*Review Article*

# Edaravone and cyclosporine A as neuroprotective agents for acute ischemic stroke

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It is well known that acute ischemic stroke (AIS) and subsequent reperfusion produce lethal levels of reactive oxygen species (ROS) in neuronal cells, which are generated in mitochondria. Mitochondrial ROS production is a self-amplifying process, termed "ROS-induced ROS release". Furthermore, the mitochondrial permeability transition pore (MPTP) is deeply involved in this process, and its opening could cause cell death. Edaravone, a free radical scavenger, is the only neuroprotective agent for AIS used in Japan. It captures and reduces excessive ROS, preventing brain damage. Cyclosporine A (CsA), an immunosuppressive agent, is a potential neuroprotective agent for AIS. It has been investigated that CsA prevents cellular death by suppressing MPTP opening. In this report, we will outline the actions of edaravone and CsA as neuroprotective agents in AIS, focusing on their relationship with ROS and MPTP.

**Key words:** Acute ischemic stroke, cyclosporine A, edaravone, mitochondrial permeability transition pore, reactive oxygen species

## INTRODUCTION

STROKE IS THE fourth leading cause of mortality and disability in Japan.[1] According to the World Health Organization, 15 million people suffer strokes worldwide each year. Of these, 5 million die and another 5 million are permanently disabled.[2] Many clinical and preclinical studies of neuroprotection in stroke have been undertaken. However, few studies have shown any clinical benefit. At present, thrombolytic therapy for acute ischemic stroke (AIS) with i.v. recombinant tissue-plasminogen activator (tPA) and endovascular thrombectomy using a stent retriever are the major effective treatments. Edaravone, a free radical scavenger, is the only neuroprotective agent for AIS used in Japan.[3]

It is well known that AIS can lead to excessive $Ca^{2+}$ influx and formation of reactive oxygen species (ROS), causing the death of neuronal cells due to mitochondrial dysfunction.[4] Edaravone was developed in Japan and approved there in 2001 as the first neuroprotective agent in the world. Clinical trials for edaravone were implemented mainly in Asian countries. It was shown that it captures and reduces excessive ROS, preventing brain damage,[5] and thus its administration to AIS patients has been recommended in Japanese guidelines for stroke management.[3] Recently, the US Food and Drug Administration granted a license for edaravone for the treatment of amyotrophic lateral sclerosis in the USA.[6] Its indication is not limited to AIS, but it is starting to be applied in neurodegenerative diseases.

There are several neuroprotective agents considered to follow edaravone. Cyclosporine A (CsA), an immunosuppressive agent, is attracting attention as a drug that suppresses mitochondrial dysfunction resulting from the formation of ROS.[7] Like edaravone, CsA's use as a neuroprotective agent has been studied by many Japanese researchers. Clinical research on CsA has been carried out mainly in France, and future development is expected.[8]

In this report we will outline the actions of edaravone and CsA as neuroprotective agents in AIS, focusing on their relationship with ROS and mitochondrial functions.

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## REACTIVE OXYGEN SPECIES FORMATION IN AIS AND REPERFUSION

ACUTE ISCHEMIC STROKE and subsequent reperfusion produce lethal levels of ROS (Fig. 1), mainly in

neurons. The main source of ROS is neural mitochondria.[9] The electron transport chain (ETC) complexes in mitochondria constantly generate superoxide anions ($O_2^{\cdot-}$) and hydrogen peroxide ($H_2O_2$; Fig. 2), while cell functions are regulated by endogenous antioxidants, such as superoxide dismutase, catalase, and glutathione peroxidase, which serve as the ROS removal system. However, when an ischemic stroke occurs, the supply of oxygen and glucose to brain tissue is disturbed by blood flow blockage, and as such, the energy to maintain neuronal ionic gradients is impaired. Rapid depletion of energy after ischemia leads to a loss of membrane potential and depolarization. Voltage-dependent $Ca^{2+}$ channels are then activated, and excitatory amino acids are released into the extracellular space. The binding of glutamate to N-methyl-D-aspartate and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors promotes a further increase in $Ca^{2+}$ entry.[10] High levels of intracellular $Ca^{2+}$, $Na^+$, and adenosine diphosphate lead to a collapse of ETC complexes in mitochondria, generating excessive ROS such as $O_2^{\cdot-}$ and $H_2O_2$.[11] Although $H_2O_2$ itself is not radical, it reacts with metal ions, such as $Fe^{2+}$ and $Cu^{2+}$, generating hydroxy radicals ($HO^{\cdot}$), which have stronger reactivity. When blood flow resumes and reperfusion occurs, excessive ROS are explosively produced, exceeding the capacity of the ROS removal system and leading to the progression of irreversible cell damage.

Electron transport chain complexes have been implicated as both sources and targets of ROS generated during ischemia reperfusion.[9] Mitochondrial ROS production is a self-amplifying process, termed "ROS-induced ROS release" (RIRR). Furthermore, mitochondrial permeability transition pore (MPTP) is deeply involved in this process[12] and may cause cell death after the AIS. Xanthine oxidase, nicotinamide adenine dinucleotide phosphate oxidase, lipoxygenases, and monoamine oxidase are also important sources of ROS in AIS and reperfusion.[9] Excessive ROS in ischemic stroke can react with protein molecules and cause protein oxidation, peptide bond cleavage, and protein degradation. Reactive oxygen species also causes lipid peroxidation, the oxidative degradation of lipids, which is more damaging than protein oxidants to cells. Reactive oxygen species breaks DNA double strands, resulting in intra- and inter-strand cross-links, protein-DNA mutations, and DNA structural changes, and precedes DNA fragmentation.[13] Increased amounts of ROS damage all cellular components, including neurons, glial cells, nerve fibers, and blood vessels (Fig. 3).[14]

In AIS, the abnormal ROS formation may trigger the detrimental process to neuronal cell death. Therefore, it is important for neuroprotection treatments to capture and reduce excessive ROS in the infarcted brain region.

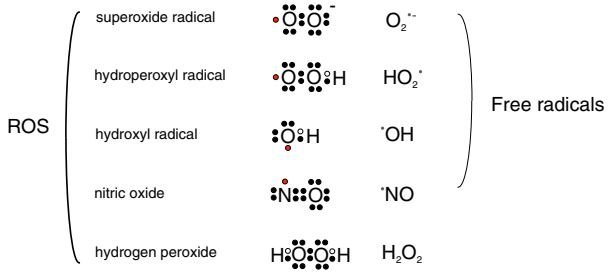


**Fig. 1.** Definition of reactive oxygen species (ROS) and free radicals. Typical molecules are expressed by common name, Lewis structure, and chemical formula. A red dot on the Lewis structure means unpaired electrons. Reactive oxygen species are species that contain one or more oxygen atom and are much more reactive than molecular oxygen. The ROS that contain nitrogen are called reactive nitrogen species. A free radical is each molecule or its fragment that can exist independently and contains one or two unpaired electrons (adapted from reference 58).

## EDARAVONE IN ACUTE ISCHEMIC STROKE

THE DEVELOPMENT OF edaravone was primarily carried out by Mitsubishi Tanabe Pharma Corporation (Osaka, Japan). They noticed that phenolic compounds had a strong radical scavenging action, which contributed to the discovery of edaravone.[5,15] Under physiological conditions, approximately 50% of edaravone exists as edaravone anion, which donates electrons to eliminate various radicals. The edaravone radical generated at that time reacts with oxygen molecules in the reaction system, changing to edaravone peroxyradical, and finally to 2-oxo-(phenylhydrazono)-butanoic acid, a reaction product unrelated to radicals. It is reported that these radical species derived from edaravone are remarkably less reactive than $HO^{\cdot}$ and lipid peroxyl radical ($LOO^{\cdot}$).[5]

## EDARAVONE, FROM LABORATORY TO CLINICAL SETTING

IN 1988, ABE *et al.*[16] reported edaravone (MCI-186) markedly attenuated ischemic and post-ischemic brain swelling in the rat middle cerebral artery occlusion (MCAO) model. Furthermore, a study modeling polyvinyl acetate-induced brain ischemia in rats showed that MCI-186 suppressed brain edema, inhibiting the changes of monoamine metabolism.[17] The effectiveness of edaravone was reported in many subsequent animal experiments. In the late 1990s, a study with the rat MCAO model showed that the infarct

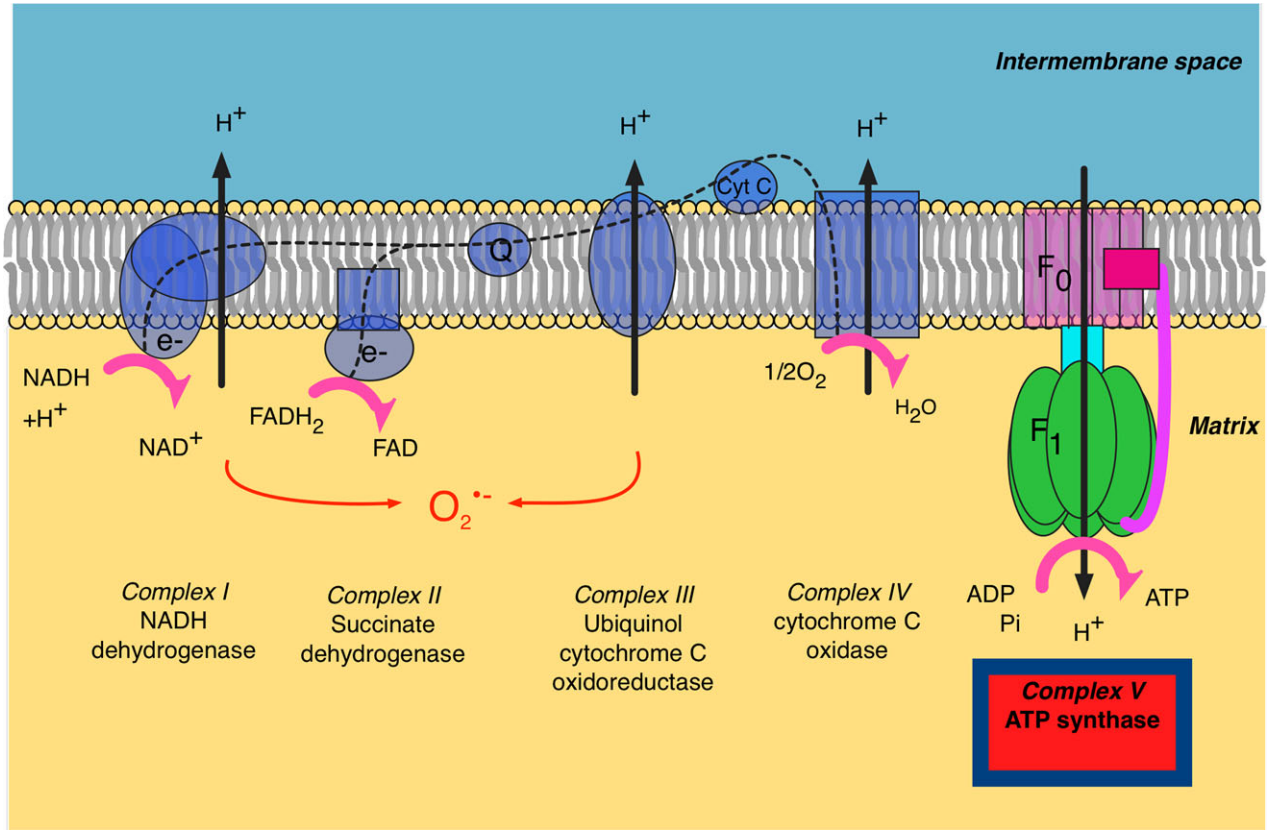


**Fig. 2.** Oxidative phosphorylation and reactive oxygen species production in mitochondria. The diagrams of the mitochondrial inner membrane show key components of the electron transport chain (ETC). Reducing equivalents of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) produced from the tricarboxylic acid cycle feed electrons to the ETC along the mitochondrial inner membrane. The electrons flow through the ETC with the following sequence: complex I and II→coenzyme Q (Q)→complex III→cytochrome C (Cyt C)→complex IV→$O_2$, during which coupled redox reactions drive $H^+$ across the inner membrane, forming the proton gradient and the negative mitochondrial membrane potential ($\Delta\Psi_m$ = −150 to −180 mV). The free energy is stored in the proton gradient, and $\Delta\Psi_m$ then drives $H^+$ through the mitochondrial ATP synthase (complex V), converting adenosine diphosphate to ATP. An estimated 0.1–1% of the electrons leak prematurely to $O_2$ at complexes I, or III, resulting in the formation of superoxide ($O_2^{\cdot-}$).[59]

volume was significantly decreased and the neurological deficits were also significantly decreased by 3 mg/kg edaravone given i.v. after MCAO.[18] In the rat forebrain ischemia model, post-ischemic treatment of edaravone significantly suppressed delayed neuronal death, indicating the effectiveness of edaravone in a reliable rat model of focal and global ischemia.[19] At the same time, clinical trials of edaravone began in Japan.

The study reported by Edaravone Acute Infarction Group in 2003 was a randomized, placebo-controlled, double-blinded multicenter trial among 250 subjects, in which 30 mg edaravone was given twice daily for 14 days in patients with AIS. The edaravone-treated group had significantly lower modified Rankin scores (MRS), which is an outcome measure of disability, compared to the placebo-treated group. The study examined all patients 3, 6, and 12 months after stroke onset, and showed that MRS was significantly lower in the treatment group.[20] In 2005, a retrospective study in 70 patients with lacunar infarction investigated patients who received 30 mg edaravone twice daily for 14 days and were evaluated on the National Institute of Health Stroke Scale as an outcome of clinical status and MRS. The authors concluded that there was a significant odds ratio effect in patients that underwent edaravone treatment.[21]

Numerous clinical trials were also completed in China and India. In 2015, two systematic reviews were published. Yang *et al.*[22] reviewed the effectiveness of edaravone on

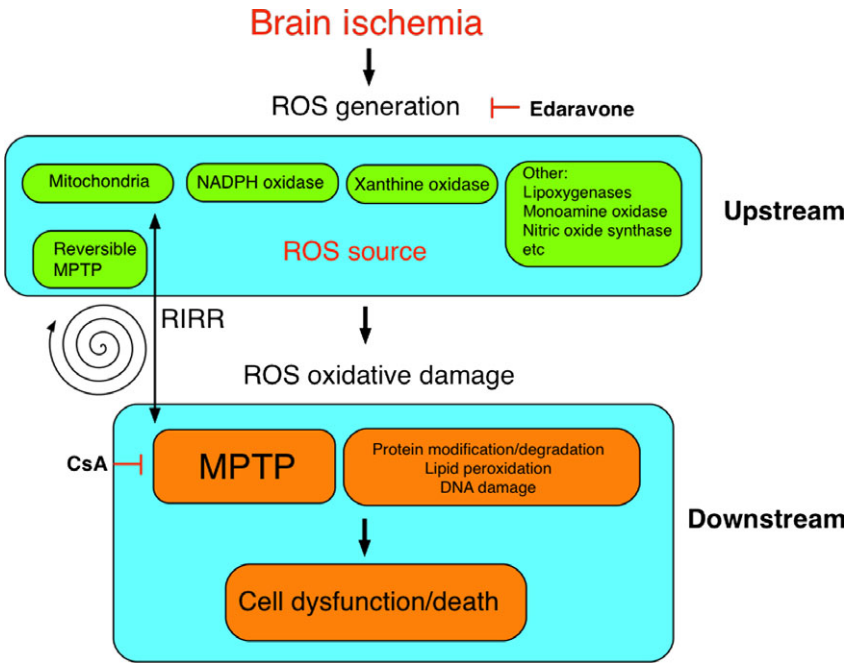


**Fig. 3.** Reactive oxygen species (ROS) generation and oxidative damage by brain ischemia. Brain ischemia induces ROS generation (upstream) and neurons are damaged by ROS (downstream), resulting in dysfunction or cell death. The mitochondria electron transport chain (ETC), together with the mitochondrial permeability transition pore (MPTP), produce cyclical ROS bursts, a process termed "ROS-induced ROS release". Edaravone scavenges upstream ROS, whereas cyclosporine A inhibits downstream MPTP opening (adapted from reference 13).

ischemic stroke and intracerebral hemorrhage (ICH), and noted that edaravone was beneficial in improving neurological impairment resulting from AIS and ICH, although currently there is not enough evidence that edaravone reduces death or long-term disability against AIS and ICH. Furthermore, Feng *et al.* extracted and investigated three clinical trials including the study by Edaravone Acute Infarction Study Group mentioned above. They found that edaravone could improve neurological impairment in the treatment of AIS.[23] However, they also noted that the observed effects might have been due to bias rather than a true biological effect; further randomized controlled studies are justified to assess the efficacy and safety of edaravone for patients with AIS. Therefore, although edaravone's effect on AIS has been recognized in Japan, it has not reached the global standard treatment, and it is necessary to undertake a large-scale clinical trial.

## CYCLOSPORINE A, A POTENTIAL NEUROPROTECTIVE AGENT FOR AIS

OVER THE PAST several decades, the immunosuppressant CsA, which is widely used in patients with organ transplantation, was found to suppress organ dysfunctions caused by ischemia–reperfusion injury. However, the first clinical trial studying CsA's effects in the human brain was in 2006, using patients with traumatic brain injury (TBI).[24]

## ANIMAL EXPERIMENTS WITH CsA

IN THE EARLY 1990s, an anti-ischemic effect of CsA in a model of transient MCAO was reported, in which the animals were treated with an oral dose of CsA for 1 week before the insult to reduce infarct volume and edema formation. Interestingly, the authors were also the first to report the effectiveness of edaravone.[25] They discussed that, as CsA has no permeability of the blood–brain barrier (BBB), its effectiveness could be the result of an immunological reaction of CsA against calcium and mitochondria. Uchino *et al.*[26] then reported that delayed neuronal death was suppressed by 10 mg/kg CsA given i.p. in a rat global ischemia model. However, as CsA does not pass the BBB, a needle insertion to the brain parenchyma which hypothetically disrupts the BBB was implemented. They concluded that the anti-ischemic effect of CsA seemed larger than that of any other drug proposed for global ischemia.

A few years later, a study reported that 20 mg/kg CsA given i.v. in a rat MCAO model reduced cortical damage.[27] In this model, researchers first injected endothelin, a potent vasoconstrictor, into the brain parenchyma, which resulted in CsA passing the BBB.

In 1999, Matsumoto *et al.*[28] reported that CsA 10 mg/kg given i.p. immediately after reperfusion and again at 24 h after reperfusion, diminished infarct size in a rat model of 2-h transient MCAO. In this model, the administration of CsA after the infarction showed anti-ischemic effects without the needle insertion to disrupt the BBB. Past experiments reported that 2 h of MCAO disrupted the BBB after 4–6 h of reperfusion in rats.[29] In our case, CsA given i.p. immediately after reperfusion reaches the brain parenchyma, and may salvage the penumbra and even the central core. However, when CsA was given immediately after occlusion, there was no effect either i.p. or i.v. These results indicate that CsA given immediately after the occlusion might not have reached adequate therapeutic concentration after BBB disruption, showing that dosage and timing of CsA should be controlled very strictly. In the rat MCAO model, the interval between BBB disruption and the time point at which ischemic damage becomes irreversible may be the exact therapeutic window for CsA.[30] This is considered to be similar in human stroke–reperfusion cases.

## ADMINISTRATION OF CsA has many restrictions

RESTRICTION OF CSA passage through an intact BBB is caused by active outward transport by p-glycoproteins in brain capillary endothelial cells.[31] One of the p-glycoproteins constituting the BBB is multidrug resistance 1a (mdr1a), also called ATP-binding cassette transporter B1.[32] We used an mdr1a knockout mouse, in which the BBB was not functional, and created an MCAO to examine the effectiveness of CsA. Cyclosporine A's effectiveness can be usually obtained at a dose of 10 mg/kg. However, while infarct size diminished at 1 mg/kg given i.p., it actually increased at 10 mg/kg. When CsA is used in the clinical treatment of cerebral ischemia, it should be noted that the effectiveness depends on its dose and method; otherwise, there is a risk of increasing brain damage.[33]

Borlongan *et al.*[34] administered 1 mg/kg CsA i.p. with 10 mg/kg methylprednisolone in rat MCAO models and undertook behavioral testing (passive avoidance test) until 3 days after onset of infarction and found significantly favorable results. The results were similar to the 10 mg/kg CsA i.p. group. This study showed that clinical benefits could be expected even at low-dose administration of CsA, and it revealed the possible validity of subsequent research in human subjects.

## CLINICAL USE OF CsA in the human brain

THE CLINICAL USE of CsA in brain damage was introduced carefully due to the difficulty of administration shown in animal experiments. As mentioned above, Empey *et al.*[24] analyzed the phase II dose-escalation trial in patients with TBI to identify the predictive dosing strategies for CsA. Within 8 h of injury, the patients were randomized into three cohorts and received one of three doses of CsA (0.625, 1.25, or 2.5 mg/kg) or placebo i.v. every 12 h for 72 h. Cyclosporine A blood concentrations and its pharmacokinetic parameters were determined. The researchers concluded that acute, severe TBI patients show a more rapid clearance and a larger distribution volume of CsA. In the subsequent clinical trial in patients with TBI, there was no improvement in the functional outcome, whereas serious side-effects of CsA were not reported in patients who received 5 mg/kg CsA infused over 24 h.[35]

Another recent clinical study in France was a multicenter, single-blinded, controlled trial, in which 127 patients aged from 18 to 85 years who underwent thrombolytic therapy after anterior-circulation stroke received i.v. bolus injections of 2 mg/kg CsA or placebo treatment.[8] Infarct volume was measured by magnetic resonance imaging after 30 days, and examined by whether the arterial occlusion was proximal or distal, as well as whether or not recanalization occurred in thrombolysis. Cyclosporine A was generally not effective in reducing infarct size. However, in patients with proximal cerebral artery occlusion and efficient recanalization, the infarct volume was significantly smaller than the placebo group. Although effectiveness was not confirmed as class I evidence, the study provided hope for larger clinical trials in the future as effectiveness was confirmed in some individuals with stroke.[7]

## ACTION MECHANISM OF CsA

THE ACTION OF CsA as a neuroprotective agent can be roughly divided into two mechanisms. One is mediated by calcineurin, which brings about initial immunosuppression, and the other is mediated by MPTP suppression, which is not related to immunosuppression.

In the immunosuppression of CsA, CsA connects with immunophilins, which are endogenous cytosolic peptidyl–prolyl isomerases. These complexes inhibit the action of calcineurin and suppress the transcription of nuclear factor-activated T cells, which then suppresses the expression of interleukin-2 and T-cell activation.[36] FK506, known as an immunosuppressive drug, is considered to have a similar action mechanism.

Apart from elucidating the immunosuppressive effect, many studies have shown that both CsA and FK506 have anti-cerebral ischemic effects,[27,37] and thus the action of both agents are inevitably combined with immunophilins, suppressing the action of calcineurin. Calcineurin activates NO synthase through dephosphorylation, which leads to increased NO levels and induces neurotransmitter release.[38] Cyclosporine A and FK506 inhibit N-methyl-D-aspartate-induced cell death of primary cortical cultures by increasing NO synthase phosphorylation, and decreasing the level of reactive NO species. Furthermore, calcineurin indirectly regulates the transcription factor cAMP response element binding protein and is involved in the neuronal viability mediated by brain-derived neurotrophic factor.[37]

However, it was suggested that suppression of MPTP, another pharmacological action of CsA, could be deeply involved in neuronal death as: (i) FK506 does not show similar improvement compared to CsA in rat hypoglycemia models;[39] (ii) N-Me-Val-4CsA without calcineurin suppression in CsA analog showed the same effect as CsA in both rat MCAO[28] and *in vitro* models.[40]

## CYCLOSPORINE A NEUROPROTECTIVE ACTION TO SUPPRESS MPTP OPENING

IN THE LATE 1970s, osmotic swelling was observed when calcium ions were loaded in mitochondria, isolated from hepatocytes.[41] This was due to the non-specific pores, called MPTP, located in the inner membrane of mitochondria.[42] The MPTP opening allows molecules of <1.5 kDa to pass through the mitochondrial inner membrane, triggering cell death.[43] It was already reported in the 1980s that MPTP is suppressed by CsA and its analogs.[44]

Acute ischemic stroke and reperfusion induce $Ca^{2+}$ influx in neurons and generate detrimental ROS. The combination of high $Ca^{2+}$ concentration and oxidative stress, together with the elevated phosphate and depletion of adenine nucleotides, provide ideal conditions for the MPTP to open.[45]

A member of the cyclophilin (CyP) family and a component of the mitochondrial matrix, CyP-D is the essential regulator of the MPTP opening.[46] Although it is unclear how CyP-D becomes activated to induce MPTP, its prolyl-isomerase activity plays a crucial role. Cyclosporine A strongly and specifically prevents MPTP opening by binding to CyP-D, inhibiting its isomerase activity and displacing it from the MPTP.[47]

The MPTP opening is generally reversible,[48] whereas at higher ROS levels, longer MPTP openings might release an ROS burst (i.e., RIRR), leading to the disruption of the inner mitochondrial membrane's permeability barrier. This causes the uncoupling of oxidative phosphorylation, osmotic swelling, and rupture of the outer membrane.[45] The concomitant release of mitochondrial pro-apoptotic proteins to the cytosol, such as cytochrome c and apoptosis-inducing factor, induce cell death. Cytochrome c activates the caspase cascade which induces apoptosis. Apoptosis-inducing factor triggers chromatin condensation and DNA fragmentation in a caspase-independent manner, also causing apoptotic cell death.[49] A recent study showed that under ischemia-

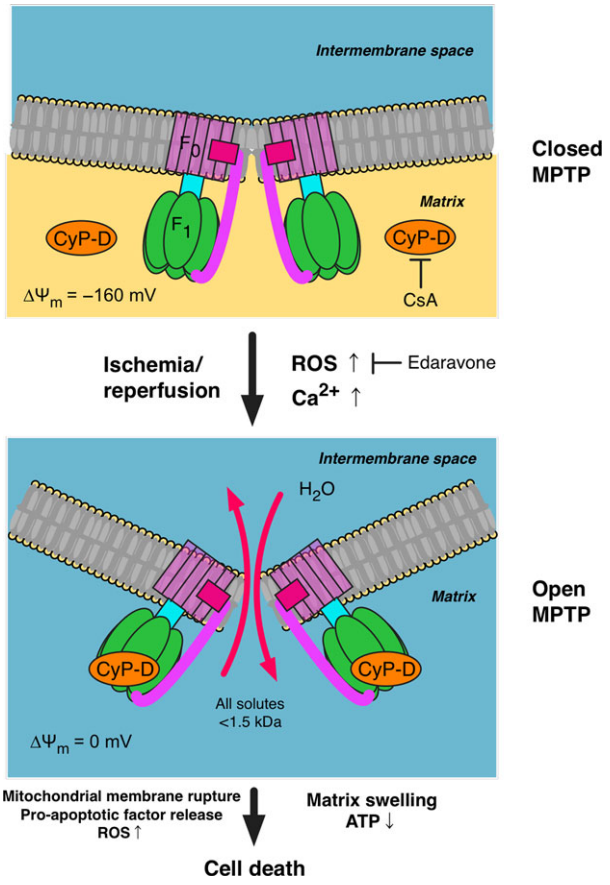


**Fig. 4.** Model of mitochondrial permeability transition pore (MPTP) structure. The MPTP is formed at the interface between two $F_1F_O$ ATP synthase (electron transport chain complex V) dimers. Phosphorylation and acetylation of cyclophilin-D (CyP-D) favor its interaction with ATP synthase and pore opening following increases in $Ca^{2+}$ and reactive oxygen species induced by ischemia and reperfusion. After the MPTP opens, molecules <1.5 kDa and water freely pass between the mitochondrial intermembrane. $\Delta\Psi_m$ turns to 0 mV, and subsequently, the mitochondrial matrix swells and membrane rupture occurs. Finally, pro-apoptotic factors are released to the cytosol. As a result, MPTP induces cell death (adapted from reference 60). CsA, cyclosporine A.

associated oxidative damage, transcriptional activator p53, together with CyP-D, opens MPTP, leading to necrosis, which CsA also suppresses.[50] These are considered to be CsA's strong anti-ischemic mechanisms (Fig. 4).

## EDARAVONE ALSO SUPPRESSES THE MPTP OPENING

TAKAYASU *et al.*[51] reported that edaravone suppressed swelling of mitochondria isolated from rat brain, which occurred due to loading $Ca^{2+}$ and $H_2O_2$, as well as CsA. Similar experiments were carried out earlier using rat hearts, and the results showed that edaravone significantly suppressed swelling due to $Ca^{2+}$ load in rat mitochondria isolated from left ventricular tissue.[52] These results suggest that inhibition of MPTP might account for the neuroprotective effect of edaravone.[51]

## MYSTERIOUS MPTP

THE MOLECULAR STRUCTURE of MPTP is still controversial, and it is one of the latest topics in molecular biology. Currently, the mainstream view is that MPTP is the dimer of the motor enzyme F1F0ATP synthase (known complex V of ETC) that rotates at high speed in the mitochondria inner membrane (Fig. 4).[53,54] ATP synthase is an important enzyme that supplies most of the ATP consumed by cells, and Boyer and Walker received the Nobel Prize in Chemistry in 1997 for finding this enzyme to be the rotary motor.[55,56] However, the essential question of why ATP synthase needs to rotate remains unsolved. It is very interesting that this mysterious enzyme forms a gate that determines the life and death of cells. Future development of research could reveal the structure and mechanisms of MPTP.

## CONCLUSION

ACUTE ISCHEMIC STROKE and subsequent reperfusion generate ROS in neuronal cells, opening MPTP, and leading to cell death. In this series of processes, edaravone suppresses ROS upstream, inhibiting harmful events occurring due to ROS, and in turn, potentially suppressing the opening of MPTP. Cyclosporine A works on the main body of the MPTP to inhibit its opening. Consequently, both edaravone and CsA inhibit neuronal cell death. Currently, the primary pharmacological treatment for AIS is endovascular thrombectomy using thrombolytic therapy by i.v. injection of tissue-plasminogen activator. Although there are great possibilities for treatments combining this method with neuroprotective agents, such treatment has not yet been established. Edaravone was recognized as a neuroprotective agent in Japan, but a larger clinical trial is required for it to be accepted internationally.

Pharmacological effects of CsA on cerebral ischemia have been firmly evaluated at the laboratory level, although there is still room for discussions such as dosage and treatment periods in clinical practice. However, as an immunosuppressive agent, CsA has an abundant history of administration in patients, and several analogs are also being developed.[40,57] There is a possibility that it will become a world-recognized neuroprotective agent when further research is advanced in the future.

## DISCLOSURE

Approval of the research protocol: N/A.
Informed consent: N/A.
Registry and the registration no. of the study/trial: N/A.
Animal studies: N/A.
Conflict of interest: None declared.

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