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REVIEWS

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Induction of Unspecific Permeabilization of Mitochondrial Membrane and Its Role in Cell Death[1]

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Abstract—Mitochondria participate in various vital cellular processes. Violation of their functions can lead to the development of cardiovascular and neurodegenerative diseases and malignancies. One of the key events responsible for mitochondrial damage—induction of Ca^{2+}-dependent mitochondrial permeability transition, due to the opening of a nonspecific pore in the inner mitochondrial membrane. Despite active studies of pore components, its detailed structure has not yet been established. This review analyzes possible constituents and regulators of the pore, the role of the pore in various pathologies, and hypotheses that explain the organization of the pores. Elucidation of these questions can help developing strategies for the treatment of a wide range of pathologies—from Alzheimer and Parkinson's disease to cancer.

Keywords: mitochondria, cell death, non-specific pore, calcium, adenine nucleotide transloacor, cyclophilin D, voltage-dependnent anion channel

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Mitochondria are unique organelles participating not only in the energy production in form of ATP, but also in maintaining Ca^{2+} homeostasis and reactive oxygen species (ROS) production. Studies performed during last two decades revealed that mitochondria possess a leading role in the regulation/initiation of cell death [1–7]. In particular, permeabilization of the outer mitochondrial membrane (OMM) and release of pro-apoptotic proteins represent a point of no return in apoptosis, one of the forms of programmed cell death. Apoptosis is an extremely important mechanism for the control of tissue homeostasis, which is responsible for the formation of organs during embryogenesis, and utilization of cell, which might bring harm to the organism. Apoptosis is an antagonist to carcinogenesis; suppression of apoptosis is regarded as one of the reasons for malignant transformation. On the other hand, uncontrolled apoptosis stimulation underlies various neurodegenerative diseases. Investigation of the possibilities of modulation of apoptotic pathways, in particular OMM permeabilization, is important for developing strategies to cure diseases caused by disturbances in this form of cell death. Since one of the strategies in fighting cancer involves sensitization of tumors to therapy, targeting mitochondria with the aim of mitochondrial destabilization, OMM permeabilization, release of pro-apoptotic factors from mitochondrial intermembrane space and cell death initiation, will uncover new possibilities of tumor sensitization to conventional therapeutic agents.

[1] The article was translated by the authors.

Abbreviations: OMM, outer mitochondrial membrane; MPT, mitochondrial permeability transition; VDAC, voltage-dependent anion channel; ANT, adenine nucleotide translocase; HK, hexokinase; CK, creatine kinase; CypD, cyclophiline D; CsA, cyclosporine A; ROS, rective oxygen species; GSK-3β, Glycogen synthase kinase 3; PiC, mitochondrial phosphate carrier; TNF-α, tumor necrosis factor α; PBR, peripheral benzodiazepine receptor; BKA, bongkrekic acid; BetA, betulinic acid.

THE OUTER MITOCHONDRIAL MEMBRANE PERMEABILIZATION PATHWAYS

There are several models describing cytochrome c release from the intermembrane space of the mitochondria into cytosol. According to one model, permeabilization takes place as a result of pore formation by pro-apoptotic Bcl-2 family proteins, such as Bax or Bak. The pore is formed after oligomerization of these proteins caused by the truncated form of the Bid protein (tBid) [8, 9]. In some instances, OMM permeabilization might be independent of Bax or Bak [10, 11]. This means that the pathway involving Bax or Bak is not the only one. OMM permeabilization might be due to induction of so-called mitochondrial permeability transition (MPT). This phenomenon was discovered by Haworth and Hunter, who showed that the

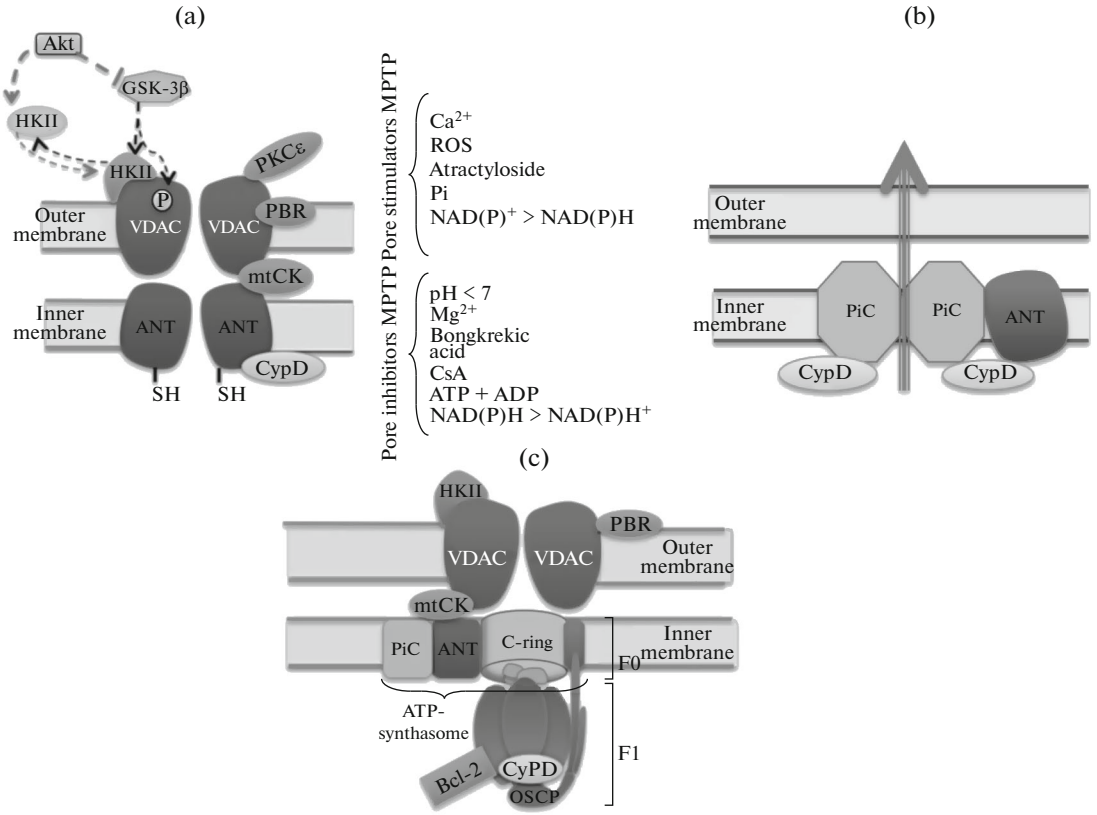

Fig. 1. Models of MPTP in which as the channel forming proteins are ANT (a) or PiC (b), located in the inner mitochondrial membrane, and VDAC in the OMM. Pore opening is regulated by CyPD, HKII, PBR, mtCK, Bcl-2 family proteins, GSK-3β, which causes HKII detachment PKCε, which can phosphorylate VDAC, activate anti-apoptotic Bcl-2 family proteins and inactivate pro-apoptotic protein Bad; c—a more current model of MPTP configuration, in which a key role plays c-subunit of F1/FO-ATP-synthase (C-ring), which interacts with ANT and PiC forming ATP-synthasome. ANT and PiC in turn can interact with other pore regulators (HKII, VDAC, PBR, mtCK). MPTP opening causes re-organization of ATP-synthasome structure, stimulating non-specific fluxes through the ring of c-subunits, swelling and deterioration of mitochondria (see the text for details).

accumulation of Ca^{2+} by mitochondria causes their damage as a result of the opening of a non-specific pore in the inner mitochondrial membrane (MPT pore, MPTP), which allows for the penetration of molecules not larger than 1.5 kDa [12]. Pore opening is accompanied by a drop of the mitochondrial membrane potential and uncontrolled entry of solutes and water into the matrix, which causes organelle swelling. The inner membrane straightens, causing rupture of the OMM and the release of proteins from the intermembrane space into the cytosol [13]. The Ca^{2+}-dependent pore opening is stimulated by inorganic phosphate, pyridine nucleotide oxidation, ATP depletion, low pH and ROS [14].

MPTP represents a multiprotein complex of about 600 kDa [15]. According to the traditional view, it consists of a voltage dependent anion channel (VDAC) in the OMM and adenine nucleotide translocase (ANT) in the inner mitochondrial membrane. VDAC and ANT form contact sites between OMM and the inner mitochondrial membranes, which under normal conditions, facilitate ADP transport into the mitochondria. In addition, parts of the MPTP are benzodiazepine receptors (PRB) and hexokinase (HK), on the surface of OMM, where they interact with VDAC, creatine kinase (CK) in the intermembrane space, and a matrix protein, cyclophilin D (CypD), a target of cyclosporine A (CsA) [14, 16, 17] (Fig. 1a).

A key factor of MPTP opening and closure is the level of Ca^{2+} in the matrix, which is determined by the balance of Ca^{2+} entry and release. Accumulation of Ca^{2+} by mitochondria is an obligatory step for MPT induction; this can be concurrently suppressed by other bivalent cations, such as Mg^{2+}, Sr^{2+}, or Mn^{2+} [18]. However, there is no direct correlation between Ca concentration and permeabilization of mitochondria, since a number of factors can modulate this process. Thus, an increase in [Pi] from 2 to 5 mM decreases the threshold level of Ca^{2+} from 5.0 to 1.8 μM [19]. A number of compounds stimulate pore opening as a result of a decrease of the Ca^{2+} threshold level, which is necessary for MPT induction. For instance, in isolated liver mitochondria from patients with Reyes syndrome, toxins do not stimulate MPT, but decrease the

threshold level of Ca^{2+} [20]. Salicylates, active metabolites in aspirin, were shown to be toxic for hepatocytes, and mitochondrial depolarization and membrane permeabilization are sensitive to CsA. The role of Ca^{2+} in salicylate toxicity was demonstrated using Ca^{2+} channel blockers, exhibiting cytoprotection [21]. It should be mentioned that the participation of pore components in the release of pro-apoptotic factors is not always related to organelle swelling and OMM rupture. It is known that only a small fraction of cytochrome c is located in the intermembrane space, whereas most of it is located inside the cristae. Scorrano and colleagues [22] demonstrated that the pro-apoptotic protein tBid could stimulate cristae remodeling, which leads to re-distribution of cytochrome c between the intermembrane space and cristae. CsA can suppress this process and is independent of the BH3 domain of tBid. Subsequent OMM permeabilization and cytochrome c release are resistant to CsA, but need the BH3 domain of tBid and another pro-apoptotic protein, Bak. According to the authors, a brief opening of MPTP is necessary for cristae remodeling and release of cytochrome c into the intermembrane space without OMM rupture [22].

Recently data were published contradicting the classical view on MPTP structure. These data raise doubts about the involvement of VDAC or ANT in pore opening. Supposedly, OMM permeabilization might be an outcome of several processes involving various proteins and protein complexes. The molecular composition of MPT might not be invariable, but is instead regulated by diverse stimuli and conditions [23]. According to the model He and Lemasters [24] proposed, the pore is formed due to aggregation of incorrectly assembled membrane integral proteins damaged by ROS or other stresses. Chaperon-like proteins block the permeability of incorrectly assembled proteins, but accumulation of Ca^{2+} induces pore opening, which can be blocked by CsA [24]. The role and contribution of various components of classical MPTP is discussed in the following section.

COMPONENTS OF MPTP AND THEIR ROLE IN CELL DEATH STIMULATION

VDAC

VDAC, which is located at the OMM, is responsible for metabolite transport into the mitochondria. The conductivity of VDAC is regulated by pyridine nucleotides, Ca^{2+}, glutamate [25–27], binding to HK [28–31], and Bcl-2 family proteins [28, 32–34]. VDAC attracted the attention of apoptosis investigators as an explanation for the release of cytochrome c from mitochondria. The size of the channel does not allow cytochrome c to penetrate freely through OMM, and a number of attempts have been made to explain OMM permeabilization by opening the VDAC after binding pro-apoptotic Bcl-2 family proteins (Fig. 2a). For example, recombinant Bax was not able to stimulate cytochrome c release from mitochondria isolated from yeasts lacking VDAC. Transfection by VADC1 restored the ability of Bax to stimulate membrane permeabilization [35]. This signifies a possible interaction of Bax and VDAC, as well as the necessity of VDAC for OMM permeabilization. VDAC antibodies prevented Bax-induced release of cytochrome c, mitochondrial depolarization, and apoptosis [36]. Based on these data, it was proposed that anti-apoptotic proteins stimulate VDAC opening, whereas binding of pro-apoptotic proteins prevents cytochrome c release [33, 37]. Subsequent investigations confirmed this. The resistance towards cytochrome c release and a drop of the membrane potential caused by Bax or MPT inducers demonstrated mitochondria with VDAC (but not ANT) knockout [32]. The existence of tertiary complex VDAC-Bax-Bcl-X_L demonstrated in vitro implies that VDAC conformation and OMM permeabilization can be regulated via a fine balance between Bax and Bcl-X_L [34]. Rostovtseva and colleagues critically analyzed the interaction of Bcl-2 family proteins with VDAC [38]. According to this analysis, Bax cannot physically interact with VDAC; Bid, but not Bax, regulates VDAC channels, inducing channel closure (Fig. 2b). The authors speculated that VDAC closure reduces metabolite exchange between mitochondria and the cytosol, leading to mitochondrial dysfunction, which plays an important role in cell death stimulation. It was shown earlier that growth factor withdrawal leads to cell death because of tBid appearance and its association with VDAC [39, 40]. This caused mitochondrial swelling, OMM permeabilization, and the release of cytochrome c and other proteins involved in mitochondrial pathway in apoptosis. Stimulation of Bcl-X_L expression and elevation of the content of this protein neutralized the effect of tBid and prevented cell death, apparently due to the binding of BclXL to VDAC and the occupation of tBid binding sites.

Deleterious consequences of VDAC closure were confirmed in experiments with the channel blocker G3139. VDAC closure caused accumulation of ROS in mitochondria, leading to MPTP opening and apoptosis induction [41, 42]. It has been shown that VDAC closure, which induces apoptosis, also favors Ca^{2+} flux into the mitochondria, which can possibly lead to permeability transition and cell death [44]. One of the models of VDAC involvement into OMM permeabilization involves its oligomerization [44]. Supposedly, Ca^{2+} stimulates oligomerization, however the precise mechanism is still unknown [45].

One of the factors involved in the regulation of mitochondrial stability towards permeabilization is the binding of HK. A number of tumors are characterized by enhanced expression of HKI (in brain) and HKII (almost in all malignancies). Interaction of VDAC with HK not only makes phosphorylation of

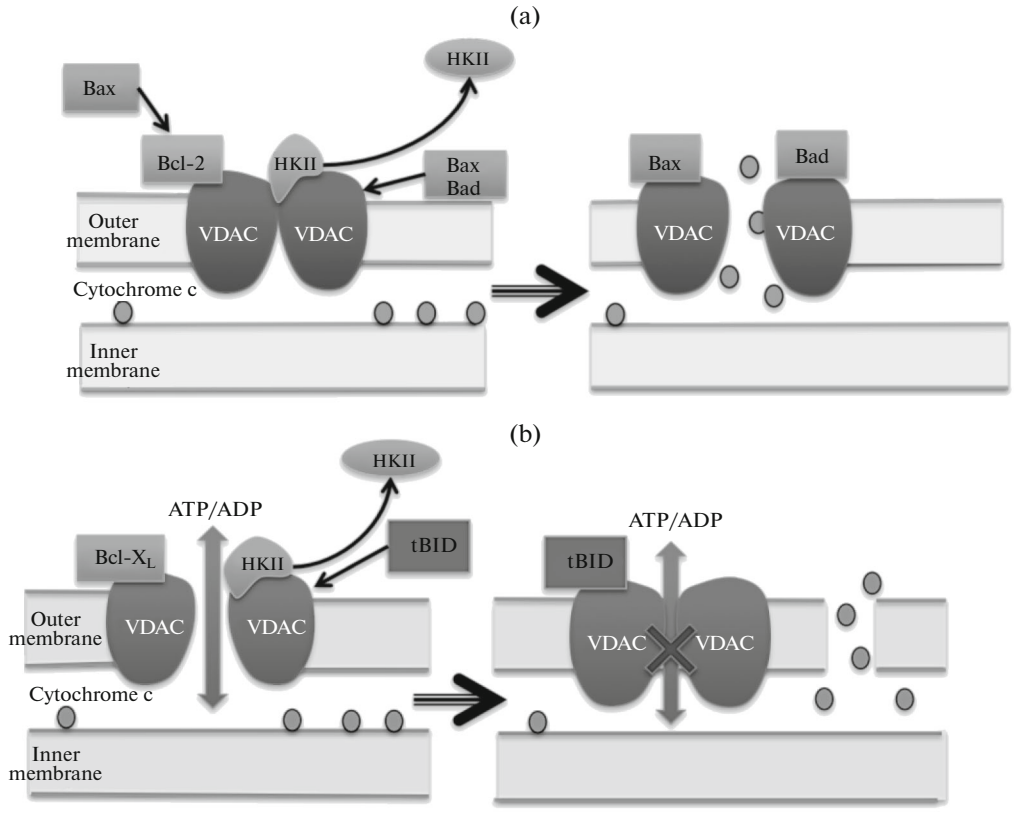

Fig. 2. Possible models of OMM permeabilization. (a) Pro-apoptotic proteins (Bax and Bak) interact with VDAC, causing its opening, whereas Bcl-X_L binds VDAC and prevents opening. (b) According to another model, anti-apoptotic molecules (HKII and Bcl-X_L) interact with VDAC, maintaining its open configuration and facilitating ADP/ATP exchange. Upon apoptosis induction, HKII or Bcl-X_L are detached from VDAC. tBid binds to VDAC causing its closure, suppressing metabolite transport, affecting mitochondrial function and subsequent OMM permeabilization.

glucose more efficient since it allows for the use of mitochondrially-generated ATP, but it also keeps VDAC in an open state, preventing OMM permeabilization. In tumor cells, hypoxia-stimulated activation of glycolysis and enhanced expression of hexokinase led to mitochondrial stabilization towards permeabilization and hence, suppressed the mitochondrial apoptotic pathways.

The mechanisms of VDAC interaction with HKI and HKII are different. HKII keeps VDAC in an open state, which is regarded as an anti-apoptotic event [30, 40, 46]. Dissociation of HKII from mitochondria causes VDAC closure, followed by mitochondrial swelling and cell death initiation, most likely because of MPTP opening [30, 47]. HKI renders an opposite effect: interaction of HKI with VDAC caused VADC closure, preventing pore opening and cytochrome c release, which protected cells from the death [48]. Interestingly, using peptides similar to N-terminal of HKII caused HKII detachment from mitochondria and opened MPTP, even in cells lacking VDAC. Apparently, the consequences of HK-VDAC interaction are determined by tissue specificity.

Interaction between HK and VDAC is regulated by various kinases and can be compromised by VDAC phosphorylation via glycogen synthase kinase 3 (GSK-3β), potentiating the therapeutic effect of anticancer drugs. GSK-3β is involved in the pathogenesis of various diseases, in particular in tumors GSK-3β modulates cell death mechanisms in response to therapy. GSK-3β stimulation is facilitated by Akt kinase suppression, which when active, suppresses GSK-3β [29]. Akt facilitates HKII binding to mitochondria [49] through phosphorylation of both HKII [50] and GSK-3β [51]. Akt-mediated HK binding to mitochondria needs glucose [52].

There are several isoforms of VDAC (VDAC1, VDAC2 and VDAC3). All the isoforms have similar capabilities of forming channels. However, if knockout of VDAC1 does affect mice phenotypes, knockout of VDAC3 induced partial embryonic lethality, whereas the absence of VDAC2 makes mice non-viable.

The role of VDAC in OMM permeabilization is not completely understood since available data are, to some extent, controversial. On the one hand, enhancing stimulation of VDAC resulted in apoptosis [53],

which supports the key role of VDAC in OMM permeabilization. The discovery of a new inhibitor of Ca^{2+}-induced MPTP, Ro68-3400, which can bind to 32 kDa protein (apparently VDAC) with high affinity, can be regarded as proving VDAC involvement in MPTP [53]. Moreover, it has been shown that monoclonal antibodies against VDAC prevented MPT-dependent release of cytochrome c caused by arsenic trioxide [55].

On the other hand, despite numerous data in favor of VDFAC participation in MPTP, there is contrary evidence as well. For example, mitochondria lacking VDAC1 revealed similar sensitivity towards Ro68-3400, as well as wild-type organelles [56]. Despite suppression of expression of all isoforms, mitochondria were still able to undergo MPTP to the same extent as wild-type mitochondria. Suppression of VDAC expression in fibroblasts with VAD1 and VDAC3 knockout slightly increased the level of apoptosis induced by H_2O_2 or staurosporine. Both wild-type mitochondria and mitochondria lacking VDAC were characterized by similar release of cytochrome c, caspase cleavage and cell death in response to overexpression of Bax, or the activation of endogenous Bax and Bid by staurosporine or TNFα. Apparently VDAC does not play a critical role in cell death mediated by MPTP or Bcl-2 family proteins [57].

Thus, the data on possible involvement of VDAC in OMM permeabilization can be grouped into three models: 1—VDAC is a part of MPT and pro-apoptotic agent stimulate release of cytochrome c as a result of pore opening; 2—homo and hetero-oligomerization of VDAC leads to the formation of bigger pores and cytochrome c release; 3—closure of VDAC-induced accumulation of mitochondrial metabolites and mitochondrial swelling by a still poorly understood mechanism, or via mitochondrial overall destruction [58].

ANT

ANT catalyzes ATP^{4-}/ADP^{3-} electrogenic exchange. A misbalance of charges allows maintaining negative membrane potential (about 180 mV) for ATP efflux into cytosol in exchange for ADP [59]. An interesting feature of ANT is its binding to cardiolipin [60]. Each molecule of ANT binds six molecules of cardiolipin, which is necessary for the normal functioning of the enzyme.

One of the first pieces of evidence of ANT participation in MPTP formation was the experiment in which ANT, incorporated in lipid membrane in the presence of Ca^{2+}, acquired properties of a non-specific pore. It was suggested that Ca^{2+} binds to negatively charged "heads" of cardiolipin in the inner mitochondrial membrane, affecting the ANT structure [61]. ATP and the inhibitor of ANT bongkrekic acid (BKA) suppressed the MPTP opening via decreasing the sensitivity of this process to Ca^{2+}. Another inhibitor of ANT, carboxyatractyloside, enhanced the sensitivity of MPTP to Ca^{2+}, stimulating its opening. Different effects of BKA and carboxyatractyloside are linked to stabilization of different conformations of ANT, matrix "m" and cytosolic "c" [62]. The sensitivity of MPTP to Ca^{2+} is markedly increased by oxidative stress, which causes oxidation of two thiol groups proximal to the adenine nucleotide-binding site [63].

Various isoforms of ANT are differently involved in apoptosis. Thus, stimulation of ANT1 and ANT3 expression was shown to cause cell death, wherein mutations affecting the ability of ANT to perform ADP/ATP exchange, as well as mutation in the N-terminal of the protein, did not influence cell death. This shows that pro-apoptotic capacity of ANT is independent of its transport activity [64–66]. In contrast, overexpression of ANT2 did not cause cell death [67]. In malignant cells, ANT2 is preferentially expressed [68, 69], which probably makes them more resistant towards therapeutic drugs.

Attempts have been made to show that mitochondrial membrane permeabilization can be regulated by the interaction of pro- and anti-apoptotic Bcl-2 family proteins with ANT [68, 69]. In this model, pore opening stimulation was explained by the interaction of ANT with Bax, which led to the formation of the channel sensitive to BKA and CsA [68, 70, 71]. According to other data, to form the pores, ANT must interact with Bax, as well as with pro-apoptotic ANT ligands, such as atractyloside and the R protein of human immunodeficiency virus [68, 71, 72]. Anti-apoptotic proteins Bcl-2 and Bcl-X_L, in contrast, increase resistance of MPTP to opening [70]. It has been found that Bcl-2 can stimulate, whereas Bax can inhibit, ANT translocase activity, which, however, does not always lead to the opening of the pores without additional stimuli such as atractylozide [69].

Despite the data indicating ANT participation in apoptosis, its role in cell death and in the formation of MPTP currently remains quite controversial. In order to investigate the role of ANT in MPTP, mice have been produced in which two isoforms of liver ANT (ANT1 and ANT2), were genetically inactivated. Despite this, mitochondria were still capable of opening MPTP. However, more Ca^{2+} than usual was required, and the ANT ligands were unable to regulate pore opening [73]. Moreover, hepatocytes with ANT knockout responded to different cell death stimuli, similar to wild-type cells. Apparently, this can be attributed to incomplete ANT isoform knockout or the existence of another isoform, ANT4, which was discovered later [74, 75]. Although ANT4 is present, although not as widespread, it is found mainly in the testes, it is possible that after knocking out two isoforms of ANT, the cells compensate for their lack by stimulating the expression of the fourth isoform.

ANT is able to interact with various matrix proteins, particularly with glutathione transferase, one of the major antioxidant enzymes in the cell [76]. Upregulation of glutathione-S-transferase occurs in the majority of tumor cells, which can determine their resistance to therapeutic agents. Interaction of ANT with glutathione transferase was weakened upon induction of apoptosis, which shows that glutathione transferase possesses the properties of endogenous repressors. In addition to glutathione-S-transferase, ANT interacts with proteins of other intracellular structures, such as the endoplasmic reticulum, resulting in the formation of polyprotein complex, alteration of which might affect the response of cells to apoptosis inducers [77]. It is possible that in some forms of cell death, ANT is not among the factors required for the permeabilization of the membrane, but its role as a regulator of MPT induction should not be denied.

CypD

If there are controversies regarding the role of VDAC or ANT in MPTP opening, CypD, without a doubt, is a key component of nonspecific pores. Cyclophilins, widespread proteins with peptidyl-prolyl *cis/trans* isomerase (PPIase) activity, mediate protein assembly. For the first time, participation of CypD in the regulation of pore opening was reported in a paper devoted to the analysis of the inhibitory effect of immunosuppressant CsA [78]. This lipophilic peptide, derived from the soil fungus Tolypocladium inflatum games, is capable of binding with high affinity to cyclophilins, particularly cytosolic cyclophilin A (17 kDa), the most widely presented in T-cells. In mitochondria CsA receptor is CypD, whose activity is inhibited by CsA in the same concentration as that necessary for MPT inhibition [79]. Suppression of pore opening does not require immunosuppressive properties of CsA [80]. Effect of CsA on the functioning of MPTP is due to its ability to inhibit the peptidyl-prolyl *cis-trans* isomerase activity of CypD. The inhibitory effect of CsA and its analogues are associated with reduced sensitivity of pores to Ca^{2+}. It is most likely that the process of interaction of CypD, Ca^{2+} and pores is multifaceted and includes MPTP protein sensitivity to oxidative stress [81].

Since CypD is a component of the pore, it is not surprising that the increase in its level could stimulate necrosis due to the opening of the pores and ATP hydrolysis stimulation by mitochondria. Suppression of CypD expression or inhibition of its activity prevents cell death. Indeed, CsA was shown to prevent necrosis caused by oxidative stress or TNFα [4, 82]. CypD deficient embryonic fibroblasts and hepatocytes possess a high resistance to necrosis induced by H_2O_2 or Ca^{2+} ionophore (A23187) [83, 84]. CypD knockout led to elevation of a threshold concentration of Ca^{2+} necessary for MPTP opening [85]. Neurons that lack CypD are more resistant to Ca^{2+}-dependent activation of MPTP and cell death in response to ischemic injury [86, 87], in case of multiple sclerosis [88] or Alzheimer's disease [89], compared to wild-type neurons. Genetic inactivation of CypD increases the level of Ca^{2+} in the cardiomyocytes matrix, activation of the Ca^{2+} dependent dehydrogenases and reduced metabolic plasticity of the cells, thereby developing hypertrophy, fibrosis and decreased cardiac function [90].

Pores opening may be the cause of both necrosis and apoptosis, but in contrast to necrosis, MPTP's role in apoptosis remains quite controversial and needs further study. Thus, pore opening was observed during reperfusion [91] dopamine-induced apoptosis during ischemia [92], and apoptosis induced by Fas or TNFα, but not by staurosporine [93]. The opening of the pores, leading to necrosis, usually occurs in a large subpopulation of mitochondria, which is accompanied by a decrease in the level of ATP in the cell as a result of stimulation of mitochondrial ATP hydrolysis. Unlike necrosis, apoptosis caused by moderate oxidative stress or relatively low concentrations of toxic substances can be accompanied by a brief opening of the MPTP in a limited subpopulation of mitochondria. Even during myocardial infarctions, cells in the core infarct zone are predominantly necrotic, whereas in the areas surrounding a myocardial infarct—apoptotic [94].

Swelling of mitochondria and release of cytochrome c following OMM permeabilization ends up by apoptosis, if the majority of mitochondria in the cell can still maintain membrane potential and perform oxidative phosphorylation. Thus, the type of cell death is determined by the size of the population of mitochondria undergoing OMM permeabilization. This explains the appearance of rings of apoptotic cells around the necrotic area at reperfusion in myocardial infarctions [95]. A similar situation occurs in the brain during transient ischemia or hypoglycemia [96].

Another piece of evidence for the involvement of MPTP in the induction of apoptosis was, obtained in the study of the effect of betulinic acid (BetA). BetA has antitumor activity in vitro and in vivo, but did not show toxicity to non-transformed cells. BetA induces release of cytochrome c resulting from the induction of MPTP opening, which is only temporarily suppressed by the anti-apoptotic members of the Bcl-2 family and is independent from Bax and Bak [97]. Preincubation with CSA prevented apoptosis induced by BetA. Apoptosis in endothelial cells induced by mitomycin C is also mediated by MPTP, since CsA, through interaction with CypD, blocked mitochondrial permeabilization and apoptosis, but at the same time, it causes necrosis that is regulated by ROS and cathepsin D1 [98].

Opening pores dissipates the mitochondrial membrane potential. CsA inhibits the pore, preventing the drop of potential in SH-SY5Y cells [99]. Using stau-

rosporine and aldehyde, 4-hydroxynonenal, as inducers of apoptosis and necrosis, respectively, in PC12 cells, demonstrated that CSA prevents both apoptosis and necrosis, underlying the important role of mitochondrial depolarization in both types of cell death [100].

The ability of CypD inhibitors to prevent apoptosis induced by various inducers suggests that suppression of the synthesis of this protein might protect cells. Nevertheless, in some cases, the cells, in the absence of CypD (e.g., thymocytes, embryonic fibroblasts, hepatocytes isolated from CypD-deficient mice), die by apoptosis in response to various inducers, such as etoposide, staurosporine, and TNF-α [83–86]. CypD-deficient mice's intestinal epithelial cells have the same sensitivity to X-ray radiation as do the control cells [83]. Moreover, in some cases, stimulation of CypD expression can prevent apoptosis induced by the overexpression of ANT1 [64]. Overexpression of CypD made HEK293 cells and C6 rat glioma cells more resistant to apoptotic stimuli. The mitochondria of these cells, in contrast to cells expressing a mutant form of CypD that is devoid of peptidyl-prolyl *cis-trans* isomerase activity, had a higher membrane potential. Protection of cells from apoptosis depends on the peptidyl-prolyl *cis-trans* isomerase activity of CypD, but inhibition of this activity does not affect the binding of ANT CypD c [101]. Increased level of CypD, observed in many tumors, may explain the resistance of these cells to apoptosis inducers [66, 102, 103]. Stimulation of CypD expression can differently affect apoptosis and necrosis. Thus, in B50 cells with elevated levels of CypD, nitric oxide (NO) stimulated necrosis, while apoptosis induced by staurosporine or NO was suppressed [102]. It should, however, be kept in mind that at high concentrations of CsA, other targets, e.g., cytoplasmic cyclophilins, can also be affected, particularly Cyclophilin A expression, which is often enhanced in tumor cells [104–108], This might affect apoptosis as well, and therefore, these data should be interpreted with caution [109–111].

The mechanisms of apoptosis suppression by CypD are not clear. It has been suggested that CypD can interact with anti-apoptotic protein Bcl-2, inhibiting cytochrome c release from mitochondria [112]. According to other reports, the degree of apoptosis suppression by CypD correlates with the amount HKII associated with the mitochondria. Apparently, CypD stabilizes binding HKII organelles through peptidyl-prolyl *cis-trans* isomerase activity, thereby providing an anti-apoptotic effect. Inhibition of CypD using CsA or knockout caused detachment of HKII from the mitochondrial membrane [101, 103]. Apoptosis induced by dissociation of HKII is associated with an alteration of the interaction between CypD and ANT [47]. Apparently, the detachment of HKII from the AMM causes conformational changes in the inner membrane, facilitating MPTP opening. Furthermore, inhibition of CypD prevented MPTP opening caused by HKII detachment [47]. Thus, considering CypD as a cell death regulator, we should distinguish the possibility of cell death stimulation due to the interaction with components of MPTP, as well as its protective role, the exact mechanism of which is still to be elucidated.

One of the MPTP regulatory mechanisms is acetylation of CypD. The mitochondrial sirtuins 3 (SIRT3, representative of evolutionarily conserved family of NAD-dependent proteins having deacetylase activity) reduces the level of CypD acetylation, mediating resistance to various types of stress. This prevents the loss of mitochondrial potential and the accumulation of ROS in cells exposed to hypoxia or staurosporine [112]. SIRT3 increases the catalytic activity of the carbonic anhydrase, preventing acidification of cells, activation of Bax and apoptotic cell death. Furthermore, deacetylation of CypD reduces binding HKII with OMM, which stimulates oxidative phosphorylation [113] and inhibits glycolysis and lactate production, thus regulating the cellular response under stress [114]. It was found that CypD deacetylation is carried at the lysine 166 residues. SIRT3 activity is required to prevent mitochondrial dysfunction and cardiac hypertrophy at aging, which allows for the development of new approaches to the treatment of heart disease and helps to delay aging [115].

Numerous data demonstrate the ability CypD to form complexes with the transcription factor p53. The basic function of p53 is to perceive stress signals, such as DNA damage, oxidative stress and ischemia. p53 controls apoptosis through both transcription-dependent and -independent mechanisms [116]. Mitochondrial localization of p53 is considered to be a major event in p53-dependent apoptosis. In response to stress, a part of p53 migrates to the mitochondria, forming a complex with mitochondrial HSP 70 that precedes the drop of membrane potential, release of cytochrome c and caspase-3 activation [117]. Later, it was shown that on OMM, p53 could bind to Bcl-2 and BclXL through its DNA-binding domain, thereby inducing OMM permeabilization [118]. Mechanisms of translocation of p53 into mitochondria, for a long time, could not be elucidated. There is evidence that p53 monoubiquitination, mediated by the E3-ubiquitin ligase Mdm2, promotes translocation of p53 into mitochondria and stimulation of apoptosis. Once delivered into mitochondria, p53 undergoes de-ubiquitination by mitochondrial protease HAUSP, which forms a complex with p53 [119]. Formation of the complex between p53-CypD can cause not only apoptosis, but also necrosis [120]. Thus, in response to oxidative stress, p53 accumulates in the mitochondrial matrix, stimulating the opening of MPTP by binding to CypD. The protective effect of CsA in these conditions, to some extent, can be explained by the prevention of the formation of the complex p53-CypD [120–124].

Permeabilization of mitochondria not only initiates apoptosis or necrosis, but also can participate in

autophagy, since the opening of a nonspecific pore causes mitochondrial depolarization and deterioration, as well as their further utilization in autophagosomes [125]. For example, tomozolomid induces mitochondrial damage and MPTP opening, which leads to autophagy, whereas inhibition of MPTP suppresses autophagy and stimulates apoptosis [126]. Another agent, tocotrienol, causes MPTP-dependent apoptosis and autophagy, while the blockade of MPTP using CsA completely prevents cell death [127]. It is shown that the effect of an antitumor agent—timosaponin A-III—on mitochondria leads to MPTP opening and stimulation of autophagy preceding apoptosis [128]. Thus MPTP plays an important role in autophagy, and inhibition CypD prevents mitochondrial permeabilization, depolarization, and autophagosomal proliferation [129, 130].

OTHER POSSIBLE COMPONENTS OF MPTP

In addition to the main components considered, there are other regulators and members of MPTP, the role and functions of which have been less studied. It has been suggested that one of the components may be MPTP mitochondrial phosphate carrier (PiC) [131, 132] (Fig. 1b). Probably, PiC binds to CypD and ANT, which can be prevented by CsA [131]. Binding PiC with ANT1 is stabilized in the presence of apoptosis inducers. Suppression of PiC expression prevented cytochrome c release from mitochondria and cell death. It is known that the pore opening, inhibitor Ro68-3400 can affect the mitochondria lacking VDAC1 [56]. Apparently, this is due to the ability of Ro68-3400, as well as another ubiquinone analogue, UQ0, to inhibit phosphate carrier (similarly to N-ethylmaleimide) at the same concentrations as the inhibition of the pores, to interact with ANT and PiC, and to induce the transition of ANT into "m" conformation [131]. Nevertheless, the issue of PiC participation remains open, since according to recent studies, suppression of PiC expression by 65–80% in HeLa cells did not affect Ca^{2+} accumulation and the opening of the pore [133].

In 2002, it was found that apoptosis induced by TNF may be suppressed not only by CsA or stimulation of Bcl-2expression, but also by oligomycin, an inhibitor of H+-ATP-synthase. The effect of oligomycin is not associated with changes in mitochondrial membrane potential or inhibiting synthesis/hydrolysis of ATP [93]. Later the participation of the mitochondrial F1/FO-ATR-synthase in stimulation of pore opening was confirmed [134–138]. ATP-synthase is capable of forming a complex with ANT and PiC, so-called ATP-synthasome [139–141], which makes ATP production more efficient. In addition to the ANT and PiC, ATP synthase interacts with CypD, acclaimed as the regulator of pore opening. CypD-ATP synthase complex formation is modulated by phosphate, which enhances CypD binding and reduces ATP synthase enzyme activity. CsA prevents formation of the complex, restoring the activity of ATP synthase [138]. Association of Bcl-2 family proteins, such as Bcl-X_L, with ATP synthase was documented in a number of studies [142–144]. This interaction increases synthetic activity of the ATP synthase [142, 144]. Similarly, binding of the processed protein Mcl-1, localized in the mitochondrial matrix, promotes ATP synthase oligomerization, activates the respiratory chain, which leads to increased membrane potential and stimulates ATP production [143].

Recently, it was shown that that the ring of c-subunits of F1/FO-ATR synthase forms a voltage-dependent channel, and the persistent opening of which led to a rapid and uncontrolled depolarization of the inner mitochondrial membrane [145]. Isolated monomers of ATP synthase, reconstituted into lipid vesicles, can generate currents upon binding to CypD in the presence of Ca^{2+}. Furthermore, vesicles enriched with ATP-synthase can generate currents that are sensitive to Ca^{2+} ions and CsA. Depletion of the c-subunit abrogates sensitivity to Ca^{2+}. Ca^{2+}-inducible mitochondrial swelling may detach subunit F1 from FO, which can be prevented by CsA. The authors assumed that the ring of c-subunits could be rearranged during MPTP induction in such a way that its diameter is increased to form a non-specific channel [145]. Thus, it can be concluded that the ring of c-subunits of F1/F0-ATP-synthase can be a central component of MPTP (Fig. 1c). The results indicate that in healthy cells, the probability of the closed state of c-subunit channel is higher, which enhances the efficiency of their function.

According to another hypothesis, proteins that form the MPTP complex may belong to a family of transporter proteins of the inner mitochondrial membrane [146]. For example, the Tim23 channel is mainly in the open state, while the intermembrane hydrophilic domain of Tim50 induces its oligomerization and closing, maintaining a permeability barrier and electrochemical proton gradient in mitochondria [147]. Tim50 downregulation increases the sensitivity of cells to death signals, as well as the stimulation of the release of cytochrome c and other pro-apoptotic proteins from mitochondria [148]. F0F1-ATPase modulates the interaction between Tim23 and complexes III–IV [149], which are also associated with CypD [138]. Hence, the regulation of MPTP by various modulators does not preclude the assumption that Tim23 and/or Tim22 can serve as components of MPTP.

THE ROLE OF MPTP IN CELLULAR PATHOLOGIES

Pore opening can occur under normal physiological conditions, especially in mitochondria located close to the calcium "hot spots" (microdomains, in

which the local concentration of calcium may be high enough to induce calcium overload and subsequent opening of pores). Apparently, the periodic acute opening of MPTP (dynamic state of the opening/closing) is important for the normal functioning of mitochondria, in particular, for the regulation of the mitochondrial Ca^{2+} content [150] or for the rapid exchange of cofactors and metabolites between the matrix and intermembrane space and cytosol. Under certain conditions, MPTP controls mitochondrial homeostasis through the increase of the permeability of the mitochondrial inner membrane for proton, water and dissolved compounds with a molecular weight of less than 1500 Da. Adenine nucleotides, matrix pH, membrane potential and the redox state regulate the physiological functions of MPTP [15]. Deregulation of the barrier properties of mitochondria can cause a number of diseases of various etiologies.

Mitochondria are involved in the pathogenesis of more than 40 human diseases [18]. CsA inhibits MPTP opening-dependent dysfunctions, including heart damage due to ischemia/reperfusion injury [151], damage to brain cells caused by ischemia, hypoglycemia [152, 153], muscular dystrophy caused by lack of collagen VI [154], amyotrophic lateral sclerosis, [155] hepatorenal toxicity [156], hepatotoxicity induced by TNF-α [157], hepatocarcinogenesis [158], and others. Ischemia alone does not stimulate MPTP opening, but rather, it creates conditions facilitating its opening at the subsequent reperfusion [159]. Prevention of MPTP opening attenuated NAD^+ depletion, caused by ischemia and reperfusion [160]. The damage during ischemia/reperfusion may switch on multiple modes of cell death. The use of caspase inhibitors [161, 162], inhibitor of necroptosis Nec1 [163], blocking the Ask1 pathway (activator of S-phase kinase) [164] led to an attenuation of damages caused by ischemia/reperfusion. It can be assumed that ischemia/reperfusion induces cell death, which can be regulated by several mechanisms. Probably, the various types of cell death can be initiated by various conditions of ischemia.

It was shown that CsA protects hepatocytes in vivo from the toxic action of ethanol, lipopolysaccharides *Streptococcus*, anti-Fas-immunoglobulins, acetaminophen or diclofenac, injury by ischemia/reperfusion and others. CsA protects nerve cells from damage caused by hyper- and hypoglycemia, ischemia, prevented photoreceptor apoptosis and also cell death caused by axotomy of motoneurons. Moreover, in mice with the *Ppif* gene (which encodes CypD) knockout, the damage caused by middle cerebral artery occlusion was significantly reduced [165].

MPTP can play a key role in muscular dystrophy with disorders related to collagen VI. Mutations in the collagen VI gene cause the development of Ullrich syndrome and Bethlem myopathy in humans. In mice with a lack of collagen VI, the phenotype is similar to Bethlem myopathy symptoms. CsA maintains the normal muscle ultrastructure and largely suppresses apoptosis [154].

Nonspecific pore formation is observed in Alzheimer's disease, when β-amyloid peptides interact with CypD after accumulation in mitochondria. Suppression of CypD expression prevented amyloid peptide-mediated activation of MAPK-dependent signaling pathways, mitochondrial damage, loss of synapses, and improved the function of synapses [166, 167]. A similar situation was observed in such aging-related neurodegenerative diseases as Parkinson's disease, in which incorrectly assembled protein α-synuclein accumulates in the cells. Interaction of synuclein oligomers with mitochondria, in particular with ANT and VDAC, can stimulate pore opening, the release of cytochrome c and activation of the apoptotic cascade. The opening of the pores and cell death were partly prevented with BKA, an inhibitor of ANT that confirms MPTP participation in the development of Parkinson's disease [168, 169].

It is known that tumor cells are considerably resistant to OMM permeabilizaiton. This resistance leads to the inhibition of mitochondria-dependent apoptosis. In tumor cells, the ratio of expression of proteins, either that are components of MPTP or are associated with it (peripheral benzodiazepine receptor, HKII, mtCK, CypD, VDAC, ANT2), is distorted [18]. For example, both CypD [66] and VDAC, which promotes binding of HK mitochondria [170] in tumors of breast, ovary, and the uterus overexpressed. It should also be noted that in many types of tumors, the expression of HKII is enhanced; the content of PBR is elevated in tumors of the breast, ovary, liver, colon, etc. In renal tumors and some other tumors, overexpression of ANT2 was observed [15], which in contrast to ANT1, does not stimulate MPT induction. Under these conditions, therapeutic strategies aimed at destabilizing mitochondria, and stimulating the MPTP opening can lead to the elimination of tumor cells that are resistant to conventional therapy.

CONCLUSIONS

Many of the mechanisms of MPTP regulation are known, but further study of the basic components and their functions as pore regulators is necessary. This will undoubtedly contribute to finding optimal strategies for the treatment of diseases associated with mitochondrial destabilization due to the opening of the pores, in particular neurodegenerative diseases. On the other hand, specific targeting of components of MPTP complex in tumors, directed towards the stimulation of mitochondrial permeabilization and their destabilization, could be used to overcome resistance of cancer cells to chemotherapy.

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