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THE EFFECT OF PERMEABILITY TRANSITION PORE OPENING ON REACTIVE OXYGEN SPECIES PRODUCTION IN RAT BRAIN MITOCHONDRIA

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The influence of mitochondrial permeability transition pore (MPTP) opening on reactive oxygen species (ROS) production in the rat brain mitochondria was studied. It was shown that ROS production is regulated differently by the rate of oxygen consumption and membrane potential, dependent on steady-state or non-equilibrium conditions. Under steady-state conditions, at constant rate of Ca^{2+}-cycling and oxygen consumption, ROS production is potential-dependent and decreases with the inhibition of respiration and mitochondrial depolarization. The constant rate of ROS release is in accord with proportional dependence of the rate of ROS formation on that of oxygen consumption. On the contrary, transition to non-equilibrium state, due to the release of cytochrome c from mitochondria and progressive respiration inhibition, results in the loss of proportionality in the rate of ROS production on the rate of respiration and an exponential rise of ROS production with time, independent of membrane potential. Independent of steady-state or non-equilibrium conditions, the rate of ROS formation is controlled by the rate of potential-dependent uptake of Ca^{2+} which is the rate-limiting step in ROS production. It was shown that MPTP opening differently regulates ROS production, dependent on Ca^{2+} concentration. At low calcium MPTP opening results in the decrease in ROS production because of partial mitochondrial depolarization, in spite of sustained increase in oxygen consumption rate by a cyclosporine A-sensitive component due to simultaneous work of Ca^{2+}-uniporter and MPTP as Ca^{2+}-influx and efflux pathways. The effect of MPTP opening at low Ca^{2+} concentrations is similar to that of Ca^{2+}-ionophore, A-23187. At high calcium MPTP opening results in the increase of ROS release due to the rapid transition to non-equilibrium state because of cytochrome c loss and progressive gating of electron flow in respiratory chain. Thus, under physiological conditions MPTP opening at low intracellular calcium could attenuate oxidative damage and the impairment of neuronal functions by diminishing ROS formation in mitochondria.

K e y w o r d s: reactive oxygen species, mitochondrial permeability transition pore, calcium, brain mitochondria.

Mitochondria are the main consumers of cellular oxygen and the main producents of reactive oxygen species (ROS) in the cell [1–3]. Under physiological conditions hydroperoxide (H_2O_2) jointly with free-radicals (superoxide-anion, O_2^{-}, hydroxyl radical, $^{\bullet}OH$, and other highly reactive species) are constantly released as the products of incomplete, one-electron reduction of oxygen at the intermediate stages of several redox-reactions in the electron transport chain [2].

The most part of ROS production is localized to complexes I and III which comprise several putative sites of ROS formation [1–3]. Flavins or flavin-binding site, iron-sulfur clusters, ubiquinone-binding site within complex I, and ubisemiquinone, Rieske iron-sulfur protein and cytochromes *b* and c_1 of complex III are considered as main sites of ROS release [3]. Relative contribution of these sites to the rate of ROS formation and total ROS production depends to the great extent on the experimental conditions and mitochondrial metabolic state, and also is tissue-specific [1]. Recent studies on the topology of ROS formation in mitochondria revealed that ROS, produced at complex I are released to the matrix, whereas ROS, generated in complex III of respiratory chain, are released on both sides of the inner membrane: towards the matrix and towards the intermembrane space from the inner (Q_i) and outer (Q_o) ubisemiquinone binding sites [4].

Primarily ROS are released as superoxide, O_2^{-}, which is readily decomposed by matrix superoxide dismutase, MnSOD, to form hydroperoxide, H_2O_2. Hydroperoxide is relatively stable and thus long-living, and could easily diffuse from the matrix to cytosol and cellular compartments [2, 4, 5]. Potential danger from hydroperoxide comes from its decomposition, catalysed in the matrix, as well as outside the mitochondria, by transition metals,

primarily most abundant Fe and Cu (which are known as Fenton and Haber-Weiss reactions), to form highly reactive and thus short-living toxic hydroxyl radical [5]. Thus, in spite of the very short life-time, ˙OH-radical would release instantly, sometimes far from the site of H_2O_2 formation, and strike several cellular targets outside the mitochondria.

Regulatory mechanisms of ROS release in mitochondria are widely discussed in the literature. In accord with contemporary views, ROS production in these organelles is controlled by the redox-state (which is the ratio of oxidized to reduced forms) of the sites which take part in the electron transport. Thus, since the one-electron reduction of oxygen requires −160 mV redox-potential, the reduced state of the sites capable for one-electron reduction of oxygen within the complexes of respiratory chain creates energetically favorable conditions for ROS formation [3]. Also, the relative concentration ratio of the acceptors to the donors of electrons as well as the ratio of the rate of ROS formation to that of ROS removal in the course of redox-reactions would serve as kinetic control of the rate and the amount of ROS produced in mitochondria. Besides, ROS production, especially under physiological conditions, is attenuated by several matrix antioxidants, such as MnSOD and glutathione [3].

The above considerations could be illustrated by the known facts that mitochondrial hyperpolarization and highly reduced state of the complexes of respiratory chain [7] as well as inhibition of electron transfer between the complexes by respiratory poisons (such as antimycin A), or, for example, cytochrome *c* depletion from intermembrane space and gating the electron flow between complexes III and IV [8,9] lead to the excess in concentrations of electron donors over that of the acceptors. Relative deficit of electron acceptors results in the increase in the lifetime of intermediate free-radical species, which are the source for ROS formation, and favors the increase in ROS production [7]. Unlike this, partial membrane depolarization (it was shown that ~10 mV drop in mitochondrial membrane potential $\Delta\Psi_m$ was sufficient to abolish ROS overproduction [1,7]) with consequent increase in the concentration of electron acceptors because of more oxidized state of the complexes of respiratory chain, and relative matrix acidification which directly inhibits superoxide formation [1,10] are thought to be beneficial for cell survival and could explain the positive therapeutic effect of the phenomenon known as "mild uncoupling" in pathological states.

It is known that excessive ROS formation could promote the opening of mitochondrial permeability transition pore (MPTP) which might further enhance ROS production by way of so-called "ROS-induced ROS-release" [11] and cause oxidative damage to brain and other tissues. "Classical" MPTP opening, which is accompanied by matrix swelling, the loss of membrane potential and the impairment of energy metabolism, the rupture of outer membrane and the release of cytochrome c together with several proapoptotic factors into cytosol, is a background of many neural disorders and a key event in triggering apoptosis in neuronal cells [12]. Thus MPTP is often considered as a therapeutical target in fighting several diseases, and MPTP blockage, for example, was proven to be effective in the prevention of destructive consequences of brain and myocardial ischemia. Nevertheless, the exact role of MPTP in regulation of ROS production in mitochondria is not quite clear. The question becomes even more complicated, taking into account that MPTP could exist in several states with different conductance [13], dependent on Ca^{2+} concentration, and the transition between these states is not well defined. Besides, there are species- and tissue-specific differences in the regulatory mechanisms of ROS production in mitochondria. [1]. The aim of this work was to study the influence of MPTP opening on ROS formation in the rat brain mitochondria.

Materials and Methods

Rat brain was homogenized in a medium (1): 320 mM sucrose in 5 mM Tris-HCl buffer (pH 7.4), 1 mM EDTA and centrifuged at 1000 g × 10 min (4 °C). BSA was added in homogenization buffer at 1.0 mg/ml. Mitochondria were sedimented by centrifugation of supernatant at 12 000 g x 20 min (4 °C), suspended in a small volume of the medium (1) without EDTA and BSA and stored on ice until use. The experiments were carried out at room temperature in the standard incubation medium: 320 mM sucrose, 1 mM KH_2PO_4, 5 mM of sodium succinate or glutamate, 5 mM Tris-HCl buffer (pH 7.4).

The data on ROS formation were obtained from dichlorofluorescein (DCF) fluorescence. Mitochondria were loaded for 20 min at 37 °C with membrane permeable non-fluorescent probe, dichlorofluorescein dihydroacetate (DCFHDA) which is known to be decomposed in the matrix to give dichlorofluorescein upon oxidation by matrix ROS, primarily hydroperoxide and superoxide. Stock suspension loaded with the probe was stored on ice. Aliquots of mitochondrial suspension were sampled in 1 ml of standard incubation medium in 1 cm cuvette of spectrofluorimeter. DCF was excited at 504 nm, and the emission was registered at 525 nm [14].

Membrane potential was estimated spectrophotometrically with use of safranine by conventional double-wavelength technique at 510/525 nm [15]. Safranine was added to incubation medium at 10 μM.

Oxygen consumption was studied polarographically, in accord with conventional protocol, in 1 ml of standard incubation medium in a closed cell with a platinum electrode at 26 °C.

All reagents were obtained from Sigma. The data represent mean ± S.D. Paired Student's *t*-test was used for estimation of significance; minimum accepted level of significance was $P < 0.05$.

Results and Discussion

After calcium uptake an equilibrium state is established referred to in the literature as resting State 2 with constant rate of Ca^{2+}-cycling across mitochondrial membrane which is supported by Ca^{2+}-influx via Ca^{2+}-uniporter and Ca^{2+}-efflux via Ca^{2+}/H^{+}- or Na^{+}/Ca^{2+}-excange pathways [16]. The constant rate of Ca^{2+}-cycling corresponds to that of oxygen consumption in State 2 because of stoichiometric proportion between the rates of potential-dependent cation uptake and the electron transport, depending on the site of substrate oxidation in the respiratory chain [16, 17]. With use of glutamate as oxidation substrate and respiration inhibition with rotenone it was shown that under steady-state conditions respiration inhibition with following membrane depolarization, and the decrease in the rate of oxygen consumption and Ca^{2+}-uptake, results in the decrease in the rate of ROS production, which thus exhibits a dependence on membrane potential (Fig. 1, *A*). Thus, the inhibition of complex I by rotenone leads to the decrease in $\Delta\Psi_m$ as well as the decrease in steady-state rate of oxygen consumption (Fig. 1, *A*, *1*, *2*) which results in the decrease in the rate of ROS production (Fig. 1, *A*, 3; *1*, *B*, *1*).

When MPTP is closed under equilibrium conditions, the steady-state rate of oxygen consumption, $J_{O2} = d[O_2]/dt = \text{const}$ (Fig. 2, *A*, *1*), is accompanied by the steady-state rate of ROS formation (R_{ROS}), in accord with the constant rate of DCFHDA oxidation and linear in time increase in the amount of oxidized fluorescent product, DCF, in mitochondrial matrix:

$$[DCF](t) = R_{ROS} \cdot t + [DCF]_0,$$

where $[DCF]_0$ is the initial DCF concentration in the matrix space, (Fig. 2, *A*, *2*). Independent of MPTP opening, gradual loss of cytochrome *c* from intermembrane space [8, 9] leads to the progressive inhibition of respiration and the deviation from equilibrium state (Fig. 2, *B*, *2*, *C*, *1*). Linear time dependence of the rates of respiration

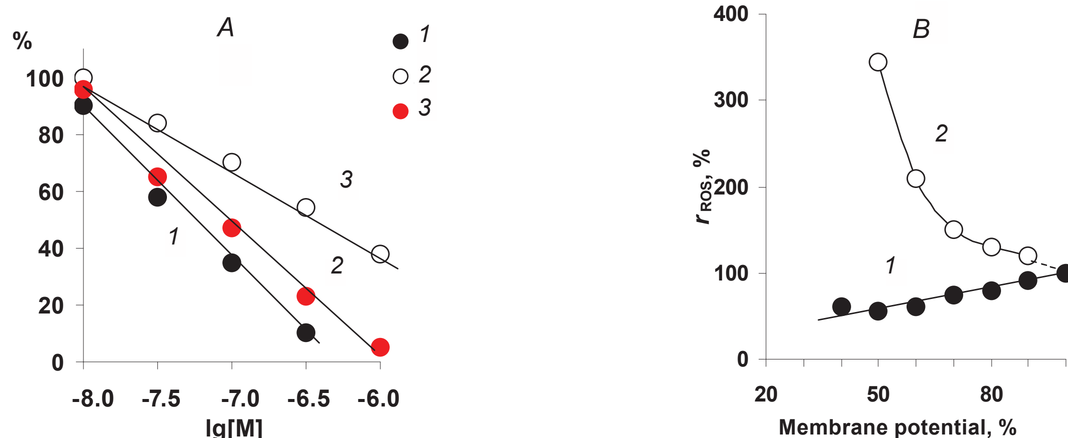

Fig. 1. The influence of membrane potential on the rate of ROS formation in the rat brain mitochondria. A: Dependence of membrane potential (1) and the rates of oxygen consumption (2) and ROS formation (3) on the concentration of rotenone. B: Dependence of the rate of ROS formation on membrane potential under steady-state (1) and non-equilibrium (2) conditions. Membrane potential was measured at different concentrations of rotenone (A, 2; B, 1) and at different times following progressive respiration inhibition (B, 2). Standard incubation medium: 320 mM sucrose, 1 mM KH_2PO_4, 5 mM of sodium glutamate, 15 μM $CaCl_2$, 1μM cyclosporine A, 5 mM Tris-HCl buffer (pH 7.4). Rotenone was added at concentrations shown on the abscissa (A). Membrane potential and the rates of oxygen consumption and ROS formation in the absence of rotenone under steady-state conditions were taken as 100% (A, B). M ± S.D., n = 3. On the abscissa axis: rotenone concentration, lg[M] (A); membrane potential in percents of maximum (B). On the ordinate axis: % change of parameters

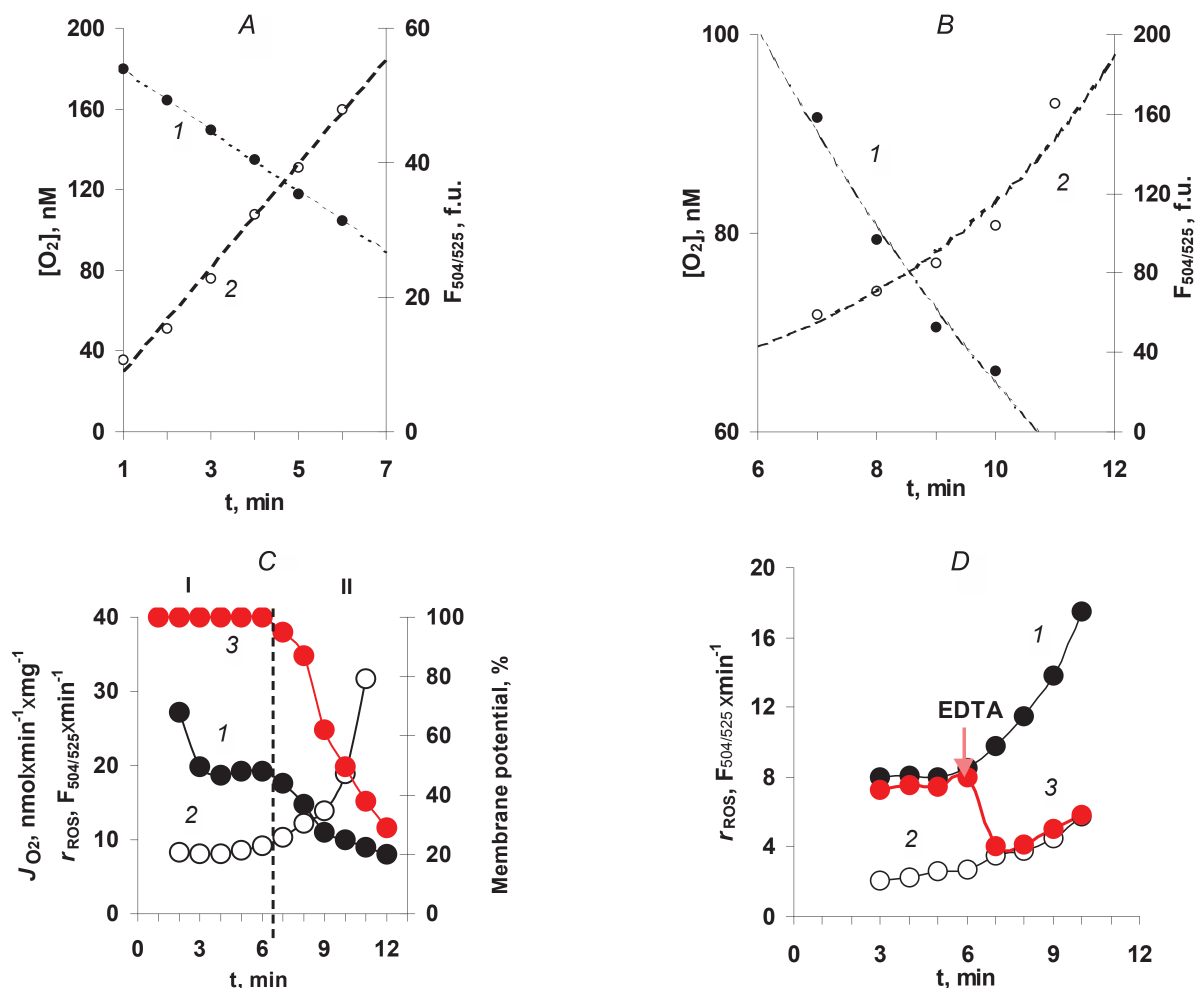

Fig. 2. The time dependence of the oxygen consumption and ROS formation in the rat brain mitochondria. A, B: The time dependence of the oxygen consumption (1) and ROS formation (2) under steady-state (A) and non-equilibrium (B) conditions. C: The time dependence of the rates of respiration (1), ROS formation (2) and membrane potential (3). The areas separated by dotted line (C) correspond to steady-state (I) and non-equilibrium (II) conditions. D: The influence of calcium on ROS production in mitochondria: the rates of ROS release in the presence of Ca^{2+} (1), in Ca-free medium (2) and after Ca^{2+} removal with EDTA (3). Incubation medium: 320 mM sucrose, 1 mM KH_2PO_4, 5 mM of sodium succinate, 5 mM Tris-HCl buffer (pH 7.4), 15 µM $CaCl_2$, 1 µM cyclosporine A, Ca^{2+}-ionophore, A-23187, and EDTA were added at concentrations 1 µM and 1 mM (D). M±S.D., n =6. Correlation coefficients, R^2, for linear approximation (A): $[O_2](t) = -15.1t + 194.8$ (1) and $F_{504/525}(t) = 7.64t + 1.32$ (2) are 0.9992 (1) and 0.9928 (2); for exponential approximation (B): $[O_2](t) = = 112{,}02e^{-0{,}11t}$ (1) and $F_{504/525}(t) = 33.9e^{0.25t}$ (2) are 0.9753 (1) and 0.9793 (2). On the abscissa axis: time, min. On the ordinate axis: oxygen concentration in the medium in nM (A, B, left axis), DCF concentration in mitochondria, in arbitrary fluorescence units (A,B, right axis), the rates of respiration in nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ and DCF formation, in fluorescence units, $F_{504/525} \cdot min^{-1}$ (C,D, left axis); membrane potential, % (C, right axis)

and ROS formation was restored by the addition of exogenous cytochrome *c* (data are not shown). Under non-equilibrium conditions of progressive gating the electron flow between the complexes III and IV of respiratory chain, respiration inhibition (Fig. 2, *B*, *C*, *1*) and membrane depolarization (Fig. 2, *C*, *3*), dependent on the presence of cytochrome *c* (data are not shown), an exponential rise in ROS formation is observed (Fig. 2, *B*, *2*), in accord with exponential increase in DCF concentration, $[DCF](t) = [DCF]_0e^{kt}$, and the decrease in oxygen consumption rate within the same time interval: $[O_2](t) = [O_2]_0e^{-kt}$ (Fig. 2, *B*, *1*, *2*).

There is much controversy in the literature concerning the influence of membrane depolarization on ROS production in mitochondria. In ac-

cord with the published [1, 7] and our own data (Fig. 1), a close dependence of ROS release on membrane potential is observed, with the decrease in ROS production following membrane depolarization, for example, in case of complex I inhibition by rotenone (Fig. 1, *B*, *1*). On the other hand, in case of cytochrome *c* depletion, gradual depolarization accompanied by the progressive inhibition of respiration together with sharp increase in ROS production was also observed by us (Fig. 2, *B*, *2*) and other authors [8, 9, 11]. In our opinion, these contradictoly facts could be, at least partially, explained, taking into account that precise sites in complexes I and III at which ROS formation preferably takes place, might differ because of the change in their redox-state, dependent on experimental conditions, possibly different in case of respiration inhibition under steady-state conditions and in the case of gradual gating the electron flow, which are far from the equilibrium state.

It is well known that the reaction order would be lowered by the excess concentrations of the reagents. Then, plausible explanation of the shift from linear to exponential time dependence of ROS formation, due to the loss of cytochrome *c* from mitochondria, and vice versa after the addition in excess of cytochrome *c*, is the change in the concentration ratio of the components of redox-reactions resulting in ROS production. Thus, the exponential decrease in concentration and the deficit of electron acceptors which arise following the loss of cytochrome *c*, would explain both the change in the reaction order of oxygen consumption (Fig. 2, *A*, *B*, *1*) and that of ROS formation which accounts for the simultaneous exponential rise in ROS production (Fig. 2, *A*, *B*, *2*), independent of membrane depolarization (Fig. 1, *B*, *2*). Consequently, depolarization might differently influence kinetics of ROS production (Fig. 1, *B*, *2*; Fig. 2, *B*), depending on the concentration of electron acceptors, capable to transfer the electrons in the respiratory chain. The gating of electron flow because of the deficit in electron acceptors, due to the loss of cytochrome *c*, possibly would favor the electron transfer from free-radical intermediates in the electron transport chain to oxygen with consequent increase in ROS formation. Addition in excess of exogenous cytochrome *c* restores the electron flow and the linearity in time-dependence of both oxygen consumption and ROS release in accord with null-order reactions kinetics (Fig. 2, *A*). Thus, a seeming contradiction between the experimental facts, concerning the influence of membrane depolarization on ROS formation, could be, at least partially, resolved, taking into account that the increase in concentration of electron acceptors with the addition of cytochrome *c* shifts the equilibrium in the redox-reactions involved in substrate oxidation towards the end product, i.e. complete two-electron reduction of oxygen, whereas depletion of mitochondria in cytochrome *c* interrupts the flow of the reaction at intermediate stages with resulting ROS formation.

Independent of equilibrium, or non-equilibrium, conditions, the rate of ROS formation exhibits a critical dependence on Ca^{2+}-cycling rate. Elimination of Ca^{2+}-cycling by the removal of Ca^{2+} with Ca^{2+}-chelators at any stage of free-radical reactions abolishes ROS overproduction and diminishes it to basal level observed in Ca^{2+}-free medium (Fig. 2, *D*, *1*, *2*). Slow DCF oxidation in the absence of Ca^{2+} in K^+-free medium (Fig. 2, *D*, *3*) is possibly due to Ca^{2+}-independent enzymatic or non-enzymatic oxidation of fluorescent probe by matrix enzymes and other oxidants [2, 3]. Thus the rate of ROS formation is limited by the rate of Ca^{2+}-uptake, leading to the conclusion that potential-dependent uptake of Ca^{2+} in energized mitochondria, is the rate-limiting step in ROS production.

When MPTP is closed, an increase in the rate of oxygen consumption with increase in the amount of added Ca^{2+} is observed (Fig. 3, *A*, *1*), consistent with the notion of stoichiometric proportion between the oxygen consumption and potential-dependent Ca^{2+}-influx [16]. The rate of Ca^{2+}-cycling is limited by the maximum rate of Ca^{2+}-uniporter and the Ca^{2+}-accumulating capacity of mitochondria [16], which could be estimated from the oxygen consumption data based on proportionality between the oxygen consumption and Ca^{2+}-transport rates [16, 17], as ~30 nmol $Ca^{2+} \cdot mg^{-1}$ protein for maximum Ca^{2+}-uptake which correspond to maximum oxygen consumption rate of 14.8 ± 1.3 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein. The rate of ROS formation shows similar dependence on Ca^{2+}-concentration (Fig. 3, *B*, *1*), with saturation at concentrations close to Ca^{2+}-accumulating capacity of brain mitochondria, i.e. ~30 nmol $Ca^{2+} \cdot mg^{-1}$ protein (Fig. 3, *A*).

With MPTP being in closed state, proportionality is established between the rate of oxygen consumption and that of ROS release (Fig. 4, *1*). This is consistent with the notion that under equilibrium conditions, provided steady-state rate of oxygen consumption and constant $\Delta\Psi_m$, ROS are released at a constant rate, depending on the rate of respiration, as by-products of the multiple redox-reactions in respiratory chain [2]. Thus, with mitochondria being in metabolic State 2, the rate of ROS production is shown to be potential-dependent (Fig. 1) and proportional to the rate of oxygen consumption (Fig. 4). It is controlled

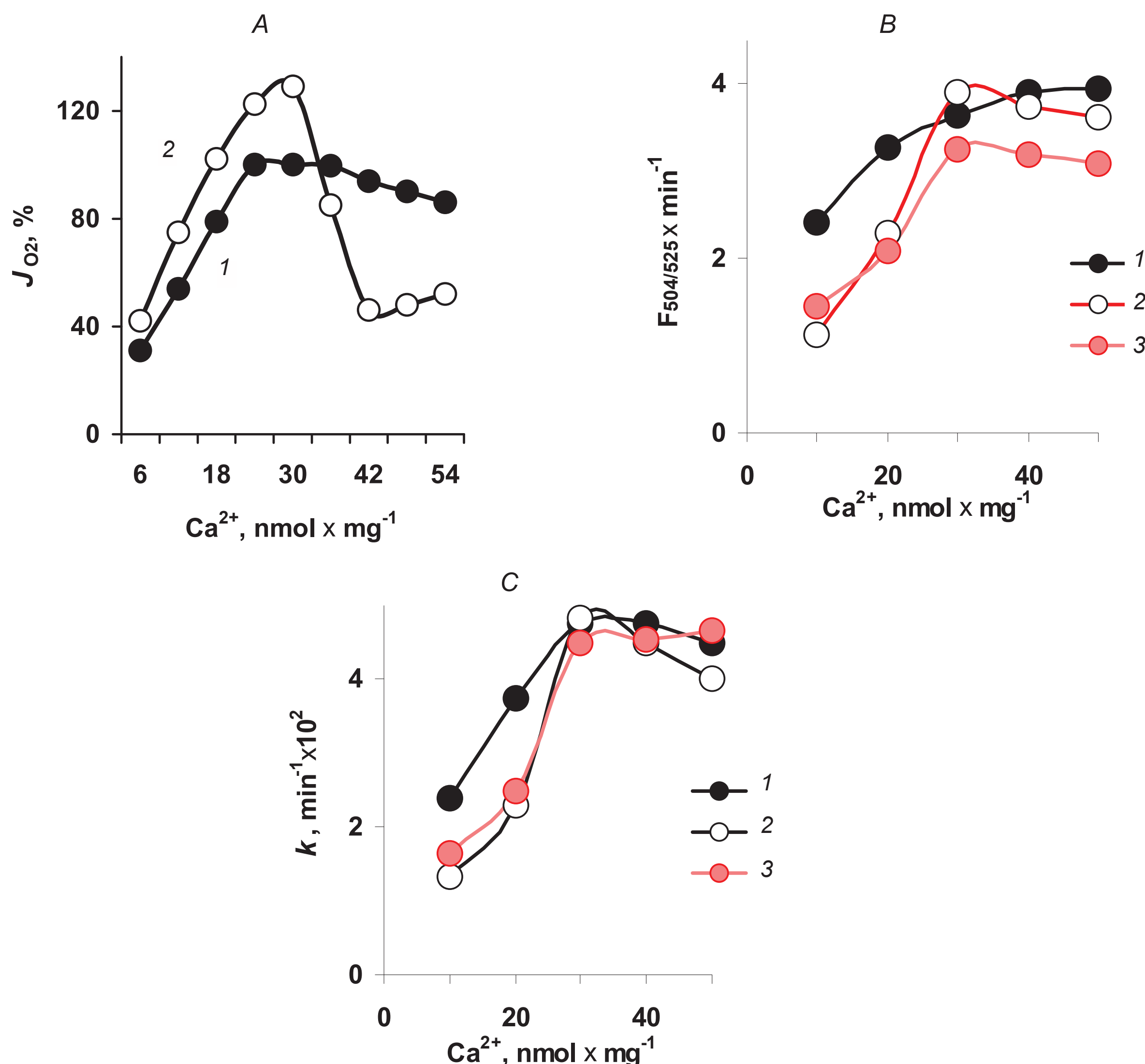

Fig. 3. The influence of MPTP opening on the oxygen consumption (A) and ROS formation (B-C) in the rat brain mitochondria. A: The influence of MPTP opening on the rate of respiration. B-C: The influence of MPTP opening on the rate of ROS formation under steady-state (B) and non-equilibrium (C) conditions. MPTP was blocked by the addition of cyclosporine A (A-C, 1). Incubation medium: 320 mM sucrose, 1 mM KH_2PO_4, 5 mM of sodium succinate, 5 mM Tris-HCl buffer (pH 7.4). Cyclosporine A (A, 1; B-C, 1,3) and A-23187 (B-C, 3) were added at 1μM; $CaCl_2$ in nmol·mg^{-1} protein was added as shown on the abscissa (A-C). M±S.D., n = 6, P < 0.05 relative to control (A-C, 1).
On the abscissa axis: the amount of added $CaCl_2$, nmol·mg^{-1} protein. On the ordinate axis: the rate of respiration, in % of maximum measured with cyclosporine A (A, 1); the rate of ROS formation, in arbitrary fluorescence units, $F_{504/525}$·min^{-1} (B); the rate constant of ROS formation, k, min^{-1}·10^{-2}, under non-equilibrium conditions (C) in presence of cyclosporine A (B-C, 1,3) and A-23187 (B-C, 3)

by the rate of potential-dependent Ca^{2+}-uptake (Fig. 2, *D*) and is limited by Ca^{2+}-accumulating capacity of mitochondria (Fig. 3, *A*, *B*, *1*).

MPTP opening differently influences ROS production in the rat brain mitochondria, depending on Ca^{2+} concentration. At low Ca^{2+} concentrations MPTP opening results in a sustained increase in oxygen consumption rate by a cyclosporine A-sensitive component, ΔJ_{O2} (Fig. 5) due to the increase in Ca^{2+}-cycling rate which, as we have shown earlier in the rat liver mitochondria [18], is supported by simultaneous work of Ca^{2+}-uniporter and MPTP as Ca^{2+}-influx and efflux pathways. Functional activity of Ca^{2+}-uniporter was proven by the fact, that an addition of ruthenium red, a specific Ca^{2+}-uniporter blocker, decreased the rate

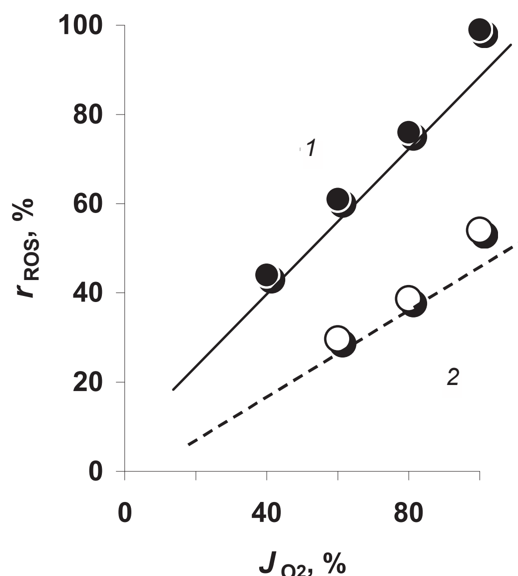

Fig. 4. The influence of ROS production on the rate of respiration under equilibrium conditions. Incubation medium: 320 mM sucrose, 1 mM KH_2PO_4, 5 mM of sodium succinate, 5 mM Tris-HCl buffer (pH 7.4). Cyclosporine A was added at 1 μM (1). M ± S.D., n = 6. The difference in the rates of ROS production in the presence (1) and the absence (2) of cyclosporine A is statistically significant (P < 0.05).
On the abscissa axis: the rate of oxygen consumption, in % of maximum measured with cyclosporine A; on the ordinate axis: the rate of ROS formation, in % of maximum measured with cyclosporine A

of oxygen consumption. Similarly, an addition of cyclosporine A, specific MPTP inhibitor, also decreased the respiration rate by a cyclosporine A-sensitive component (Fig. 5) which corresponds to MPTP contribution to transmembrane exchange of Ca^{2+} and oxygen consumption.

In accord with established proportionality in the rates of oxygen consumption and ROS formation (Fig. 4, *1*), an increase in ROS production would be expected with cyclosporine A-sensitive increase in respiration rate (Fig. 3, *A*, *2*; Fig. 5). But on the contrary, instead of the increase in ROS production, its decrease, also sensitive to cyclosporine A under steady-state as well as non-equilibrium conditions, was observed (Fig. 3, *B-C*, *2*). With MPTP opening at low Ca^{2+} concentrations, a similar linear dependence between the ROS release and oxygen consumption was obtained, except that less ROS production was detected at even oxygen consumption rates (Fig. 4, *2*).

To explain the observed decrease in ROS production with MPTP opening at low calcium in the rat brain mitochondria, it should be taken in consideration that MPTP opening is accompanied by partial membrane depolarization (of about ~40%, as determined with safranine [15]). Then, taking into account the dependence of ROS production on $\Delta\Psi_m$ (Fig. 1, *B*, *1*), membrane depolarization at constant electron transport rate could serve as a plausible explanation for the decrease in ROS formation due to MPTP opening at low calcium, provided steady-state conditions, because of simultaneous change in the redox-state of the complexes of respiratory chain.

At high Ca^{2+} concentrations a "classical" pattern of MPTP opening is observed, i.e. progressive respiration inhibition, cyclosporine A-sensitive decrease in the rate of oxygen consumption (Fig. 3, *A*, *2*, Fig. 5), and the increase in ROS production, due to the fast cytochrome c loss (Fig. 3, *B-C*, *2*) and gating the electron flow in respiratory chain

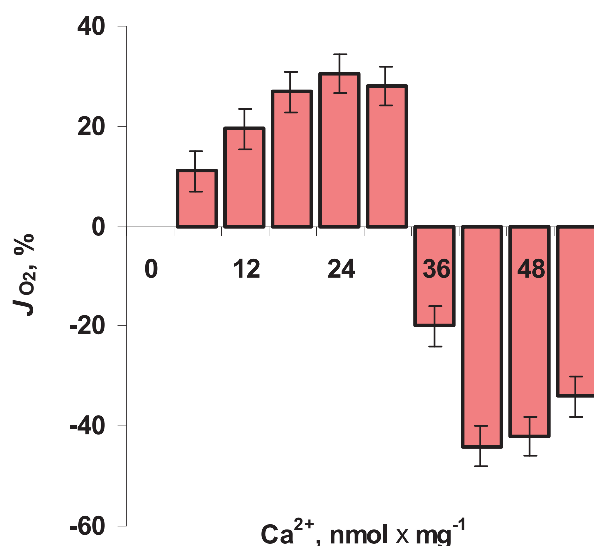

Fig. 5. The contribution of MPTP opening to the steady-state rate of oxygen consumption. The part of MPTP in the rate of respiration (% of maximum in the absence of cyclosporine A) was calculated as the difference in the respiration rates in the absence and the presence of cyclosporine A. Incubation medium: 320 mM sucrose, 1 mM KH_2PO_4, 5 mM of sodium succinate, 5 mM Tris-HCl buffer (pH 7.4). $CaCl_2$ was added as shown on the abscissa. M ± S.D., n = 6, P < 0.05 relative to control (0%) in the absence of cyclosporine A.
On the abscissa axis: the amount of added calcium, in nmol·mg^{-1} protein. On the ordinate axis: the difference in respiration rate, ΔJ_{O2}, in the absence and the presence of 1 μM cyclosporine A, in % of maximum

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which was shown to be favorable for ROS formation (Fig. 2, *B*, *2*).

At low calcium the effect of MPTP opening on oxygen consumption and ROS production in the rat brain mitochondria is similar to that of Ca^{2+}-ionophore, A-23187 (Fig. 3, *A-C*, *2*, *3*) under equilibrium, at constant electron flow (Fig. 3, *B*), and non-equilibrium (progressive respiration inhibition) conditions (Fig. 3, *C*). It is most possible that in case of A-23187 an additional cause to diminish ROS production (Fig. 3, *B-C*, *3*) is matrix acidification [1], due to Ca^{2+}/H^{+}-exchange which directly inhibits superoxide formation [1,10] and ROS metabolism. In case of MPTP opening, an increase in Ca^{2+}-cycling rate and uncoupling of mitochondria would also decrease the uptake of Ca^{2+} [18] and would result in relative matrix acidification, but whether MPTP in the rat brain mitochondria could contribute to oxygen consumption as a cyclosporine A-sensitive Ca^{2+}/H^{+}-exchange (as it is the case with the rat liver mitochondria [18]) needs a more detailed study. It is necessary to take into account possible multiple conductance states of MPTP with different regulation of membrane permeability, membrane potential and other mitochondrial functions [13]. The regulatory effect of MPTP opening on ROS production in mitochondria is also complicated because of concomitant water uptake and osmotic matrix swelling even at low calcium [18], K^{+}-influx under physiological conditions (~150 mM of cytosolic K^{+} [15]), more easy release of cytochrome c from intermembrane space due to the rupture of the outer membrane [19] and the interruption of electron flow which is favorable for ROS production [8] (Fig. 2, *B*, *2*).

As it was mentioned earlier, MPTP opening, even at low calcium, is accompanied by partial membrane depolarization, which is in accord with the observed decrease in ROS production (Fig. 1, *B*, *1*; Fig. 4, *2*). Thus, membrane depolarization and “mild uncoupling” due to Ca^{2+}-cycling, as well as relative matrix acidification because of cyclosporine A-sensitive Ca^{2+}/H^{+}-exchange would make the effect of MPTP opening at low Ca^{2+} concentrations similar to that of Ca^{2+}-ionophore (Fig. 3, *B-C*, *2*, *3*).

The role of K^{+}-uptake in mitochondria in the regulation of ROS production will be studied later. With relevance to physiological conditions, it is important that reversible regulation of ROS production by MPTP open state was observed. In accord with the data (Fig. 6), MPTP blockage after its opening resulted in the increase of ROS release. In view of the obtained results, this could be explained by blockage of cyclosporine-sensitive component of Ca^{2+}-cycling (Fig. 5), prevention of

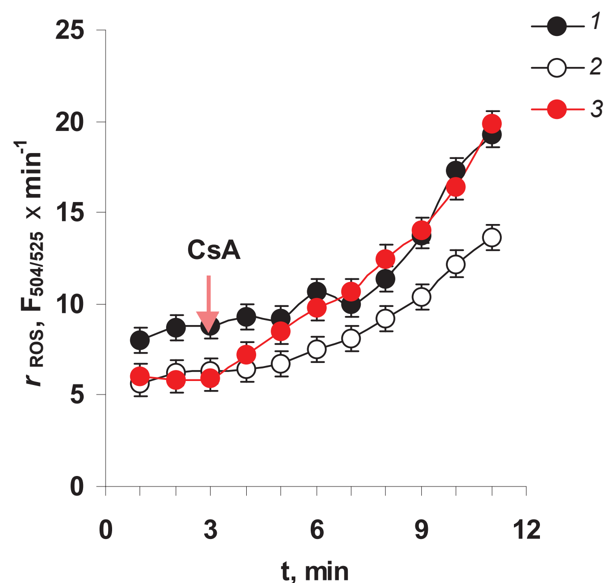

Fig. 6. The influence of reversible MPTP opening on the rate of ROS production in rat brain mitochondria. Incubation medium: 320 mM sucrose, 1 mM KH_2PO_4, 5 mM of sodium succinate, 5 mM Tris-HCl buffer (pH 7.4), 15 μM $CaCl_2$. 1 μM cyclosporine A was added to the medium to prevent MPTP opening (1), or after MPTP opening as shown by the arrow (3). M ± S.D., n = 3. On the abscissa axis: time, min. On the ordinate axis: the rate of ROS formation, in fluorescence units, $F_{504/525} \cdot min^{-1}$

cytochrome *c* loss and membrane repolarization (Fig. 1, *B*, *1*), which all together lead to the increase in the rate of ROS formation. These data well correlate with our earlier observations that blockage of MPTP in vivo increased the level of ROS produced in mitochondria [20].

Thus, it was shown that kinetics of ROS production in the rat brain mitochondria is dependent on the kinetics of oxygen consumption and the electron transport in the respiratory chain. Under steady-state conditions a linear dependence between the rate of respiration and that of ROS formation was established, which was shown to be potential-dependent. Under non-equilibrium conditions of progressive gating the electron transport due to the deficit of cytochrome *c*, a non-linear, exponential, time-dependence of ROS formation was observed, as well as the increase in ROS production together with respiration inhibition and membrane depolarization. Different influence of MPTP opening on ROS production could also be explained by different regulation of electron transport and oxygen consumption kinetics, depending on calcium concentration, with the maintenance of steady-state conditions at low calcium and the

transition to non-equilibrium state with the increase in Ca^{2+} concentration, possibly due to the change in the conductance state of MPTP [13] with consequent increase in membrane permeability and mitochondrial volume. Obtained results give convincing evidence that under physiological conditions, at low intracellular level of calcium, MPTP opening could attenuate oxidative stress and the impairment of neurons by diminishing ROS formation in mitochondria.

ВПЛИВ ВІДКРИТТЯ МІТОХОНДРІАЛЬНОЇ ПОРИ НА ГЕНЕРАЦІЮ АКТИВНИХ ФОРМ КИСНЮ В МІТОХОНДРІЯХ МОЗКУ ЩУРІВ

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Досліджено вплив відкриття мітохондріальної пори (mitochondrial permeability transition pore, MPTP) на генерацію активних форм кисню (АФК) у мітохондріях мозку щурів. Показано, що в залежності від стаціонарних чи нерівноважних умов продукція АФК по-різному регулюється швидкістю споживання кисню і мембранним потенціалом. За умов стаціонарної рівноваги, що відповідає постійній швидкості циклічного транспортування Ca^{2+} та споживання кисню, продукція АФК є потенціалзалежною і знижується разом з пригніченням дихання і мітохондріальною деполяризацією. При цьому постійна швидкість утворення АФК прямо пропорційна швидкості споживання кисню. Порушення стаціонарного стану внаслідок вивільнення цитохрому *с* й поступового гальмування дихання призводить до експоненціального зростання в часі продукції АФК. Незалежно від стаціонарних чи нерівноважних умов, швидкість утворення АФК контролюється швидкістю потенціалзалежної акумуляції Ca^{2+}, яка є швидкістьлімітуючою стадією утворення АФК. Показано, що відкриття циклоспоринчутливої пори по-різному впливає на продукцію АФК залежно від концентрації Ca^{2+}. За дії низьких концентрацій Ca^{2+} зниження швидкості утворення АФК корелює з частковою деполяризацією мітохондрій, незважаючи на циклоспоринчутливе прискорення дихання внаслідок одночасної роботи Ca^{2+}-уніпортера й MPTP як шляхів входу і виходу Ca^{2+}. Ефект відкриття циклоспоринчутливої пори за дії низьких концентрацій Ca^{2+} близький до ефекту Ca^{2+}-іонофора А-23187. За дії високих концентрацій Ca^{2+} відкриття пори призводить до швидкого порушення стаціонарного стану внаслідок вивільнення цитохрому *с*, блокування транспортування електронів і зростання продукції АФК. Отже, відкриття пори в умовах низького рівня внутрішньоклітинного Ca^{2+} може пом'якшувати оксидативний стрес і ушкодження нейронів шляхом пригнічення продукції АФК у мітохондріях.

Ключові слова: активні форми кисню, мітохондріальна пора, кальцій, мітохондрії мозку.

ВЛИЯНИЕ ОТКРЫТИЯ МИТОХОНДРИАЛЬНОЙ ПОРЫ НА ПРОДУКЦИЮ АКТИВНЫХ ФОРМ КИСЛОРОДА В МИТОХОНДРИЯХ МОЗГА КРЫС

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Изучено влияние открытия митохондриальной поры (mitochondrial permeability transition pore (MPTP) на продукцию активных форм кислорода (АФК) в митохондриях мозга крыс. Показано, что в зависимости от стационарных либо неравновесных условий, продукция АФК по-разному регулируется скоростью потребления кислорода и мембранным потенциалом митохондрий. В условиях стационарного равновесия, соответствующих постоянной скорости циклического транспорта Ca^{2+} и потребления кислорода, продукция АФК является потенциалзависимой и снижается вместе с подавлением дыхания и митохондриальной деполяризацией. При этом постоянная скорость образования АФК прямо пропорциональна скорости потребления кислорода. Переход к неравновесным условиям вследствие высвобождения цитохрома *с* из митохондрий и прогрессирующего подавления дыхания приводит к нарушению прямопропорциональной зависимости скорости образования АФК от скорости дыхания и к экспоненциальному возрастанию во времени продукции АФК. Независимо

от равновесных либо неравновесных условий, скорость образования АФК контролируется скоростью потенциалзависимой аккумуляции Ca^{2+}, являющейся стадией, лимитирующей скорость образования АФК. Показано, что открытие МРТР по-разному регулирует продукцию АФК в зависимости от концентрации Ca^{2+}. При низких концентрациях Ca^{2+} открытие МРТР приводит к снижению продукции АФК вследствие частичной митохондриальной деполяризации, несмотря на циклоспоринчувствительное повышение скорости дыхания за счет одновременной работы Ca^{2+}-унипортера и МРТР как путей входа и выхода Ca^{2+}. Открытие МРТР при низких концентрациях Ca^{2+} близко к эффекту Ca^{2+}-ионофора А-23187. При высоких концентрациях Ca^{2+} открытие МРТР приводит к повышению продукции АФК за счет быстрого перехода к неравновесным условиям вследствие выхода цитохрома *c* и прогрессирующего торможения транспорта электронов в дыхательной цепи. Таким образом, в физиологических условиях открытие МРТР при низких внутриклеточных концентрациях кальция может смягчать окислительный стресс и нарушение нейрональных функций путем снижения продукции АФК в митохондриях.

Ключевые слова: активные формы кислорода, митохондриальная пора, кальций, митохондрии мозга.

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