УДК 577.152.3

O.V. Akopova, L.I. Kolchinskaya, V.I. Nosar, V.A. Bouryi, I.N. Mankovska, V.F. Sagach

Cytochrome *c* as an amplifier of ROS release in mitochondria

The influence of exogenous cytochrome c on reactive oxygen species (ROS) formation and its dependence on mitochondrial permeability transition pore (MPTP) opening is studied in rat liver mitochondria. Fluorescent probe dichlorofluorescein (DCF) was used. It was shown that MPTP activation by increasing concentrations of Ca^{2+} in the medium results in the increase in mitochondrial ROS production and oxygen consumption, but the decrease in matrix calcium retention, dependent on the amount of added Ca^{2+}. Cytochrome c in the incubation medium does not much influence ROS formation when MPTP opening is blocked by cyclosporine A. However, in the presence of cytochrome c MPTP opening is accompanied by dramatic increase in ROS production. Steep rise in DCF fluorescence because of matrix ROS formation is sensitive to MPTP opening and is not resulted from the direct interaction between the probe and cytochrome c outside the mitochondria. To explain obtained data the hypothesis is put forward that MPTP could serve for ROS exchange between the matrix and the medium where heme iron of cytochrome c would act as a catalytic center to enhance ROS production. We suppose that apart of its conventional function, cytochrome c which is not involved in electron transport, could serve in such way as the amplifier of ROS production which in turn would provide a background for the development of apoptosis due to MPTP opening.
Key words: mitochondria, Ca^{2+}, mitochondrial permeability transition pore, ROS, cytochrome c.

INTRODUCTION

At the beginning of 90th it was well established that not only NO and its derivatives, but also free-radical and other highly reactive oxygen derivatives (reactive oxygen species, ROS) are active participants in cellular signal transduction [19]. However, mitochondria, the potent source of ROS, received relatively little attention, and their role in cellular ROS signaling for long time have been underestimated. In the late 90th convincing evidences were obtained that mitochondria could behave as excitable organelles which convey and exchange Ca^{2+} and other signals [12].

Ca^{2+}-signalling between the mitochondria and other cellular structures such as plasma membrane and sarcoplasmic reticulum (SR) is now well established fact in cell biology. Effectiveness in the transduction of Ca^{2+}-signals is provided by the Ca^{2+}-transporting system of mitochondria, as well as intracellular localization of the organelles – near the plasma membrane, near SR and near the core. Signal transduction is facilitated also by the existence of the contact sites between mitochondria and the neighboring structures [16].

There is increasing amount of evidence that not only Ca^{2+} but also ROS signals could propagate between the cells [20]. Well known is the phenomenon of ROS-induced ROS-release [22] which is tightly connected to the opening of mitochondrial permeability transition pore (MPTP) and spreading of ROS between mitochondrial populations. It is tempting to speculate a possibility of ROS (as well as Ca^{2+}-) signaling between mitochondria, SR, plasma membrane, and the core, as the foreground for the induction of cellular apoptosis. The mechanisms of ROS signaling in the cell

are not well understood, in spite of their intensive study over last decades.

Impaired Ca^{2+}-signalling, the opening of mitochondrial pore accompanied by the uncoupling and the decreased effectiveness of ATP synthesis, massive release of ROS and the release of cytochrome *c* from the intermembrane space into cytosol are often considered as the first step in the induction of cellular apoptosis [17]. Although cytochrome c is indispensable participant in the electron transfer between the complexes of respiratory chain, its role in regulation of the level of ROS generated as by-products in the course of oxygen reduction have not been fully elucidated. It is well known that positively charged cytochrome c is retained by weak electrostatic forces on the outer surface of the inner membrane and is at equilibrium with unbound protein in the bulk solution of intermembrane space [10]. Normally, cytochrome *c* serves for electron transport between III and IV complexes of respiratory chain. After the rupture of the outer membrane due to MPTP opening, cytochrome *c* would release from the intermembrane space into cytosol and adsorbed protein would dissociate from the surface due to the decrease of its concentration in the bulk solution. The loss of cytochrome *c*, in turn, would lead to the inhibition of respiration which could be reversed by the addition of exogenous cytochrome *c* [13]. However, it is known that gating of electron flow between the complexes of respiratory chain upon the loss of cytochrome *c* makes the conditions highly favorable for ROS production by mitochondria because the transition of the sites of ROS production "upstream" of the inhibition of electron flow to more reduced state favors one-electron reduction of oxygen with consequent increase in ROS formation [18].

In its normal function cytochrome *c* could exhibit antioxidant properties as an acceptor of electrons [14]. The role of cytochrome *c* outside the mitochondria in regulation of the redox reactions in the cell, however, is controversial. In the literature, there is plenty of evidence for prooxidant as well as antioxidant action of cytochrome *c* [6,7,14]. Dramatic increase in ROS production, not dependent on MPTP opening, after the loss of cytochrome *c* in mitochondria was shown by many authors [7,21]. Release of cytochrome *c* from mitochondria could be provoked by different stimuli, such as calcium and potassium uptake [8,9], translocation of proapoptotic protein Bax to mitochondrial outer membrane [21] and many others.

MPTP is often considered as the way of a massive ROS release from mitochondria [22]. After MPTP opening the release of ROS from the matrix takes place together with the release of cytochrome c from the intermembrane space. The role of cytochrome *c* in mitochondrial ROS production under conditions of MPTP opening, however, received relatively little attention in the literature. The aim of our work was to estimate the influence of exogenous cytochrome *c*, which does not take part in the electron transfer in the respiratory chain, on ROS formation in mitochondria and its dependence on the permeability transition pore opening.

MATERIALS AND METHODS

Adult Wistar rats with average body weight 200 g were used. Rat liver was homogenized in a medium (1): 250 mM sucrose in 20 mM Tris-HCl buffer (pH 7,4), 1 mM EDTA and centrifuged at 700g x 8 min (4°C). Mitochondria were sedimented by centrifugation of supernatant at 11000g x 15 min (4°C), suspended in a small volume of medium (1) without EDTA and stored on ice until use. The experiments were carried out at 37°C in standard incubation medium: 120 mM KCl, 1 mM KH_2PO_4, 4 mM of sodium glutamate, 20 mM Tris-HCl buffer (pH 7.4). Cytochrome c was added at 1 μM.

The data on ROS formation were obtained from dichlorofluorescein (DCF) fluorescence.

Non-fluorescent probe, (2',7'-dihydrodichlorofluorescein diacetate, DCFHDA) at 200 μM was added to stock mitochondrial suspensions and loaded for 20 min at 37°C. Then stock suspension was placed on ice, and the aliquots were sampled in 1cm cuvette of spectrofluorimeter. The probe was excited at 504 nm, and the emission registered at 525 nm [23]. Ca^{2+}-uptake was studied spectrophotometrically with arsenazo-III by conventional double-wavelength technique at 654/690 nm as described earlier [1]. Mitochondrial swelling was studied based on light absorbance at 525 nm [1,2]. Oxygen consumption was studied polarographically, according to conventional protocol, in 1 ml of standard incubation medium in a closed cell with a platinum electrode at 26°C.

Cytochrome c was inactivated by heating at 90C. Inactivation was proven polarographically by the inability of inactivated protein to restore respiration inhibited by cytochrome c loss.

All reagents were from Sigma. The data represent mean ± S.D. Paired Students t-test was used for estimation of significance; minimum significance level was $P<0.05$.

RESULTS AND DISCUSSION

Membrane permeable compound DCFHDA is widely used as a probe on ROS formation in cells and organelles [23]. In mitochondria it is readily deacetylated to form membrane-impermeable non-fluorescent derivative, H_2DCF which is then further oxidized to highly fluorescent dichlorofluorescein (DCF) by mitochondrial ROS. To be ascertained that all fluorescent signal detected in the course of Ca^{2+}-uptake comes from inside the mitochondria the experiment was carried out in parallel in mitochondria preloaded with DCFHDA and in the course of DCFHDA loading together with Ca^{2+}-uptake.

The time dependence of ROS formation in mitochondria induced by Ca^{2+}-uptake is shown on fig.1. In mitochondria preloaded with the probe DCF fluorescence rises immediately after the beginning of Ca^{2+}-uptake. The time dependence of the change in DCF fluorescence due to ROS formation is close to that of Ca^{2+}-uptake (fig. 1A, 1,2). On the contrary, under conditions of DCFHDA loading together with Ca^{2+}-uptake, the fluorescence rises only

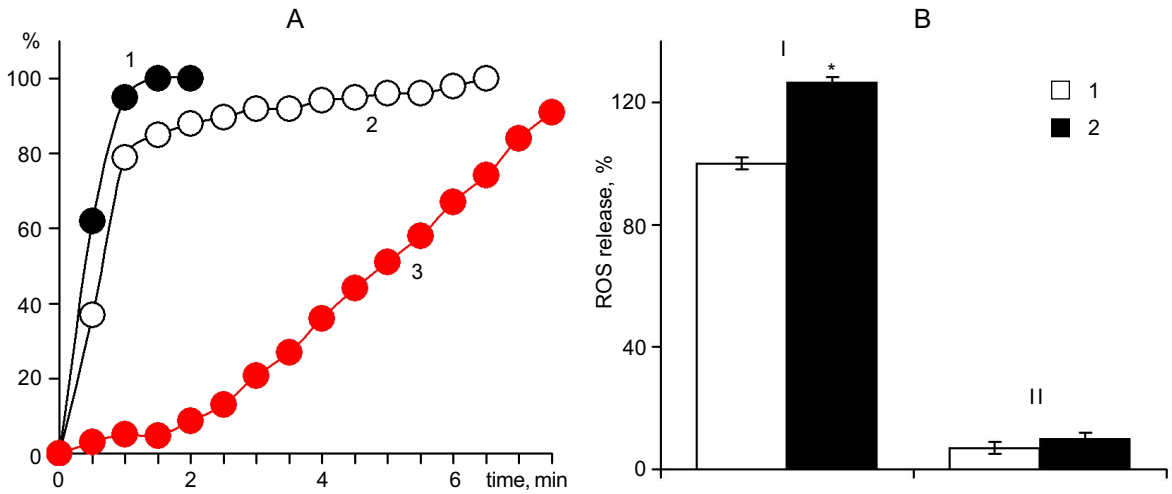

Fig. 1. The time dependence of Ca^{2+}-uptake (A, 1) and DCF fluorescence (A, 2-3) in mitochondria in cases of closed (B, 1) and open (B, 2) state of mitochondrial pore. Mitochondria were preloaded with the probe (A, 2; B, I) or loaded simultaneously with Ca^{2+}-uptake (A, 3; B, II). In case B, fluorescence was determined after 1 min incubation of mitochondria. Incubation medium: 120 mM KCl, 1 mM KH_2PO_4, 4 mM of sodium glutamate, 20 mM Tris-HCl, 30 μM Ca^{2+} buffer (pH 7.4). Cyclosporine A was added at 1 μM (A; B, 1). In case of DCFHDA loading in the course of Ca^{2+}-uptake, 20 μM of the probe was added into incubation medium. M±m, n=4; circles represent M±m (A); * $P<0.05$ relative to control (B, I, 1). On the abscissa: time, min (A); on the ordinate: percent change of parameters (A); % change in fluorescence (B)

after prolonged lag-phase (fig. 1A, 3). According to the data, over time of DCFHDA loading DCF concentration in mitochondria is too low to give a detectable signal (fig. 1A, 3), in spite of the observation that most part of ROS production takes place in the course of Ca^{2+}-uptake (fig. 1A, 1,2) and ROS formation in mitochondria due to Ca^{2+}-uptake is completed long before the rise in fluorescence signal in case of probe loading together with added Ca^{2+} (fig. 1A, 2,3). Thus, the rate of fluorescence increase is limited by the rate of DCFHDA loading, and reflects kinetics of the probe loading in mitochondria. The data obtained show that in spite of high concentration of DCFDA in the medium, all fluorescence registered in the course of Ca^{2+} accumulation is due to DCF formation within mitochondria (fig. 1A, 1,2; 1B, I). The probe outside the mitochondria does not give any detectable signal, at least within the timeframes of the experiment (fig. 1A, 3; 1B, II). This observation remains valid, independent of the open/closed state of the mitochondrial pore (fig. 1B, I-II, 1,2) or the presence of cytochrome c in the incubation medium. Thus DCFHDA under conditions of the experiment is a reliable probe on ROS formation in the matrix, inside the mitochondria, and supposed oxidation of this probe by cytochrome c outside the mitochondria [6] would not give detectable signal interfering with mitochondrial ROS production.

The time dependences of the change in mitochondrial matrix volume and ROS production, as well as matrix Ca^{2+}-retention, and the differences in the rates of oxygen consumption and ROS production, dependent on the open/closed state of MPTP at different amounts of added calcium, are shown on fig. 2. As show the data, MPTP opening (in the absence of cyclosporine A) leads to the matrix swelling (fig. 2A, 2), the increase in ROS production (fig. 2B, 1), decrease in Ca^{2+}-uptake and Ca^{2+}-retention capacity (fig. 2C, 2,3) as well as the increase in the rate differences of oxygen consumption and ROS production in metabolic state 2, dependent on the presence or the absence of cyclosporine A (fig. 2D). According to data, MPTP activation by the increasing amounts of added calcium results in the increase in MPTP contribution to oxygen consumption and ROS production (fig. 2D), in spite of the diminishing in Ca^{2+}-retention capacity and the amount of matrix Ca^{2+} (fig. 2C, 2,3).

When pore opening is blocked by cyclosporine A, all added Ca^{2+} is taken up by mitochondria (fig. 2C, 1). In the absence of the MPTP blocker, Ca^{2+} which is initially sequestered by mitochondria, is then released through mitochondrial pore (fig. 2 C, 2,3) and MPTP opening results in the decrease of Ca^{2+} content in mitochondria. The greater is the amount of Ca^{2+} taken up by mitochondria, the faster is its release and the less Ca^{2+} remains in the matrix due to MPTP activation. With the increase in the amount of added Ca^{2+} in the medium, progressive decrease in matrix of Ca^{2+} content is observed (fig. 2C, 2,3). However, in spite of the less matrix calcium, MPTP activation is accompanied by the increase in mitochondrial ROS production (fig. 2 B,D, 1) and oxygen consumption rate (fig. 2D, 2).

To explain such reverse dependence between the Ca^{2+}-uptake and matrix Ca^{2+} content, on the one hand, and matrix Ca^{2+} and ROS formation, on the other, high proton conductance of the mitochondrial pore should be taken into consideration which facilitates Ca^{2+}-release and would account for the increase in oxygen consumption rate in rat liver mitochondria (fig. 2D, 2). The background for observed increase in respiration rate is the increase in proton conductance due to MPTP opening which, as we have shown earlier [1], leads to the activation of cyclosporine A-sensitive Ca^{2+}/H^{+}-exchange and the increase in the rate of Ca^{2+}-cycling. Main components of Ca^{2+}-cycling under MPTP opening are potential-dependent Ca^{2+}-uptake through the Ca^{2+}-uniporter, accompanied by oxygen consumption in a stoichiometric proportion, and cyclo-

sporine A-sensitive release of Ca^{2+}, in exchange for cyclosporine A-sensitive proton influx into matrix space [1]. After the efflux of Ca^{2+} from the matrix under steady-state conditions Ca^{2+}-uptake is continually equilibrated with Ca^{2+}-efflux in the course of Ca^{2+}-cycling, which could be reversed by the addition of cyclosporine A [2]. Acceleration of the transmembrane exchange of Ca^{2+} results in the increase in the respiration rate and electron transport, consistent with the increase in ROS production in mitochondria (fig. 2B,D, 1).

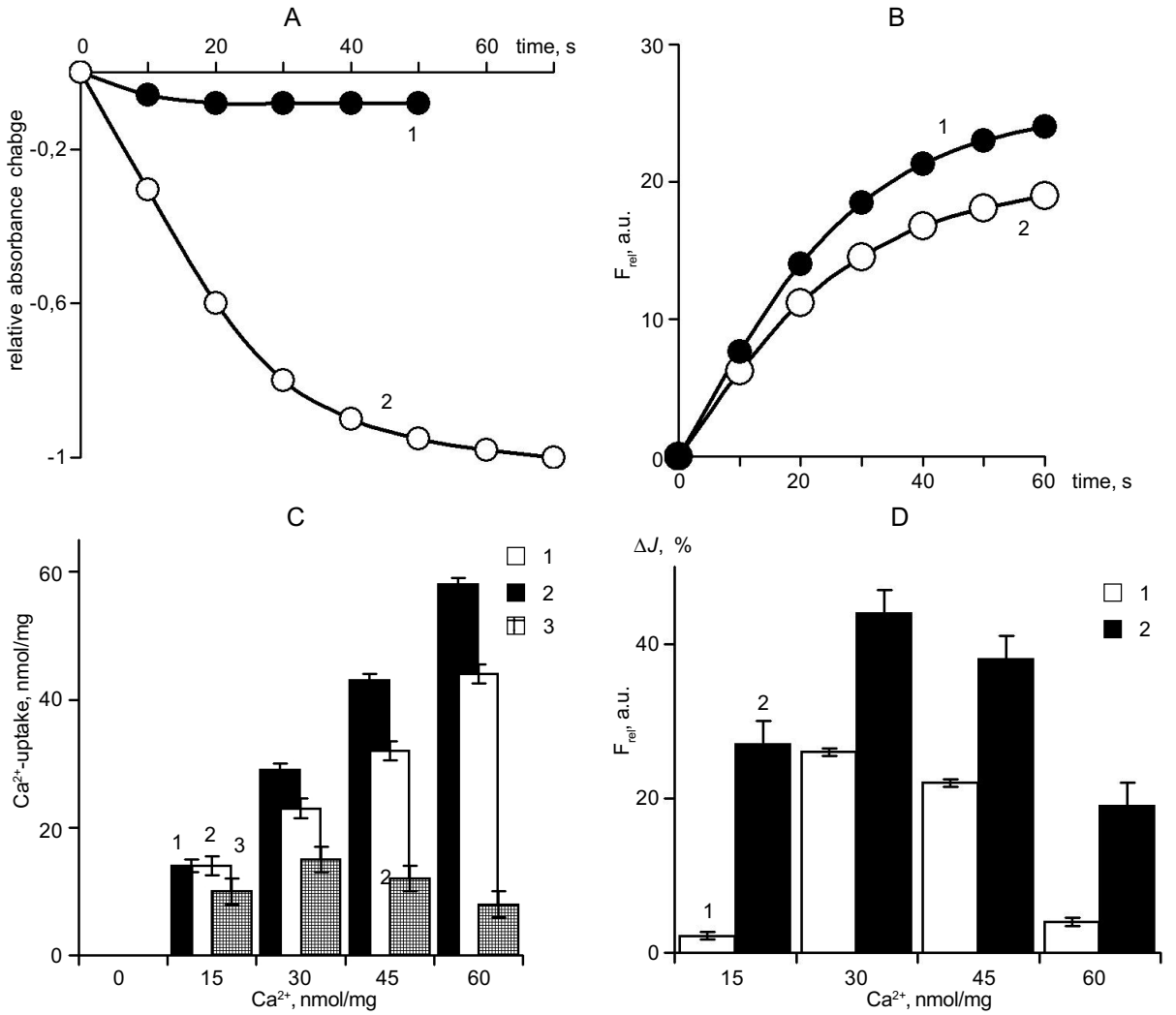

Fig. 2. The influence of MPTP opening in mitochondria on mitochondrial volume (A, 2), DCF fluorescence (B, 1) and Ca^{2+}-uptake (C, 2,3). Incubation medium: 120 mM KCl, 1 mM KH_2PO_4, 4 mM of sodium glutamate, 20 mM Tris-HCl buffer (pH 7.4). Cyclosporine A was added at 1μM (A, 1; B, 2; C, 1). Relative fluorescence, F_{rel}, is the differences between fluorescence at different times and initial fluorescence values at zero time. Ca^{2+}-uptake in mitochondria was determined in the presence of cyclosporine A (C, 1) or in the absence of cyclosporine A after 1 and 3 min incubation (C, 2,3). MPTP contribution to the rates of ROS production and respiration, ΔJ, was estimated as the rate differences in the absence and the presence of cyclosporine A, in % of maximum values (D, 1,2). $CaCl_2$ was added into incubation medium at 30 μM (A,B) or at concentrations shown on the abscissa (C,D). M±m, n=6, P<0.05 relative to control: Ca^{2+}-uptake in the presence of cyclosporine A (C,1) and zero rate differences in the presence of cyclosporine A (D).
On the abscissa: time, s (A,B), amount of added Ca^{2+} in nmol/mg protein (C,D). On the ordinate: relative absorbance change at 520 nm (A); relative fluorescence, in arbitrary units (B); Ca^{2+}-uptake, in nmol/mg protein (C); rate differences, ΔJ, in % of maximum (D)

It is well known that charge transfer across mitochondrial membrane is in a stoichiometric proportion with the electron transport and oxygen consumption in the respiratory chain [3]. Then, the increase in the rate of transmembrane Ca^{2+}-exchange due to MPTP opening would result in the increase in the rates of one- as well as two-electron reduction of oxygen. Thus, the rate of ROS production would be proportional to the rate of oxygen consumption, and the increase in the rate of Ca^{2+}-cycling would result in the increase in ROS production, both sensitive to cyclosporine A, in spite of the decrease in matrix Ca^{2+}-content in mitochondria. The data obtained show the increase in ROS production with the increase in Ca^{2+}-uptake either in presence or in the absence of cyclosporine A (fig. 2B). Under MPTP opening ROS production in rat liver mitochondria is higher than in the case of MPTP blockage (fig. 2B, 1). It well correlates with the increase in electron transport due to the acceleration of Ca^{2+}-cycling with MPTP opening and suggests a direct proportionality between the rate of ROS formation and the rate of electron flow under experimental conditions.

Increase in ROS production induced by Ca^{2+}-, as well as K^{+}-uptake, was observed by many authors [5,8] but has not received a satisfactory explanation. A decrease in ROS formation would rather be expected under conditions of acceleration of Ca^{2+}- (and proton) cycling after MPTP opening which is close to the phenomenon of so-called “mild uncoupling” of respiratory chain [4]. As it was shown in the literature, the increase in ROS release often correlates either with high proton potential [15] or with the inhibition of electron flow [18,21] and, in both cases, with more reduced state of the sites responsible for taking up the electrons from free radicals.

As a plausible explanation of the rise in ROS production after Ca^{2+}-uptake and MPTP opening which was observed independently by many authors [5], we assume that amount of ROS released from mitochondria would be not only under control of the redox-state of the sites and species that accept and donate electrons [18], but possibly, under kinetic control dependent on the equilibrium between the rates of ROS formation and scavenging. It means that under certain conditions the rate of ROS formation should be much faster than that of ROS removal. Thus, observed increase in ROS release after MPTP opening would be a direct result of the increase in the rate of electron transport in the respiratory chain due to activation of Ca^{2+}-cycling under MPTP opening. Increase in electron transport rate, in turn, would limit the rate of ROS production due to the increase in the rate of electron transfer in the respiratory chain. Consequently, linear increase in time of oxidized fluorescent product, DCF, is observed, in accord with constant rate of ROS formation in the course of substrate oxidation and oxygen reduction (fig. 3A).

However, relative to moderate increase in ROS release due to MPTP opening alone (fig. 3B, 1), dramatic increase in ROS production in the presence of exogenous cytochrome *c* in the incubation medium is observed (fig. 3B, 2). The data obtained first 10 min from the beginning of Ca^{2+}-uptake show, with a reliable approximation, a linear in time increase in DCF formation both in the absence and the presence of cytochrome c (fig. 3A, 1,2), and the transition to steep exponential time dependence of ROS production in case of MPTP opening in the presence of cytochrome c (fig. 3B, 2). In this last case the rate of ROS formation in mitochondria with cytochrome c added into incubation medium far exceeds that of mitochondria without addition of cytochrome c (fig. 3C, bars 2,4).

It is known from the literature [6] that DCFDA in alkaline medium could undergo deacetylation with following direct oxidation by cytochrome c to DCF. Sharp increase in ROS production which is observed in the presence of cytochrome c in the incubation medium only under MPTP opening, however, could not be explained by a direct oxidation

of the probe by cytochrome c, because both of them are separated by the mitochondrial membrane which serves as a barrier to prevent the direct contact of cytochrome *c* with the probe inside the mitochondria. Moreover, it should be taken into account that presence of cytochrome *c* in the medium in case of MPTP blockage does not result in such steep rise of DCF fluorescence (fig. 3A, 2) which would also suggest another mechanism of DCF formation than direct oxidation of the probe by cytochrome *c*.

There is sufficient amount of evidence [14] that cytochrome *c* could behave as an effective antioxidant taking up one electron from superoxide and thus scavenging superoxide-anion. It should be pointed out, however, that not only superoxide but large amounts of hydroperoxide and other ROS are released from mitochondria [21], and these ROS products also would interact with cytochrome *c*. An interaction of released ROS with cytochrome *c* by a mechanism of Fenton reaction type would result not only in novel ROS formation but possibly in the amplification of ROS products.

Both forms of a heme protein cytochrome *c*, that is oxidized (ferricytochrome) and reduced (ferrocytochrome), could interact with ROS in the reactions of Fenton or Haber-Weiss-type [11]:

$$\cdot O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow \cdot OH + OH^- + Fe^{3+} \quad (2)$$

$$2\cdot O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

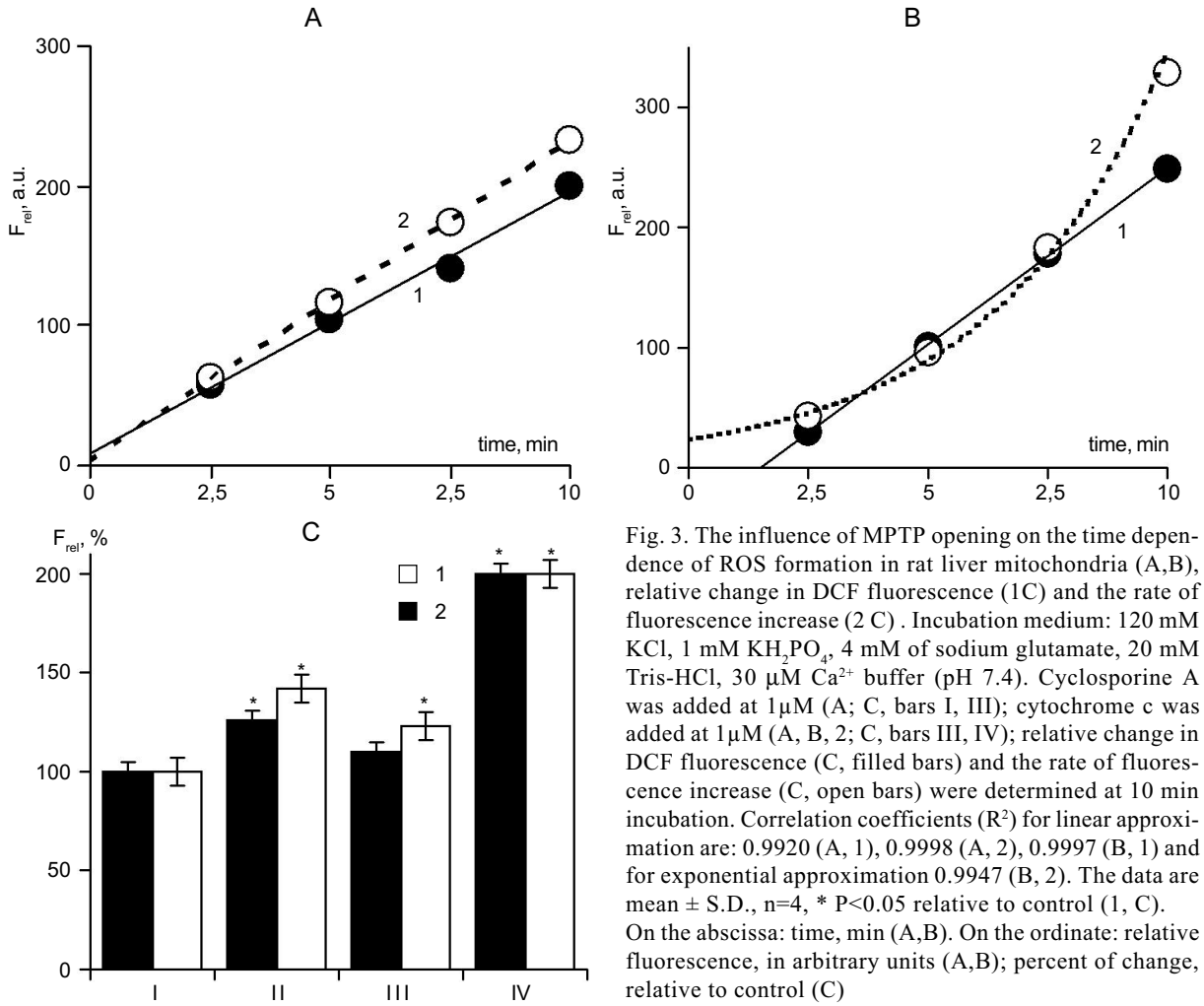

Fig. 3. The influence of MPTP opening on the time dependence of ROS formation in rat liver mitochondria (A,B), relative change in DCF fluorescence (1C) and the rate of fluorescence increase (2 C) . Incubation medium: 120 mM KCl, 1 mM KH_2PO_4, 4 mM of sodium glutamate, 20 mM Tris-HCl, 30 μM Ca^{2+} buffer (pH 7.4). Cyclosporine A was added at 1μM (A; C, bars I, III); cytochrome c was added at 1μM (A, B, 2; C, bars III, IV); relative change in DCF fluorescence (C, filled bars) and the rate of fluorescence increase (C, open bars) were determined at 10 min incubation. Correlation coefficients (R^2) for linear approximation are: 0.9920 (A, 1), 0.9998 (A, 2), 0.9997 (B, 1) and for exponential approximation 0.9947 (B, 2). The data are mean ± S.D., n=4, * $P<0.05$ relative to control (1, C). On the abscissa: time, min (A,B). On the ordinate: relative fluorescence, in arbitrary units (A,B); percent of change, relative to control (C)

Ferricytochrome could accept one electron from superoxide (1) and thus behave itself as a scavenger of superoxide and an effective antioxidant [14]. But on the other hand, reduced form of cytochrome c, ferrocytochrome, could react with hydoperoxide (2) which in large quantities is released from mitochondria [21]. Cytochrome *c* which is reduced in the reaction with superoxide (1) could then interact with hydroperoxide (2) resulting in the formation of highly toxic hydroxyl-radical. In the course of this last reaction heme iron of cytochrome c would undergo transition to more oxidized form which, in turn, could again take up electrons from superoxide, after which ferrocytochrome could once again interact with hydroperoxide. It is worth mentioning that a large quantity of hydroperoxide, the main source of hydroxyl-radical, is supplied by superoxide itself which would be readily disproportionated in the relatively (to the matrix) acidic milieu of the intermembrane space (3). Thus, in such interactions with ROS which in large quantities are released from mitochondria, cytochrome c would behave as a catalyst which itself undergoes repeated transitions from oxidized to reduced form, being both a scavenger of superoxide and a potent generator of ROS. It should be expected that total amount of ROS released in the course of such Fe-cycling would depend on a complex equilibrium between redox states of the heme iron of cytochrome *c*, the quantity of individual free radicals, and the rate of their release from mitochondrial matrix. Most probably, that proposed catalytic Fe-cycling mechanism would result not only in novel ROS formation but possibly in the amplification of ROS products.

To explain observed rise in matrix ROS formation induced by cytochrome *c* outside the matrix space, we propose that mitochondrial pore would facilitate ROS exchange between the matrix and the medium. ROS generated in the matrix would be at first released through the pore into intermembrane space where ROS metabolism could be manifold activated by Fe-complex of cytochrome c which would act as a catalyst in the amplification of ROS production by Fenton-type reactions. In turn, even transient increase in ROS concentration would lead to their flow back into matrix space through the same mitochondrial pore which is in its open state and would increase the level of ROS inside the mitochondria. It worth mentioning that catalase added to incubation medium was not able to prevent the rise in ROS production, thus, free-radical reactions, resulting in ROS production are localized to the matrix and intermembrane space, but not to the bulk solution. Thus, we suppose that apart of its conventional function, cytochrome *c* could serve in such a way as an amplifier of ROS production in mitochondria which would provide a background for the development of apoptosis induced by MPTP opening.

CONCLUSIONS:

1. Exogenous cytochrome c could serve as an amplifier of ROS production in mitochondrial matrix in a way sensitive to MPTP opening.

2. Such amplification of ROS production takes place only under conditions of ROS release from mitochondria through mitochondrial pore to interact with cytochrome *c* outside the matrix space.

3. Heme iron complex of cytochrome *c* would act as a catalyst in amplification of ROS production by Fenton-type reactions.

4. Increase in the ROS concentration outside the matrix, even transient, would result in their flow back to the matrix, increase in probe oxidation and DCF fluorescence inside the mitochondria.

5. MPTP opening is necessary for ROS exchange between the mitochondrial matrix and the heme iron of cytochrome *c*. If MPTP is closed, cytochrome *c* would not significantly increase ROS production in mitochondria. Thus, exogenous cytochrome *c* might serve as potent amplifier of ROS signaling between

mitochondria in several pathological states accompanied by MPTP opening.

О. В. Акопова, Л.И. Колчинская, В.И. Носарь, В.А. Бурый, И.Н. Маньковская, В.Ф. Сагач

ЦИТОХРОМ С КАК УСИЛИТЕЛЬ ГЕНЕРАЦИИ АКТИВНЫХ ФОРМ КИСЛОРОДА В МИТОХОНДРИЯХ

Изучено влияние экзогенного цитохрома *c* на образование активных форм кислорода (АФК) под действием Ca^{2+} в митохондриях печени крыс в условиях открывания и блокирования митохондриальной поры (МП). О генерации АФК судили по изменению флуоресценции дихлорофлуоресцеина (ДХФ). Показано, что активация МП приводит к повышению потребления кислорода и продукции АФК, несмотря на снижение содержания Ca^{2+} в матриксе с повышением концентрации катионов в среде инкубации. В присутствии цитохрома *c* в инкубационной среде происходит прогрессирующее возрастание количества АФК, чувствительное к открыванию поры. Показано, что наблюдаемое резкое нарастание флуоресценции ДХФ соответствует генерации АФК в матриксе и не вызвано непосредственным взаимодействием цитохрома *c* с зондом. В условиях подавления МП присутствие цитохрома с в инкубационной среде не влияет существенным образом на содержании АФК в митохондриях. Полученные результаты свидетельствуют, что наблюдаемое влияние экзогенного цитохрома с на продукцию АФК обусловлено открыванием МП. Предполагается, что в условиях открывания поры цитохром *c* играет роль катализатора в реакциях превращения АФК, высвобождающихся из митохондриального матрикса, причем обмен свободнорадикальных продуктов между матриксом и средой осуществляется через пору, находящуюся в открытом состоянии. Подобный механизм амплификации продуктов превращения кислорода с участием гемового железа цитохрома *c* и образованием токсичных свободнорадикальных производных может создавать благоприятные предпосылки индукции клеточного апоптоза и развития окислительного стресса вследствие избыточной генерации АФК.

Ключевые слова: митохондрии, Ca^{2+}, митохондриальная пора, активные формы кислорода, цитохром с.

О.В. Акопова, Л.І. Колчинська, В.І. Носар, В.А. Бурий, І.М. Маньковська, В.Ф. Сагач

ЦИТОХРОМ С ПОСИЛЮЄ ГЕНЕРАЦІЮ АКТИВНИХ ФОРМ КИСНЮ У МІТОХОНДРІЯХ

Досліджено вплив екзогенного цитохрому с на утворення активних форм кисню (АФК) під дією Ca^{2+} у мітохондріях печінки щурів за умов відкриття та блокування мітохондріальної пори (МП). Утворення АФК реєстрували за зміною флуоресценції дихлорофлуоресцеїну (ДХФ). Показано, що активація МП призводить до підвищення споживання кисню та продукції АФК, попри зниження вмісту Ca^{2+} у мітохондріях із збільшенням концентрації катіонів у середовищі інкубації. За наявності екзогенного цитохрому *c* в інкубаційному середовищі відбувається стрімке підвищення продукції АФК, яка є чутливою до відкриття пори. За умов блокування останньої цитохром *c* суттєво не впливає на утворення АФК. Показано, що флуоресцентний сигнал відповідає генерації АФК у мітохондріальному матриксі, і зростання флуоресценції ДХФ не зумовлене безпосередньою взаємодією зонду з цитохромом *c*. Одержані результати свідчать, що вплив екзогенного цитохрому *c* на продукцію АФК у мітохондріях спричинений відкриттям МП. Запропоновано механізм, згідно з яким цитохром с може відігравати роль каталізатора у реакціях перетворень АФК, що вивільнюються з мітохондрій, причому обмін вільнорадикальних продуктів між матриксом і середовищем відбувається за посередництвом пори, яка знаходиться у відкритому стані. Подібний механізм ампліфікації продуктів перетворення кисню за участі гемового заліза цитохрому *c* з утворенням токсичних вільнорадикальних похідних може створювати сприятливі передумови для подальшої індукції клітинного апоптозу і розвитку оксидативного стресу внаслідок надлишкової продукції АФК.

Ключові слова: мітохондрії, Ca^{2+}, мітохондріальна пора, активні форми кисню, цитохром *c*.

Ин-т физиологии им. А.А.Богомольца НАН Украины, Киев

REFERENCES

1. Akopova O.V. The role of permeability transition pore in transmembrane Ca^{2+}-exchange in mitochondria //Ukr. Biochim. J. – 2008. – **80**, №3. – P.40-47.
2. Akopova O.V. The influence of ATP-dependent K^{+}-channel opener on the opening of mitochondrial permeability transition pore in rat liver mitochondria // Ibid. – 2011. – **83**, №3. – P.37-47
3. Beavis A.D. Upper and lower limits of the charge translocation stoichiometry of mitochondrial electron transport //J. Biol. Chem. – 1987. – **262**, № 13. – P. 6165-6173.
4. Brand M.D., Affourtit Ch., Esteves T., Green K., Lambert A.J., Miwa S., Pakay J., Parker N. Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins //Free Radical Biol. Med. – 2004. – **37**, №6. – P.755-767.
5. Brookes P.S., Yoon Y., Robothham J.L., Anders M.W., Sheu Sh.-Sh. Calcium, ATP, and ROS: a mitochondrial love-hate triangle //Amer. J. Physiol. – 2004. – **287** – P.C817-C833.
6. Burkitt M.J., Wardman P. Cytochrome c is a potent catalyst of dichlorofluorescein oxidation: implications for the role of reactive oxygen species in apoptosis //

Biochem. Biophys. Res. Commun. – 2001. – **282** – P.329-333.
7. Cai J., Jones D.P. Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome c loss // J. Biol. Chem. – 1998. – **273**. – P.11401-11404.
8. Costa D.T.A., Quinlan C.L., Andrukhiv A., West I.C., Jaburek M., Garlid K.D. The direct physiological effects of $mito_{KATP}$ opening on heart mitochondria //Amer. J. Physiol. – 2006. – **290**. – P. H406-H415.
9. Gogvadze V., Robertson J.D., Enoksson M., Zhivotovsky B., Orrhenius S. Mitochondrial cytochrome c release may occur by volume-dependent mechanisms not involving permeability transition //Biochem. J. – 2004. – **378** – P.213-217.
10. Hackenbrock C.R. Chemical and physical fixation of isolated mitochondria in low-energy and high-energy states //Proc. Natl. Acad. Sci. USA. – 1968. – **61**. – P.598-605.
11. Halliwell B. Oxidants and human disease: some new concepts //FASEB J. – 1987. – **1**, №5. – P.358-364.
12. Ichas F., Jouaville L.S., Mazat J.-P. Mitochondria are excitable organelles capable of generating and conveying electrical and calcium signals //Cell. – 1997. – **89**. – P.1145-1153.
13. Jacobs E.E., Sanadi D.R. Phosphorylation couples to electron transport mediated by high potential electron carriers //Biochim. Biophys. Acta. – 1960. – **38**. – P.12-33.
14. Korshunov S.S., Krasnikov B.F., Pereverzev M.O., Skulachev V.P. The antioxidant functions of cytochrome *c* //FEBS Lett. – 1999. – **462**. – P.192-198.
15. Korshunov S.S., Skulachev V.P., Starkov A.A. High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondia //FEBS Lett. – 1997. – **416** – P.15-18.
16. Kroemer G., Petit P., Zamzami N., Vayssiere J.-L., Mignotte B. The biochemistry of programmed cell death //FASEB J. – 1995. – **9**. – P. 1277-1287.
17. Knowles M.K., Guenza M.G., Capaldi R.A., Marcus A.H. Cytoskeletal-assisted dynamics of the mitochondrial reticulum in living cells //Proc. Natl. Acad. Sci. USA. – 2002. – **99**. – P.14772-14777.
18. Kushnareva Y., Murphy A.N., Andreyev A. Complex I-mediated reactive oxygen species generation: modulation by cytochrome c and $NAD(P)^+$ oxidation-reduction state //Biochem. J. – 2002. – **368**. – P.545-553.
19. Lander H.M. An essential role for free radicals in signal transduction //FASEB J. – 1997. – **11**. – P.118-124.
20. Pletjushkina O.Yu., Fetisova E.K., Lyamzaev K.G., Ivanova O.Yu., Domnina L.V., Vysokih M.Yu., Pustovidko A.V., Alekseevsky A.V., Alekseevsky D.A., Vasilyev Yu.M., Murphy M.P., Chernyak B.V., Skulachev V.P. Hydrogen peroxide produced inside mitochondria takes part in cell-to-cell transmission of apoptogenic signal //Biochemistry (Moscow). – 2006. – **71**, N1. – P.75-84.
21. Starkov A.A., Polster B.M., Fiskum G. Regulation of hydrogen peroxide production by calcium and Bax //J. Neurochem. – 2002. – **83**. – P.220-228.
22. Zorov D.B., Juhaszova M., Sollott S. Mitochondrial ROS-induced ROS release: an update and review // Biochim. Biophys. Acta. – 2006. – **1757**. – P.509-517.
23. Zorov D.B., Kobrinsky E., Juhaszova M., Sollott S. Examining intracellular organelle function using fluorescent probes //Circulat. Res. – 2004. – **95**. – P.239-252.

O.O. Bogomolets Institute of Physiology, National Academy of Sciences of Ukraine, Kyiv
E-mail: luko@biph.kiev.ua

Received 12.09.2011