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 [17]. The total protein content in the mitochondrial fraction was 2 mg/ml.

Investigation of the fluorescence spectra of C-956, as well as changes in its fluorescence response when interacting with cells, was carried out 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. The measurements were performed using a fluorometric method on the Quanta Master 40 PTI (Canada) spectrofluorimeter with FelixGX 4.1.0.3096 software.

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. For the visualization of mitochondria and cell nucleus the fluorescent dyes MitoTracker Orange CMH_2TMRos (200 nM) and Hoechst

33342 (50 nM) were used, respectively [18]. The loading of immobilized myocytes with fluorescent probes and compound C-956 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-H_2TMRos, and the fluorescence was registered in the spectral range 560–615 nm. The autofluorescence of myocytes and fluorescence of C-956 were excited using excitation laser at wavelength 405 nm, and registered with the BP 420–480 filter. The myocytes were also excitated by an ultraviolet lamp at 360 nm and fluorescence was recorded using a F Set 01 wf filter. The study of the C-956 distribution kinetics in the cell was conducted in the Time Series mode, and the ROI (Region of Interest) function was used for the quantitative analysis, which allowed obtaining a graph of the fluorescence intensity of the time averaged for the selected region.

Assessment of the content of ionized calcium in the mitochondria. Loading of the mitochondria by the probe Fluo-4 AM at a concentration of 2 μM was carried out in the medium that contained (mM): Hepes – 10 (pH 7.4, 37°C), sucrose – 250, and 0.1% bovine serum albumin for 30 min at 37°C. To improve the process, the dye was mixed with Pluronic F-127 (0.02%). The relative values of Ca^{2+} content in the matrix of mitochondria, loaded with Fluo-4 AM (λ_{ex} = 490 nm, λ_{fl} = 520 nm) was investigated using the fluorometric method on spectrofluorometer Quanta Master PTI 40 (Canada) with software FelixGX 4.1.0.3096. The medium, from which energy-dependent accumulation of Ca^{2+} was carried out by the mitochondria, had a composition (mM): Hepes – 20 (pH 7.4, 37°C), sucrose – 250, K^+-phosphate buffer – 2 (pH 7.4, 37°C), $MgCl_2$ – 3, ATP – 3, sodium succinate – 5, concentration of Ca^{2+} – 80 μM. Energy-dependent Ca^{2+} accumulation was performed for 5 minutes, after which the suspension aliquot (100 μl) was diluted in the medium of Ca^{2+} release (2 ml) of the composition (mM): Hepes – 20 (pH 6.5, 37°C), sucrose – 250, K^+-phosphate buffer – 2 (pH 6.5, 37°C), sodium succinate – 5, cyclosporin A – 0.005 [15].

Study of NO biosynthesis by isolated mitochondria using DAF-FM and flow cytometry. The measurements were performed using COULTER EPICS XL^{TM} (Beckman Coulter, USA) cytometer with an argon laser (λ_{ex} = 488 nm, λ_{fl} = 515 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. The working medium was composed of (mM): Hepes – 20 (pH 7.4, 37°C), K^+-phosphate buffer – 2 (pH 7.4, 37°C), KCl – 120, sodium pyruvate – 5, sodium succinate – 5, L-arginine – 0.05, Ca^{2+} – 0.1, NADPH – 0.01, BH_4 – 0.01, aliquot of the mitochondria fraction contained 20 μg of protein [19].

Registration of NADH/FAD fluorescence in mitochondria using spectrofluorimetry method. The registration of the relative values of NADH fluorescence (λ_{ex} = 350 nm, λ_{fl} = 450 nm) and FAD fluorescence (λ_{ex} = 450 nm, λ_{fl} = 533 nm) in the fraction of the myometrium mitochondria was performed using a fluorometric method on the Quanta Master 40 PTI (Canada) spectrofluorimeter with FelixGX 4.1.0.3096 software. The studies were carried out in a medium of the following composition (mM): Hepes – 20 (pH 7.4, 37°C), K^+-phosphate buffer – 2 (pH 7.4, 37°C), KCl – 120, sodium pyruvate – 5, sodium succinate – 5, aliquot of mitochondrial fraction contained 100 μg protein [20].

Registration of DCF-fluorescence in mitochondria. The loading of mitochondria by reactive oxygen species sensitive DCF-DA fluorescence probe at a concentration of 25 μM was performed in a medium containing 10 mM Hepes (pH 7.4, 37 ° C), 250 mM sucrose, 0.1% bovine serum albumin for 30 min at 24°C. The Pluronic F-127 dye was added (0.02%) to improve the loading process. DCF-fluorescense in isolated mitochondria was studied using the flow cytometry method on the COULTER EPICS XL^{TM} (Beckman Coulter, USA) equipped with an argon laser (λ_{ex} = 488 nm, λ_{fl} = 525 nm (Fl1 channel). The studies were carried out in a medium of the following composition (mM): Hepes – 20 (pH 7.4, 24°C), K^+-phosphate buffer – 2 (pH 7.4, 24°C), KCl – 125, NaCl – 25, sodium pyruvate – 5, sodium succinate – 5. The reaction was initiated by adding aliquots (20 μl) of 5 mM pyruvate + 5 mM succinate. Incubation time 35 min. Calix[4]arene at a concentration of 50 μM were added for 15 min. The protein content in the aliquot of the mitochondria fraction was 20-25 μg.

Synthesis of calix[4]arene C-956 (5.11,17.23-tetra(trifluoro)methylphenylsulfonylimino)-methylamino-25,27-dioctyloxy-26,28-dipropoxy calix[4]arene) was carried out and characterized using NMR and IR spectroscopy in the Department of Phosphoran Chemistry, Institute of Organic Chemistry, National Academy of Sciences of Ukraine

under the leadership of Volodimir Kalchenko, Academician of National Academy of Sciences of Ukraine. Calix[4]arene were dissolved in DMSO. When conducting experiments, the aliquots of the solvent introduced into the incubation medium served as the control.

The statistical analysis of the data obtained was carried out using a package of standard IBM PC programs, using the well-known methods and Student's t-criterion.

In the work the following reagents were used: Hepes, glucose, sucrose, sodium succinate, sodium pyruvate, bovine serum albumin, poly-L-lysine, collagenase type IA, ATP, Pluronic F-27, DAF-FM **(**4-amino-5- methylamino-2',7'-difluorescein, diaminofluorescein-FM**)**, DCF-DA (2',7'-dichlorodihydrofluorescein diacetate), EGTA, $CaCl_2$, L-arginine, NADPH, BH_4, cyclosporin A (Sigma, USA); Hoechst 33342, soybean trypsin inhibitor (Fluka, Switzerland); MitoTracker Orange CM-H_2TMRos, Fluo-4 AM (Invitrogen, USA). Any other reagents are produced in Ukraine.

The solutions were prepared on bidistilled water, which had a specific electrical conductivity of not more than 2.0 μcm. The electrical conductivity of the water was recorded using a conductometer OK-102/1 (Hungary).

Results

It is established that the calix[4]arene C-956 (Fig. 1) has its own fluorescence in the blue-violet region. It has been experimentally proved that for this compound λ_{ex} is 283 nm, and λ_{fl} is 365 nm (Fig. 2). The fluorescence of C-956 increases linearly with increasing concentrations in a working cell-free medium (Fig. 3). In order to increase the nonspecific permeability of the plasma membrane, we used a detergent digitonin (0.01%), which, at this concentration, does not affect the individual functional parameters of the intracellular membranes of the uterine myocytes, in particular Ca^{2+} -transport ability [21]. It is proved that it does not change the nature and intensity of the fluorescence signal from the C-956 (Fig. 3).

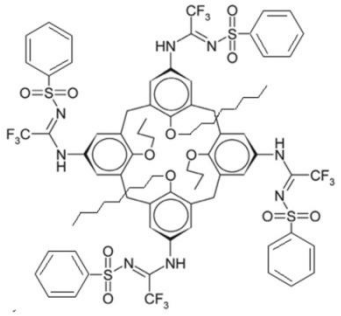

Figure 1: Structural formula of the calix[4]arene C-956

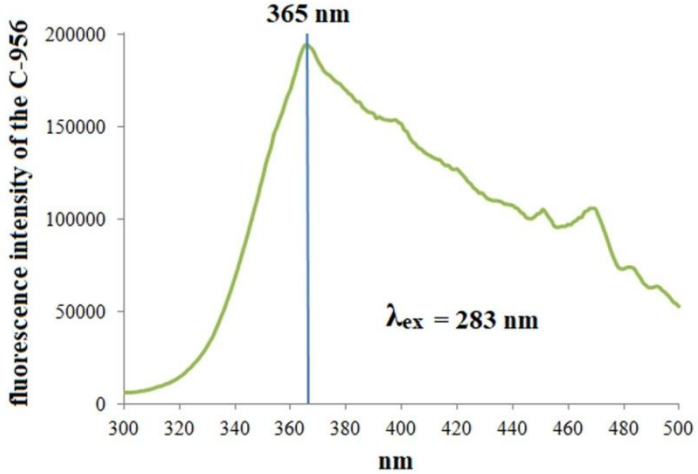

Figure 2: Fluorescence spectrum of 100 μM calix[4]arene C-956 in the working medium

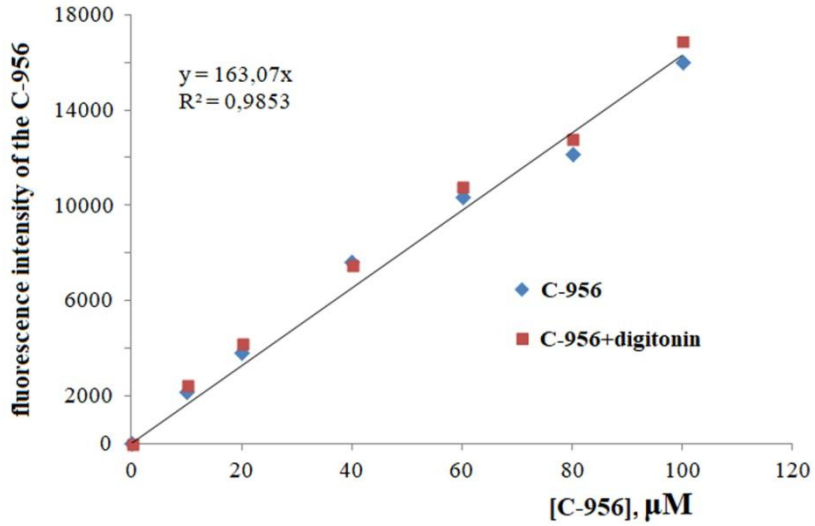

Figure 3: Fluorescence of C-956 in the working medium and in the presence of 0.01% digitonin

When C-956 interacts with myocytes, the intensity of the calix[4]arene fluorescence changes: it first increases as a result of its interaction with cells, then decreases (Fig. 4, A). Initial growth and subsequent bleaching of C-956 fluorescence depends on its concentration: the initial fluorescence growth decreases and bleaching increases with increasing concentration of C-956 (Fig. 4, A; Fig. 5, A). The effectiveness of the C-956 interaction with the cytoplasm increases and the degree of bleaching of its fluorescence decreases in the conditions of the plasma membrane desruption in the presence of 0.01% digitonin (Fig. 4, B; Fig. 5, B). The absence of the digitonin effect on the initial rise of C-956 fluorescence was been demonstrated (Fig. 5, A). The curve of the initial rate of fluorescence C-956 bleaching from its concentration has the character similar to the bell-shaped (Fig. 6).

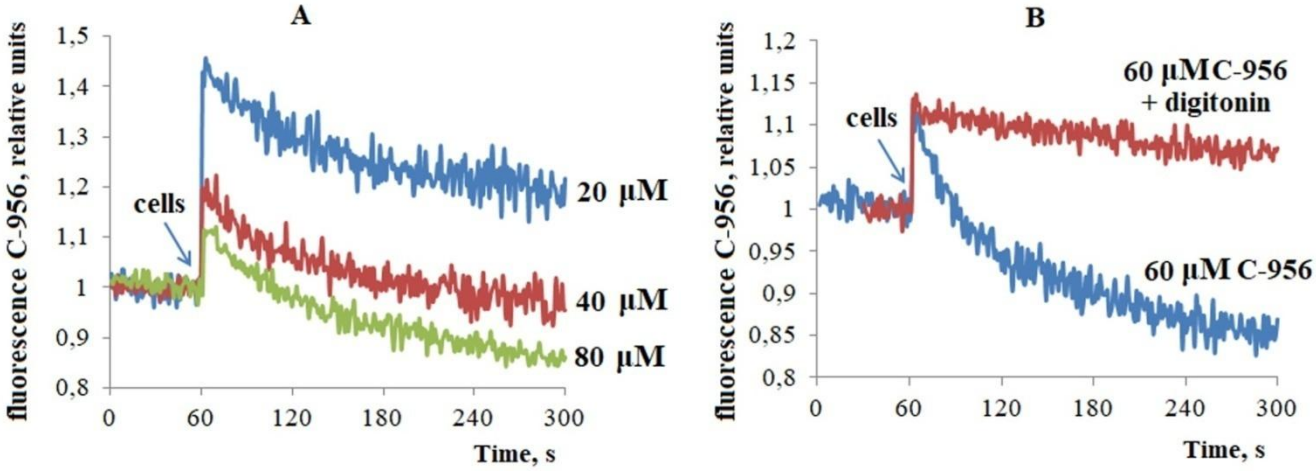

Figure 4: The C-956 interaction kinetic with cells at it different concentrations (A) and the influence of 0.01% of digitonin on this process (B). The arrows mark the time of cells insertion. The result of a typical experiment. At least 5 independent experiments were conducted. Spectrofluorimetry method

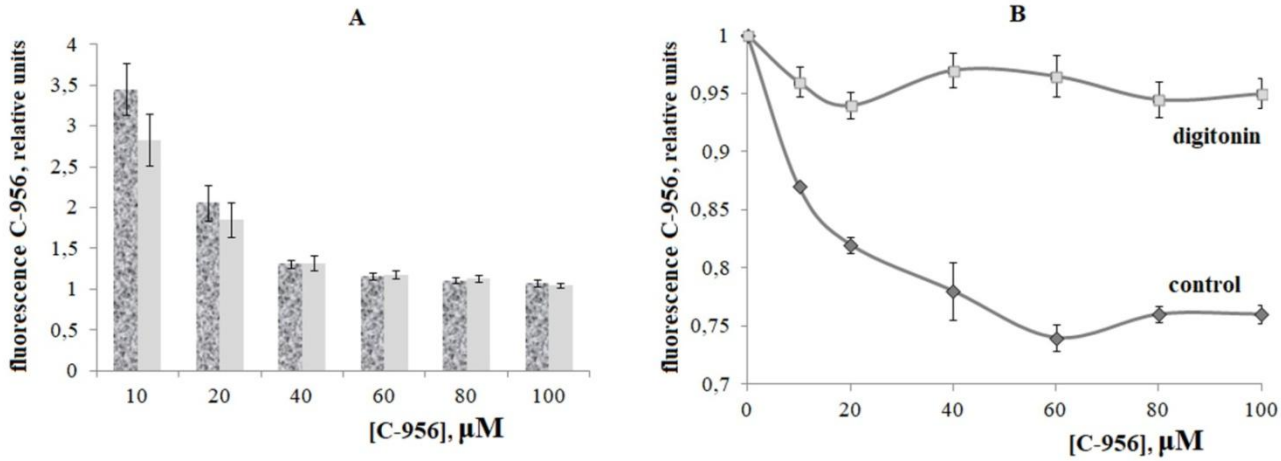

Figure 5: Concentration dependence of fluorescence changes of C-956 in the control and in the presence of 0.01% digitonin. A - the initial phase of the fluorescence flare of C-956 in its interaction with the cells: left bars - without digitinin, right bars - digitonin added. B - bleaching phase of C-956 fluorescence after its interaction with cells. Data are shown as mean of 5 independent measurement ± SD. Spectrofluorimetry method

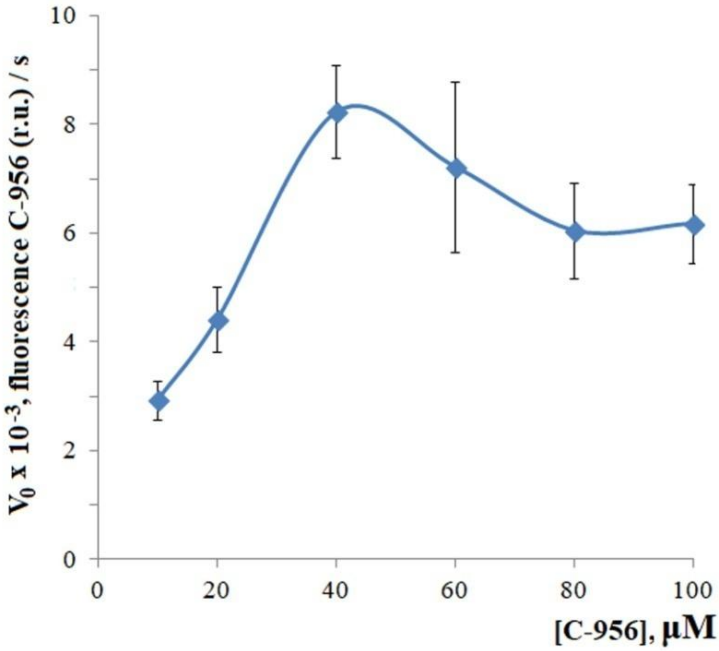

Figure 6: The dependence of the initial rate of C-956 fluorescence bleaching on its concentration when interacting with cells. The method of the initial rate of bleaching calculating is based on the rebuilding of experimental results in the previously proposed coordinates: ln [(F_0 – F')/(F – F')]; t, (s), where F_0 is the initial fluorescence, F is the fluorescence at the appropriate time interval, and F' is the constant level of fluorescence reached over time [28]. Data are shown as mean of 5 independent measurement ± SD.

A similar distribution of blue fluorescence in the cell and fluorescence of the mitochondrial dye MitoTracker Orange CM-H_2TMROS (red) are observed in the analysis of the optical slice of the cell from the cytoplasm area outside the nucleus (Fig. 7). The blue fluorescence of myocytes is due to the presence of adenine nucleotides in the cytoplasm, the content of which is particularly high in mitochondria. The intensity of the signal in the blue region doubles after

entering the calix[4]arene C-956. Moreover, the colocalization of the blue and red signals in the cytoplasm is observed. These results indicate the potential interaction of C-956 with mitochondria.

Qualitatively similar spectrofluorimetric results were obtained by confocal microscopy using an ultraviolet lamp (excitation at 360 nm). Adding C-956 aliquots at a concentration of 20 μM induced an initial rise of fluorescence followed by bleaching. The gradual concentration of C-956 increase in the medium reduced the initial fluorescence intensity corresponding to the spectrofluorimetry results (graphic data is not given).

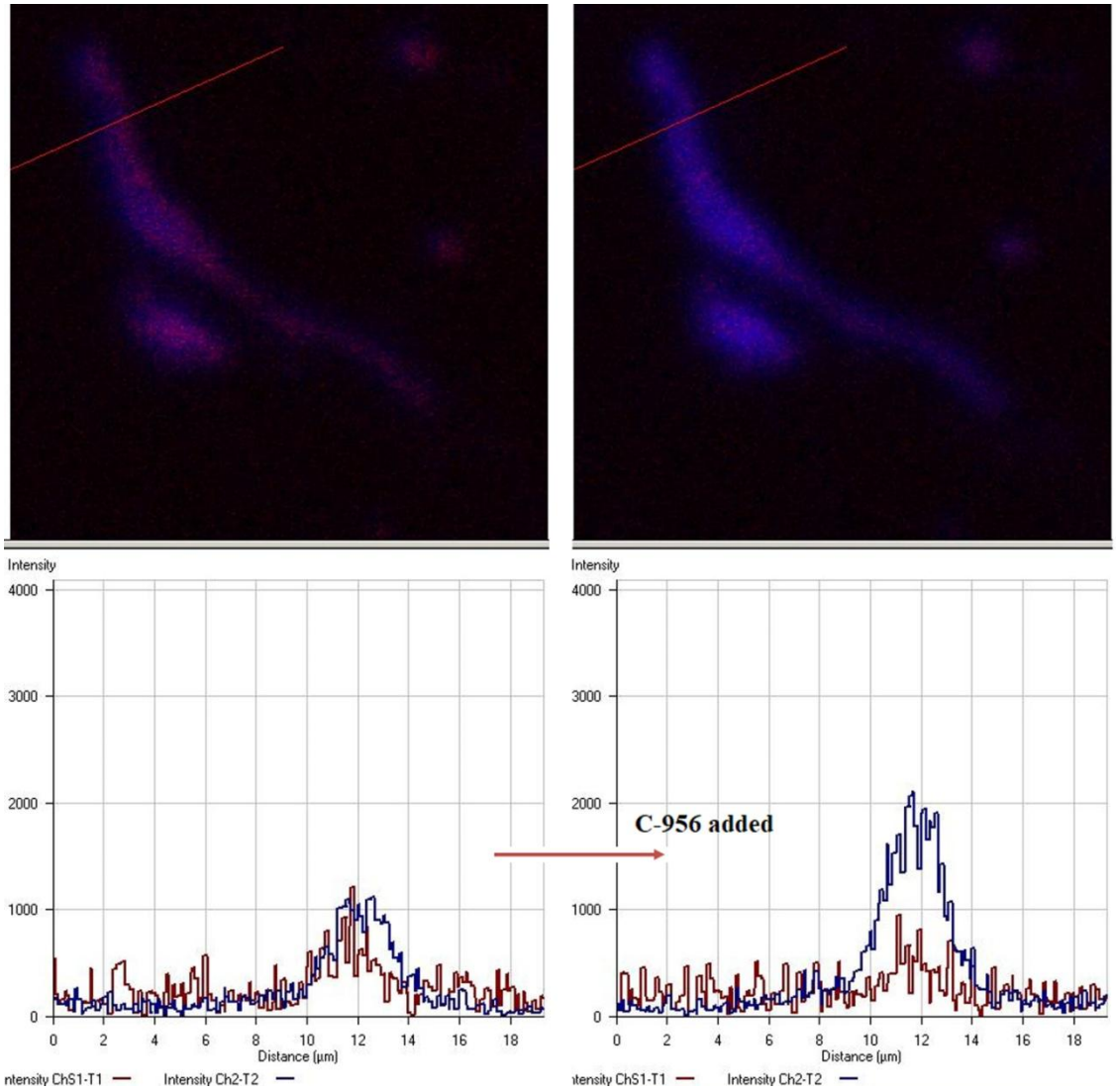

Figure 7: Comparison of distribution profile of the 20 μM C-956 (blue) and specific for mitochondria 200 nM MitoTracker Orange CM-H_2TMROS (red) in myocyte. The area outside the nuclear was selected for analysis (the nuclear was visualized using 50 μM Hoechst dye previously). The result of a typical experiment. At least 5 independent experiments were conducted. Method of confocal microscopy

The macrocycle C-956 with high efficiency suppresses the H^+-Ca^{2+} exchange in mitochondria (Fig. 8), but the electrophoretic accumulation of Ca^{2+} is resistant to its action.

The braking of the ΔpH-dependent release of Ca^{2+} from mitochondria results a rise in its matrix concentration and an increase in Ca^{2+}-dependent processes, in particular, the synthesis of nitric oxide. Indeed, there is a strengthening of biosynthesis NO by isolated mitochondria in the working environment in the presence of respiratory substrates and cofactors under the action of 50 μM C-956 (Fig. 9).

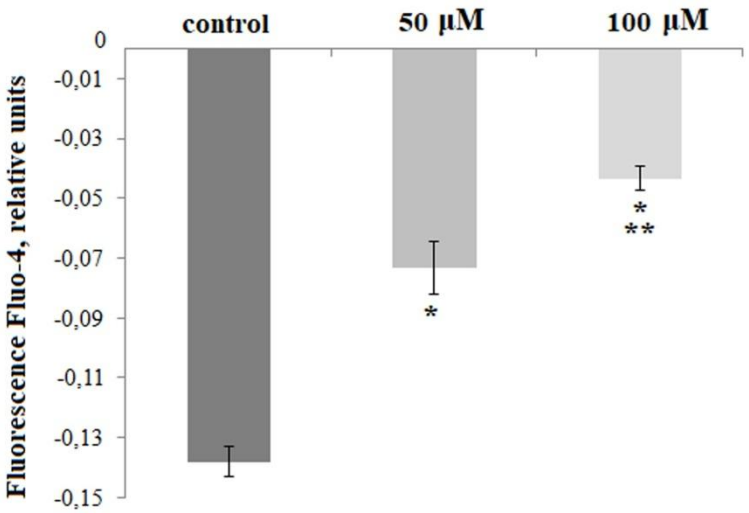

*Figure 8: Decreasing of ΔpH-dependent release of Ca^{2+} from isolated mitochondria at the action of 50, 100 µM C-956; *P<0.05 vs control, **P<0.05 vs 50 µM C-956. Data are presented as mean±SD, n=5. Spectrofluorimetry method*

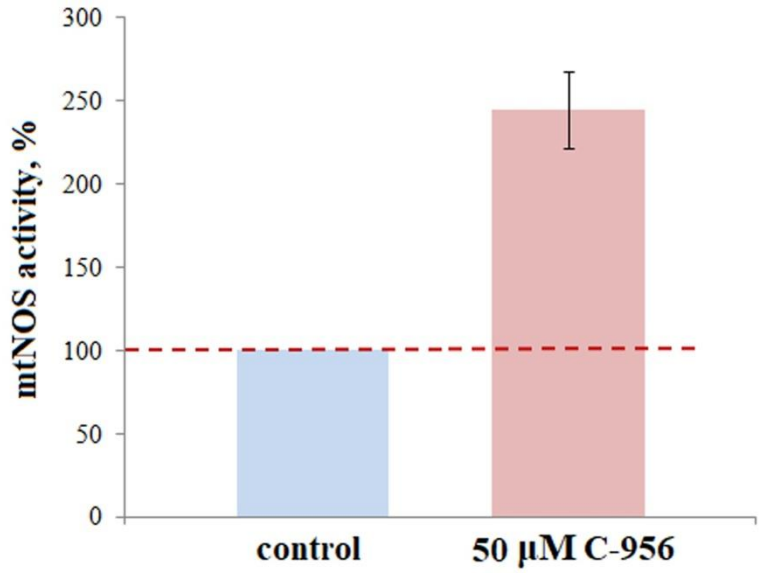

Figure 9: Stimulation of the NO-synthase activity by 50 µM C-956 in isolated mitochondria. Data are presented as mean±SD, n=5. Method of flow cytometry

C-956 suppressed the intensity of NADH/$FADH_2$ oxidation in mitochondria (Fig. 10) in accordance with the notions of the electron transport chain inhibition under the nitric oxide action. In addition, the studied calix[4]arene almost does not affect DCF-fluorescence of mitochondria at least at a 50 µM concentration (Fig. 11), which suggests that it does not significantly increase the generation of reactive oxygen species under experimental conditions.

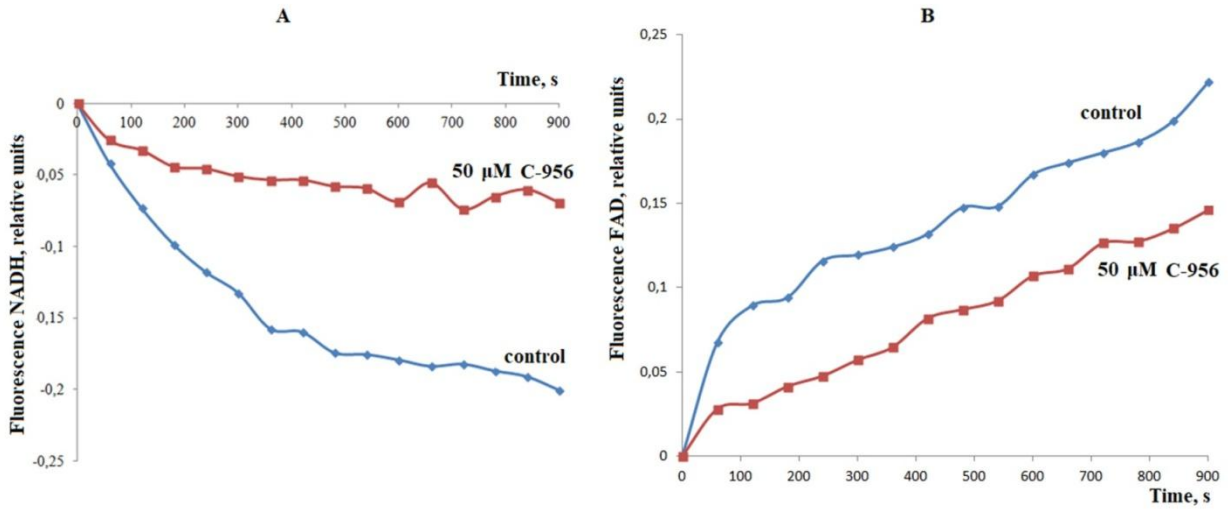

Figure 10: Decreasing of the oxidation of endogenous NADH and $FADH_2$ in isolated mitochondria with the action of 50 µM C-956. The result of a typical experiment. At least 5 independent experiments were conducted. Spectrofluorimetry method

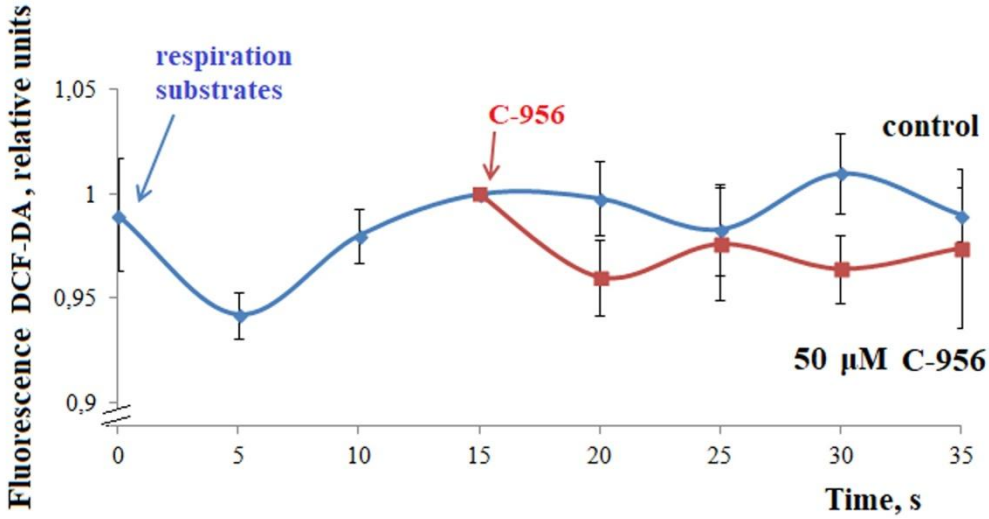

Figure 11: DCF-fluorescence in isolated mitochondria in control and in the presence of 50 µM C-956. Data are shown as mean of 5 independent measurement ± SD. Method of flow cytometry

Discussion

Structural formula of the studied calix[4]arene is presented in Figure 1. The basis of its structure is a macrocycle polyphenolic ring, the so-called "calix[4]arene chalice", which has substitutes of different chemical nature on the upper crown. It should be noted that the purely "calix[4]arene chalice" and the substituents do not separately affect the cation-transport systems and the polarization of the subcellular structures of the myometrium [22]. From our point of view, these effects are due to the additive action: "calixarene chalice" and substitutes. Calix[4]arenes are hydrophobic compounds, which allows the ability to penetrate the lipid bilayer plasmalemma inside the cell. Calix[4]arene C-956 exists four sulfonylamide groups with phenyl residues and two elongated aliphatic chains in the structure that facilitates interaction of C-956 with hydrophobic parts of subcellular membranes and penetration into a lipid bilayer. It was shown that hydrophobicity directly correlates with calix[4]arenes biochemical efficiency [23].

Calix[4]arene C-956 has fluorescence in the blue-violet region (Fig. 2), which may be explained by the presence in the molecule of the system of conjugate π-orbitals. The initial grows of the fluorescence signal is observed when C-956 interacts the cells (Fig. 4). The obtained data indicate that the effects of followed reducing fluorescence (Fig. 4) are occurred due to the interaction of C-956 molecules among themselves: the higher concentration - the more intensive self-bleaching. The effectiveness of the C-956 interaction with the cytoplasm increases and the degree of bleaching of its fluorescence decreases under conditions of the digitonin plasma membrane desruption (Fig. 4, Fig. 5). The absence of the digitonin effect on the initial rise of C-956 fluorescence (Fig. 5, A) may indicate the fact of C-956 penetration into the cytoplasm. The more intensive distribution in the intracellular volume of C-956 prevents aggregation of its molecules on the outer surface of the cells and the degree of calix[4]arene fluorescence bleaching decreases in the presence of a detergent.

Bell-shaped curve dependence of the initial rate of fluorescence C-956 bleaching from its concentration (Fig. 6) can be explained as follows. C-956 is adsorbed on the membrane surface, followed by the interaction between the calix[4]arene molecules leads to an increase in the bleaching rate depending on its concentration. But at higher concentrations, the simultaneous process of penetration into the cytoplasm increases and the rate of self-bleaching is somewhat reduced.

The colocalization of the blue (C-956) and red (MitoTracker) signals in the cytoplasm indicate the potential interaction of C-956 with mitochondria (Fig. 7). Consequently, calix[4]arene C-956 is adsorbed by the plasmatic membrane of the uterine myocytes, penetrates into the myoplasm and interacts with the mitochondria.

Ca^{2+} is a universal secondary messenger and regulatory cation that ensures the wide range of cellular reactions in eukaryotes. Calcium ions play a fundamental role in providing smooth muscle contraction, since the change in their intracellular concentration is a key element in electromechanical and pharmacomechanical coupling of excitation and contraction. Endoplasmic reticulum and mitochondria act as Ca^{2+} depot [8, 24]. Mitochondria play a key role in the processes of Ca^{2+}-signaling. Due to their effective accumulation, especially in the contact areas of the endoplasmic reticulum/plasma membrane, and mitochondria, where the local concentration of the cation can reach tens and even hundreds micromoles, the latter can modulate Ca^{2+} signal, in particular its amplitude and time characteristics [8, 25-26].

Due to the importance of biochemical processes within mitochondria for the functioning of the whole cell, as well as the direct relationship between mitochondrial dysfunction and the development of smooth muscle pathologies, the search for non-toxic and selective modifiers of the mitochondrial Ca transport systems is a focus of research interest. The inhibitory effect on certain calix[4]arenes on Ca^{2+}, Mg^{2+}-ATPase of the sarcolemma and sarcoplasmic reticulum, and ATP-hydrolase activity of myosin preparations from the myometrium has been revealed [2, 4]. The ability of some supramolecular macrocyclic calix[4]arenes, in particular, C-90 and C-956 (C-90 is analogue of the C-956), to inhibit the activity of the plasma membrane and sarcoplasmic reticulum Ca^{2+}-pumps [4] in smooth muscle draws interest to these compounds as possible regulators of Ca^{2+} transport in mitochondria. We have assumed that selected calix[4]arenes are capable of interacting with Ca^{2+} transport systems in the smooth muscle mitochondria and altering their metabolism and energetic.

Two systems that regulate the Ca^{2+} concentration in the matrix have been identified in the myometrial mitochondria: a Ca^{2+}-uniporter and an H^+-Ca^{2+}-exchanger. We demonstrated a system of inverse H^+-Ca^{2+} exchange (ΔpH-dependent calcium ion transport), which did not depend on gradients of Na^+ and K^+, in our previous studies conducted on isolated mitochondria from the rat myometrium. It was activated by physiological pH (pH_a≈7.0) and was performed in 1:1 stoichiometric ratio [16]. Previously, it has been shown that the use of a fluorescent probe Fluo-4 AM enables to quite reliable testing of the changes in Ca^{2+} concentration in isolated mitochondria matrix, and their inner membrane has barrier function as to calcium ions. A lot of electron microscopic studies, functional tests (assessment of calcium-transporting processes), and data of photon correlation spectroscopy clearly indicate the presence of the intact inner membrane in isolated mitochondria of the myometrium [16]. According to modern concepts, molecular structure that ensures the functioning of H^+-Ca^{2+} exchanger in the inner mitochondrial membrane, is protein LETM1. We have shown that acidulation of outside mitochondrial environment (pH 6.5)

stimulates Ca^{2+} release from isolated mitochondria that had been previously accumulated by the organelles in energy-dependent process. Anti-LETM1 significantly suppressed ΔpH-induced Ca^{2+} release from the mitochondrial matrix [27].

In this work we have found that calix[4]arene C-956 at a concentration of 50 and 100 μM effectively blocked the ΔpH-induced Ca^{2+} release from isolated rat myometrium mitochondria (Fig. 8) and did not affect the potential dependent accumulation of this cation. In the previously work we have found that apparent inhibition constant (K_i) of calix[4]arene C-956 action on the H^+-Ca^{2+}-exchanger of the inner mitochondrial membrane of rat uterine myocytes is 35.1 ± 7.9 μM [28]. Therefore, we used an active concentration of C-956 in further studies 50 μM. The results of our studies indicate that the action of calix[4]arene C-956 on Ca^{2+}-transporting systems of the mitochondria is directed toward increasing Ca^{2+} concentration in the matrix.

The unique property of the calix[4]arenes is their specific structure-dependent effect on the cation-transport systems of subcellular organelles. In particular, we showed that the high-affinity inhibitors of Na^+, K^+-ATPase namely C-97, C-99 and C-107 depolarized the plasma membrane of rat uterine myocytes and transiently hyperpolarized the internal mitochondrial membrane [22]. The latter effect testifies to the penetration of calix[4]arenes into the cell and their direct interaction with mitochondria. The indicated calix[4]arenes were shown to effectively block the potential-dependent Ca^{2+} accumulation and reduce the cation concentration in the myometrium mitochondria, probably, stimulating the H^+-Ca^{2+}-exchanger [29].

To date, it has been found that NO modulates energy, metabolic and transport processes in mitochondria. The inner membrane of mitochondria is an effective target of nitrocompounds, since the enzymes localized therein contain high levels of thiol residues, iron-sulfur centers, heme groups, and the membrane itself is the site of the formation of superoxide anion [30-31]. Nitric oxide is capable of regulating the activity of mitochondria electron transport chain, reversibly suppressing cytochrome *c*-oxidase, and controlling the value of mitochondrial matrix pH [32]. At low nanomolar concentrations NO limits the intensity of respiration and oxidative phosphorylation, which is a physiologically significant reaction [33]. NO regulates Ca^{2+} homeostasis in mitochondria and, accordingly, Ca^{2+}-dependent processes, primarily the activity of a range of dehydrogenases [34]. We have firstly demonstrated the formation of NO in mitochondria of uterine smooth muscle cells 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 [19]. The optimal work of mtNOS in isolated myometrium mitochondria requires the presence of respiration substrates, Ca^{2+}, NADPH and L-arginine. This enzymatic reaction was dependent on the intensity of exogenous Ca^{2+} entry to the matrix (activation constant for Ca^{2+} is 44.4 ± 14.5 μM) and suppressed by N^G-nitro-L-arginine – nonspecific constitutive NO-synthase inhibitor. It has been assumed that C-956 increases mtNOS activity in the isolated myometrium mitochondria because of its possibility to rises matrix Ca^{2+} concentration. Indeed, 50 μM C-956 was enhanced the NO-synthase mytochondria activity almost twise (Fig. 9) in the presence of 5 mM succinate+pyruvate, 100 μM Ca^{2+}, 50 μM L-arginine, 10 μM NADPH and 10 μM tetrahydrobiopterin (for protected against superoxide-anion formation).

NO own biosynthesis in mitochondria can lead to a decrease in the activity of mitochondrial dehydrogenases and the corresponding inhibition of the work of the electron transport chain. To test this assumption, it is necessary to examine the effect of the C-956 on the functional activity of the electron transport chain. As noted above, the marker of the activity of the latter is the redox state of adenine nucleotides ($NADH/FADH_2$). The dependence of changes in the fluorescence signal of NADH/FAD in the norm and in the conditions of the specific blocking of the complexes *I* and *II* of the respiratory chain, as well as after adding protonophore, was studied in our previously works in order to demonstrate the correspondence between the work of the electronic transport chain and the redox status of adenine nucleotides [20]. Consequently, our previous results testify to the possibility of examining the functional activity of the electron transport chain of isolated myometrium mitochondria by using experimental data as to the changes in the own fluorescence of NADH and FAD coenzyme. These results correspond to the views of other authors [14]. The NADH fluorescence decreases and FAD fluorescence increases in the presence of pyruvate and succinate (5 mM) in the incubation medium due to the functioning of the complex *I* and *II* of the respiratory chain (Fig. 10). The

analysis of the fluorescence response of NADH/FAD suggests that the effect of calix[4]arene on the functional activity of the electron transport chain is associated with the inhibition of the complex *I* and *II* activity (Fig. 10). This effect can be explained by Ca^{2+}-dependent enhancement of NO biosynthesis in mitochondria under the action of C-956 with subsequent decline of the inner membrane respiratory chain functioning.

It is known that the excessive uptake of Ca^{2+} and exceeding NO production by the mitochondria may cause reactive oxygen species generation in these organelles. The main mechanism of their production is the respiratory chain stimulation by calcium ions [35-36]. But NO-dependent electron transport chain inhibition may be protected factor against reactive oxygen species production. Indeed, C-956 practically does not affect on DCF-fluorescence and, consequently, production of reactive oxygen species, including peroxynitrite, by mitochondria (Fig. 11). These data may indicate a corrective role of C-956 in relation to the smooth muscle mitochondrial metabolism. Inhibition of ΔpH-dependent release of Ca^{2+} from the matrix is accompanied by an increase in the synthesis of NO, which in turn limits the Ca^{2+}-dependent activation of the electron transport chain and the hyperproduction of reactive nitrogen and oxygen species, which should lead to mitochondrial dysfunction.

Conclusions

Calix[4]arene C-956 is adsorbed by the plasmatic membrane of rat uterine myocytes, penetrates into the myoplasm and interacts with mitochondria. It inhibits the ΔpH-dependent Ca^{2+} release from the mitochondrial matrix and stimulates biosynthesis of nitric oxide by these sub-cellular structures. The C-956 suppresses the oxidation of endogenous NADH/$FADH_2$ in the electron transport chain in the presence of respiratory substrates and does not affect the generation of reactive oxygen species in mitochondria. Further study of the effect of C-956 and its analogues on the Ca^{2+} transport, bioenergetic and the synthesis of reactive nitrogen and oxygen species in the smooth muscles mitochondria may bring the possibility of using these low-toxic compounds for correction of mitochondria functional activity.

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Conflict of Interest

Authors declare no conflict of interest.

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