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Invited Review

Molecular mechanisms of neuroprotective action of immunosuppressants - facts and hypotheses

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Abstract

Cyclosporin A (CsA) and FK506 (Tacrolimus) are short polypeptides which block the activation of lymphocytes and other immune system cells. Immunosuppressants exert neuroprotective and neurotrophic action in traumatic brain injury, sciatic nerve injury, focal and global ischemia in animals. Their neuroprotective actions are not understood and many hypotheses have been formed to explain such effects. We discuss a role of drug target - calcineurin in neuroprotective action of immunosuppressants. Protein dephosphorylation by calcineurin plays an important role in neuronal signal transduction due to its ability to regulate the activity of ion channels, glutamate release, and synaptic plasticity. *In vitro* FK506 protects cortex neurons from NMDA-induced death, augments NOS phosphorylation inhibiting its activity and NO synthesis. However, *in vivo* experiments demonstrated that FK506 in neuroprotective doses did not block excitotoxic cell death nor did it alter NO production during ischemia/reperfusion. Tissue damage in ischemia is the result of a complex pathophysiological cascade, which comprises a variety of distinct pathological events. Resident non-neuronal brain cells respond rapidly to neuronal cell death and may have both deleterious and useful role in neuronal damage. There is increasing evidence that reactive gliosis and post-ischemic inflammation involving microglia contribute to ischemic damage. We have demonstrated that FK506 modulates hypertrophic/proliferative responses and proinflammatory cytokine expression in astrocytes and microglia in vitro and in focal transient brain ischemia. Our findings suggest that astrocytes and microglia are direct targets of FK506 and modulation of glial response and inflammation is a possible mechanism of FK506-mediated neuroprotection in ischemia.

Keywords: FK506 • cyclosporin A • calcineurin • neuroprotection • microglia • proinflammatory cytokines • ischemia

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Introduction

Cyclosporin A (CsA) and FK506 (Tacrolimus) are short polypeptides which have revolutionized transplantology due to ability to block the activation of lymphocytes and other immune system cells. They are routinely used to impede graft rejection in transplantation of organs and bone marrow. In cells CsA and FK506 bind to specific intracellular proteins called immunophilins: CsA binds to cyclophilin and FK506 binds to FKBP (FK506-binding protein). Both types of complexes bind to regulatory subunit of calcineurin (phosphatase dependent on Ca^{2+} and calmodulin) and thus inhibit its activity (Fig. 1) [1-3]. Rapamycins are macrocyclic lactones that possess immunosuppressive, antifungal and antitumor properties. Rapamycin associates with immunophilin FKBP12, and this complex binds and inhibits the function of mTOR (mammalian target of rapamycin) a serine/threonine (Ser/Thr) kinase. In cells of the immune system calcineurin dephosphorylates the transcription factor NFAT (Nuclear Factor of Activated T Cells) that regulates expression of cytokines such as Interleukin-2, Interleukin-3, GM-CSF (Granulocyte Macrophage Colony Stimulating Factor), TNF (Tumor Necrosis Factor), and Fas ligand [1]. Calcineurin is the crucial enzyme engaged in an activation of immune system cells and thus inhibition of its activity is the main mechanism of immunosuppressive action of CsA and FK506 [2-3].

The immunophilins are abundant in the brain where they are colocalized with the Ca^{2+}-activated phosphatase calcineurin [4]. In addition to their immunosuppressive properties, CsA and FK506 may exert a powerful neuroprotective and neurotrophic action [5,6].

Neuroprotective and neurotrophic effects of CsA and FK506

CsA and FK506 display neuroprotective effect in focal and global ischemia in rats [7-11], gerbils [12,13] and primates [14]. FK506 not only reduces the infarct size but also alleviates the neurological deficits. Cyclosporin A has been shown to decrease the infarct size and the edema when administrated orally before or during a transient ischemia [7], while FK506 was still active as neuroprotectant when given 2 h after ischemia [15,16]. FK506 showed a neuroprotective effect in a primate model of stroke and the therapeutic time window of FK506 was at least 3 h after the onset of middle cerebral artery occlusion (MCAo) [14].

CsA and FK506 are effective in reducing the axonal damage associated with traumatic brain injury (TBI) in a rat impact-acceleration model of TBI [17-19]. FK506 and another immunophilin ligand V-10,367 may mediate neuroprotection and improve axonal regeneration following damage to peripheral nerve fibres [20]. Locally applied FK506 enhanced the sprouting of axotomized central intrinsic neurons such as retinal ganglion cells *in vivo* after optic nerve crush. FK506, CsA, rapamycin and their nonimmunosuppressive analogues promote neurite outgrowth both in PC12 cells and sensory neuronal cultures of dorsal root ganglia with potencies resembling their immunosuppressive homologues [21-23]. FK506 and its derivative L-685,818 treatment of rats with crushed sciatic nerves enhances both functional and morphologic recovery [23].

Systemic administration of immunophilin ligands provides neurotrophic influences to dopaminergic neurons in rodent models of Parkinson's disease (PD), resulting in the initiation of clinical trials in patients with PD. The non-immunosuppressive immunophilin ligand, GPI-1046, promotes the regeneration of dopamine cells in association with functional recovery in rodent models of Parkinson's disease (PD), however, it does not have neuroprotective and regenerative effects in MPTP-treated primates [24, 25].

Treatment with FK506, but not rapamycin reduces the number of oligodendroglia undergoing cell death in rats with a contusion injury to the spinal cord and increases the number of surviving oligodendroglia in dorsal white matter [26]. FK506 and another immunophilin ligand V-10,367 improves functional recovery after spinal cord injury in rats [27]. FK506 was found to be neuroprotective in a model of antiretroviral toxic neuropathy [28]. The neuroprotective effects of immunosuppressants are not understood and many hypotheses have been formed to explain such effects.

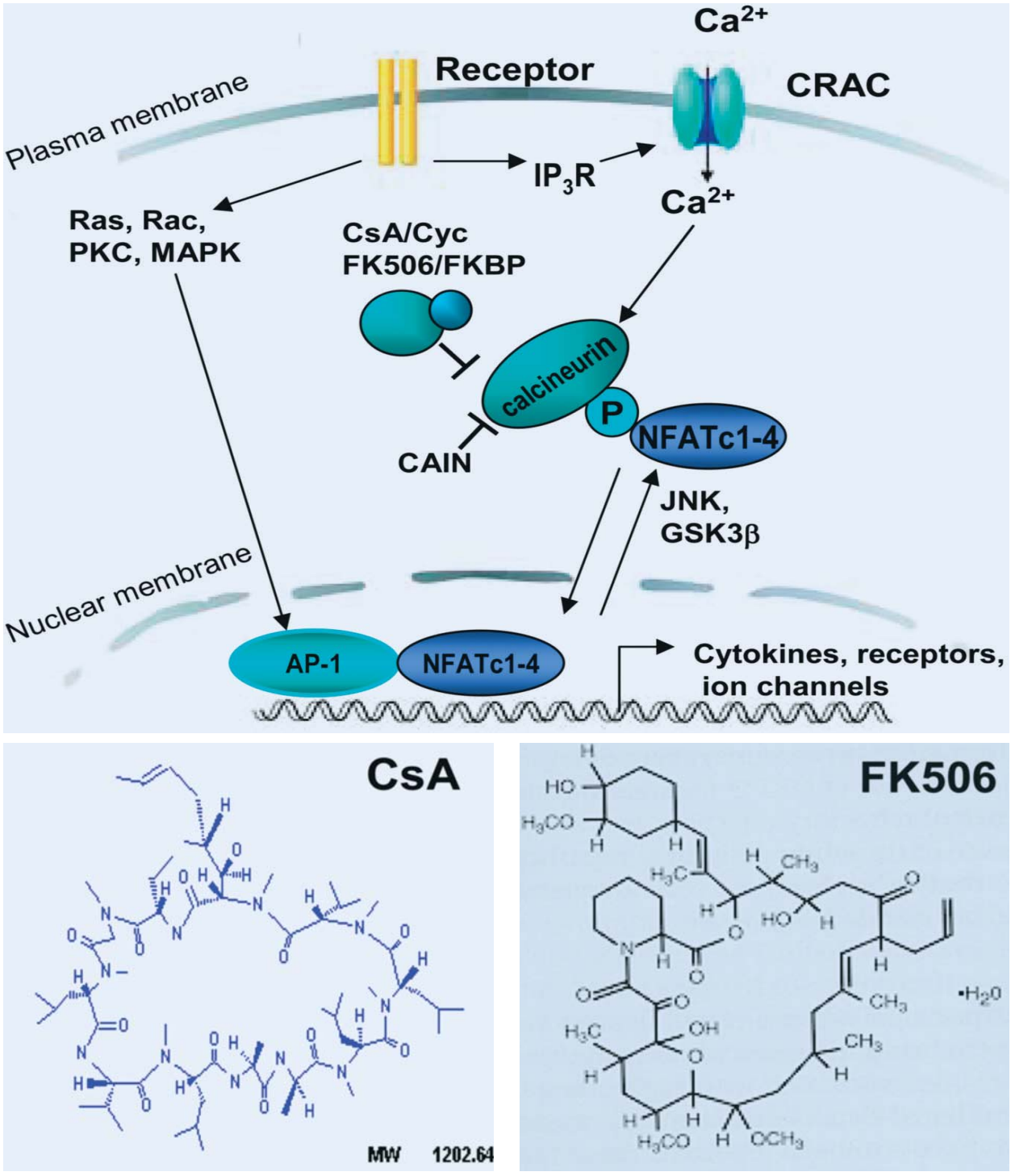

Fig. 1 Immunosuppressant structure and action in the immune system cells. A. Chemical structure of CsA and FK506. **B.** CsA binds to cyclophilin and FK506 to FKBP (FK506-binding protein), and such complexes bind to regulatory subunit of calcineurin (phosphatase dependent on Ca^{2+} and calmodulin). Calcineurin is positively regulated by calcium ions and negatively regulated by an endogenous inhibitor CAIN. In the immune system cells, calcineurin dephosphorylates NFAT (Nuclear Factor of Activated T Cells) transcription factor that regulates expression of cytokines, their receptors, and ion channels. NFAT complex is composed of NFAT proteins and AP-1 transcripion factor activated *via* Ras/PKC/MAPK pathway (PKC- protein kinase C; MAPK -mitogen activated portein kinases). JNK (c-Jun N-terminal MAP kinase) and GSK3β (glycogen synthase kinase 3 β) phosphorylate NFAT proteins counteracting effects of calcineurin.

The role of calcineurin and immunophilins in the regulation of neuronal survival

The activity of many structural and regulatory proteins depends on their phosphorylation state, which is related to adverse action of kinases and protein phosphatases. Calcineurin is one of the key enzymes regulating the level of phosphorylation hence activity of many proteins in neurons [29,30]. This phosphatase is important in neuronal signal transduction as well, given its abundance in the nervous tissue and the necessity for tightly controlled calcium-regulated phosphorylation. The brain contains 3-10 times higher concentrations of calcineurin than most studied tissues [31]. However, physiological function of high level of calcineurin expression in the brain remains largely unknown. We discuss only a few functions of the phosphatase that can be relevant for its neuroprotective potential.

Calcineurin as a regulator of neuronal survival

Protein dephosphorylation by calcineurin may play an important role in neuronal signal transduction due to its ability to regulate the activity of ion channels, glutamate release, and synaptic plasticity [29]. Calcineurin has been shown to regulate the activity of N-methyl-D-aspartate (NMDA) receptor channels by both altering their ion gating properties and promoting desensitization in cultured hippocampal neurons [32-34]. In cultured fetal rat cortices, the inhibition of calcineurin with CsA (5-20 μM) increased the rate of spontaneous neuronal firing, which has been ascribed to the enhanced release of glutamate by the presynaptic cells [33].

A considerable amount of evidence suggests that excitotoxic overactivation of glutamate receptors, especially those of the NMDA-type, contributes to neuronal cell death. Since NMDA receptors are highly permeable to Ca^{2+}, influx of extracellular Ca^{2+} is considered to be the primary event responsible for glutamate toxicity [35]. Although the mechanism of Ca^{2+}-induced neurotoxicity is not completely understood, calcineurin, a Ca^{2+}-regulated protein, may be associated with the neurotoxicity [36].

Calcineurin can control the level of neurotransmitter release by modifying the function of synaptic vesicles. Dynamin I and synapsin I, proteins participating in neurotransmitter release, become activated upon phosphorylation. Dephosphorylation by calcineurin makes them inactive [37]. Calcineurin up-regulates activity of nitric oxide synthase (NOS) and augments the level of nitric oxide (NO)-stimulated neurotransmitter release. In PC12 cells (rat adrenal pheochromocytoma cell line) NOS is inactive when phosphorylated. Calcineurin, through dephosphorylation of NOS, activates it, which leads to increased NO level and induces neurotransmitter release. The participation of calcineurin in regulation of NOS activity and indirectly in neurotoxicity suggested the potential role of this enzyme in regulation of neuronal cell viability. It was shown that FK506 and CsA inhibited NMDA-induced death of primary cortical cultures that was associated with an increase in the level of NOS phosphorylation, inhibition of enzyme activity and decrease in the level of cGMP and reactive NO species (Fig. 2) [38].

FKBP-calcineurin complex participates in the regulation of the calcium level in a cell through IP_3R (a inositol trisphosphate receptor). FKBP12 protein (FK506 binding protein) is a part of an IP_3R/ryanodine receptor in the endoplasmic reticulum. Calcineurin through FKBP12 binds to the IP_3 receptor and, after activation by calcium ions and calmodulin, dephosphorylates IP_3R (in a site phosphorylated by PKC) which causes a decrease in the calcium efflux. FK506 makes FKBP-calcineurin complex to dissociate from IP_3R, which triggers calcium efflux to cytoplasm [29]. However, relevance of this phenomenon to the neuronal survival has not yet been tested.

In 1995, a novel role of calcineurin as a mediator of cell death signalling pathway has been described [39]. Wang and co-workers [40] have demonstrated that calcineurin directly affects the viability of cells through an activation of the proapoptotic Bad protein. The viability of cells is regulated by the level of pro-survival proteins from the Bcl-2 family, which play a crucial role in regulation of homeostasis and maintenance of the proper mitochondrial function. Upon the increase of calcium level, activated calcineurin dephosphorylates Bad, which in turn triggers its translocation

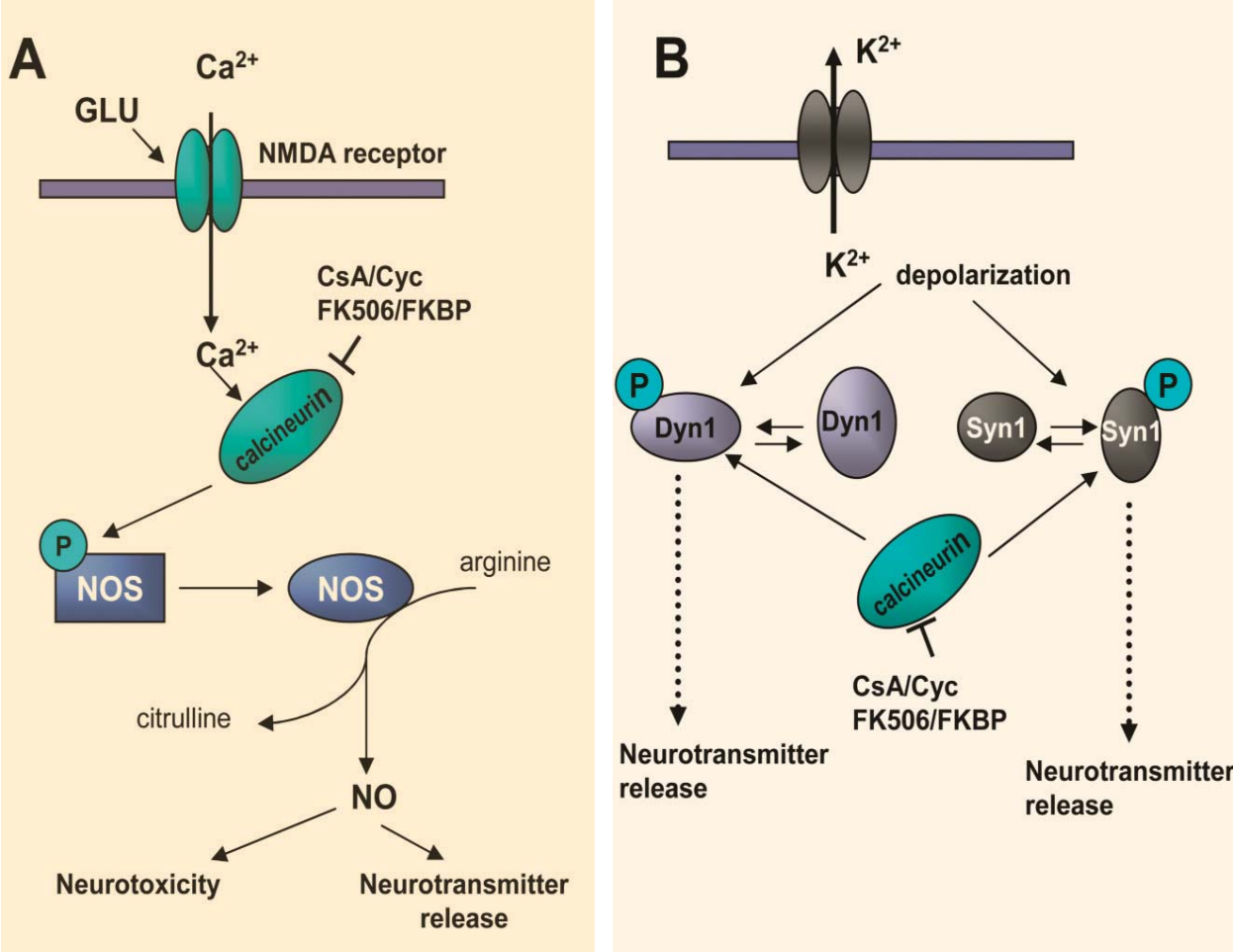

Fig. 2 Role of calcineurin in the regulation of neuronal function. A. NOS (nitric oxide synthase) is inactive when phosphorylated. Calcineurin dephosphorylates and up-regulates NOS activity thus augmenting the level of nitric oxide (NO)-stimulated neurotransmitter release. Increase of NOS activity plays a role in excitotoxic neuronal death. **B.** Dynamin I and synapsin I, proteins participating in neurotransmitter release, become activated upon phosphorylation. Dephosphorylation by calcineurin makes them inactive and modulates neurotransmitter release.

to mitochondria, where Bad binds Bcl-xL protein and thus impedes its pro-survival action. Mitochondrial dysfunction may lead to a decrease in mitochondrial potential and rupture of the outer-membrane resulting in release of proapoptotic proteins. Inhibition of calcineurin and subsequent lack of Bad dephosphorylation causes retention of Bad in cytoplasm where it is bound by 14-3-3 adaptor protein.

Calcineurin can also regulate indirectly neuronal survival by influencing the expression of antiapoptotic genes through a transcription factor CREB. Applying a short impulse/stimulus leads to simultaneous activation of kinases that phosphorylate CREB (mainly CaMKII), as well as the phosphatase, which dephosphorylates CREB. It results in transient CREB activation, not sufficient to activate gene expression. After the prolonged synaptic activation, inactivation of phosphatase allows for maintaining of CREB in its phosphorylated form and leads to activation of CREB-dependent transcription [41]. One of CREB-regulated genes is gene coding for Brain Derived Neurotrophic Factor (BDNF), a neurotrophin that plays an important role both in neuronal plasticity as well as in the regulation of neuronal viability [42, 43].

Immunophilins in regulation of neuronal survival

Immunophilins are intracellular binding proteins for FK506 and CsA, but they are also abundant peptidylprolyl-cis-trans-isomerases (PPIases) that facilitate protein folding and are important in protein-protein interactions. They are also thought to take part in response of cells to oxidative stress [44]. Since CsA and FK506 inhibit PPIase activity of immunophilins as well as calcineurin, the question arises concerning the role of immunophilins in cell survival. To distinguish between inhibition of calcineurin and inhibition of immunophilins, nonimmunosuppressive analogs of CsA were used, e.g. NIM 811 (methyl-cyclosporin), that does not inhibit calcineurin but interacts with immunophilins. Since NIM 811 turned out to be as potent as CsA in prevention of TNF-α-induced apoptosis in cultured rat hepatocytes, it seems that inhibition of immunophilins alone might be sufficient to obtain protective effect. It has been shown that endothelial cells pretreated with submicromolar concentrations of both CsA and NIM 811 became resistant to toxic, higher concentrations of CsA [45].

One possible explanation refers to the postulated mechanism of neuroprotection by inhibition of mitochondrial permeability transition pore (mPTP) opening that is exerted through interaction of CsA or NIM 811 with cyclophilin D (Cyp D) [46]. Cyclophilin D is one of the main components of mPTP along with voltage-dependent anion channel (VDAC) localized in the outer membrane and adenine nucleotide translocator (ANT) localized in the inner membrane. Furthermore mPTP comprises: hexokinase, mitochondrial creatine kinase, glycerol kinase, and pro- and antiapoptotic proteins from the Bcl-2 family. The VDAC dimer constitutes the main channel and ANT and other proteins take part in regulation of the opening. The mPTP opening is triggered by increase of Ca^{2+} level in mitochondrial matrix, decrease of mitochondrial potential beneath the critical value on both sides of the inner membrane and oxidative stress [47].

CsA as well as NIM811 are specific inhibitors of mPTP, that after binding to Cyclophilin D, cause dissociation of this complex from ANT, which leads to mPTP closure. *In vitro*, CsA in micromolar concentrations inhibits formation of mPTP and prevents changes in permeability of mitochondrial membranes, dissipation of transmembrane potential and as a consequence, impedes subsequent events leading to apoptosis [48]. On the other hand, it has been reported that CsA increases the expression level of CypA [44, 49] and that overexpression of CypD protects rat glioma C6 cells from staurosporin-induced cell death and oxidative stress [50]. Moreover it was found that a neuroprotective dose of FK506 also increases FKBP expression and activity in ischemic rat brain [51]. These results contradict the previously mentioned explanation and might suggest that cytoprotective effect is achieved rather by cellular mobilization of cyclophilins by submicromolar concentrations of an immunosuppressant rather than through their inhibition. However, FK506 does not bind Cyclophilin D and does not inhibit mPTP opening [52]. Rapamycin, which binds with a similar affinity to FKBP12 but does not inhibit calcineurin, did not give neuroprotection and even abolished neuroprotective effects of FK506 when co-administered [15]. It suggests that inhibition of calcineurin is indispensable for neuroprotective effects of FK506.

Neuroprotective action of immunosuppressants in brain ischemia

Comparison of data from different laboratories shows neuroprotective effect in a model of transient focal cerebral ischemia (Table 1). CsA was neuroprotective in a dose of 20 mg/kg and protective effect was observed only when CsA was administrated before or instantly after ischemia [53-54]. FK506 (1 and 10 mg/kg) also turned out to be neuroprotective when applied 2 h after ischemia in a model of transient focal cerebral ischemia induced by endothelin-1 [15, 16]. Weaker protective effect of CsA, in comparison to FK506, results from the fact that CsA crosses the blood-brain barrier poorly and may only accumulate when this barrier is partially damaged [55-57].

Mechanisms of brain injury after ischemia

Stroke results from instant disruption of blood flow to the brain. It can be caused by cerebral

Table 1 Comparison of drug dose and time of drug application on neuronal damage induced by MCAo (middle cerebral artery occlussion) or excitotoxins.

Drug	Neuro-protection	Dose (mg/kg)	Neuronal damage	Drug treatment	References
CsA	+++	15	MCAO	5 days before to 2 days after MCAo	7
FK506	+++	0.1–1	MCAO/ injection endothelin-1	1 min, 1 godz.	15
FK506	+++	1 and 10	MCAO/	1 min, 120 min	16
CsA	++	20	injection endothelin-1	1 min	
FK506	–––	1 and 10	Injection NMDA, AMPA Quinonate	1 min	16
FK506	+++	0.1, 1, 5	MCAO	30 min after MCAO	55
SDZASM981	+++	1, 5		30 min before reperfusion	
Rapamycin	–––	2			
FK506	+++	1	MCAO	1 min	52
FK506	+++	0.3	MCAO	30-60 min	8
CsA	+++	10	MCAO	5 min	54
CsA	+++	2 x 20	MCAO	1 min and 24 h after reperfusion	53

infarction when one of the arteries supplying blood to the brain is abruptly clogged and blood flow to the brain is insufficient (below critical threshold) or can be caused by cerebral haemorrhage that is due to bleeding from an instantly broken vessel [58]. About 85-90% of strokes represent cerebral infarction. As a result of this process, neurons in the severely ischemic core are depleted of oxygen and trophic substances and in consequence become destroyed. Around the core zone, a much more extensive area of moderate ischemia, called ischemic penumbra arises supplied with blood by collateral arteries, where activity of neurons deteriorates, but rapid morphological changes do not occur [58]. Primary neuronal death within the core of infarct that occurs within a short time after brain damage (especially in striatum) is probably due to necrosis, while delayed neuronal death occurring over days and months bears features of apoptotic process [59-61]. Apoptosis, contrary to necrosis, is a slower process, requiring energy supply, switching on the gene transcription and protein synthesis. Delayed neuronal death is characterized by cell shrinkage, chromatin condensation, upregulation of proapoptotic Bcl-2 family members and activation of caspases [62]. The occurrence of delayed cell death creates favorable conditions for a therapeutic window, giving a chance for timely pharmacological intervention in order to salvage normal activity of neurons in the area of moderate ischemia [63, 64].

Tissue damage in ischemia is the result of a complex pathophysiological cascade, which comprises a variety of distinct pathological events. An increase in the GLU concentration and excessive stimulation of NMDA receptors may lead to death of other cells in a chain reaction. Activation of NMDA receptors and metabotropic glutamate receptors contribute to calcium overload, which initiates an activation of proteolytic enzymes that degrade cytoskeletal proteins, as well as extracellular matrix proteins [65]. Activation of phospholipase A2 and cyclooxygenase generates free-radicals species producing lipid peroxidation and membrane damage. Moreover, oxygen free radicals serve as signalling molecules that trigger

inflammatory reaction and cell death. Tissue damage is enhanced by NO, generated by the calcium dependent enzyme NOS. NO reacts with superoxide anion to form the highly reactive species, peroxinitrate. Although excitotoxicity is a primary trigger of neuronal death, elevated GLU level is maintained for a few up to dozen hours after ischemia, while neuronal death is going on weeks or months after damage [66]. GLU receptor antagonists have a small influence on the scale of delayed neuronal death in the penumbra and display neuroprotective action only when they are applied before or during ischemia [64].

Molecular mechanisms underlying neuroprotection by CsA and FK506 in brain ischemia

Previous studies of molecular mechanisms underlying neuroprotection evoked by CsA and FK506 in brain ischemia concentrated on their influence on excitotoxic neuronal death. As mentioned above, excessive stimulation of NMDA receptors causes NOS activation and an increase in the level of reactive nitrogen oxide species. In primary cerebral cultures and hippocampal slices, and in animal models of ischemia, glutamate toxicity is mediated at least in part by NOS. Experiments with specific inhibitors of neuronal NOS (7-nitroindasole) and with knock-out animals, defective with respect to NOS isoforms, suggest an important role of NOS activation in neurotoxicity during ischemia [67,68].

Since FK506 inhibited NMDA-induced death of cortical cultures *in vitro* that was associated with an increase of the level of neuronal nitric oxide synthase (NOS) phosphorylation, inhibition of enzyme activity and decrease of the level of cGMP and reactive NO species [38], it has been suggested that the FK506 neuroprotective action may be mediated by suppression of NOS activation and NO production that occurs during ischemia or early reperfusion. However, FK506 at doses that provide neuroprotection did not alter NO production during ischemia and early reperfusion after MCA occlusion in rats [69].

An antiexcitotoxic mechanism is unlikely to mediate the neuroprotective action of FK506 in focal cerebral ischemia. Butcher *et al.* [16] has shown that FK506, at doses that reduced the volume of ischemic cortical damage by 56-58%, did not decrease excitotoxic damage induced by quinolinate, NMDA, AMPA, or striatal quinolinate lesions. The contrasting efficacy of FK506 in ischemic and excitotoxic lesion models cannot be explained by drug pharmacokinetics, because brain FK506 content rose rapidly after both treatments and sustained for 3 days.

Some data are consistent with a hypothesis of calcineurin involvement in regulation of neuronal survival by influencing the expression of antiapoptotic genes. Miyata and co-workers [70] demonstrated that phosphorylation of CREB was kept augmented throughout the time course examined in cyclosporin A-treated animals, while it ceased without CsA. BDNF mRNA expression was increased in the CA1 sector of cyclosporin A-treated animals. The protein expression of BDNF and TrkB appeared to be upregulated in cyclosporin A-treated animals, whereas it was transiently upregulated but decreased to the marginal level of expression without cyclosporin A.

The role of microglia activation and reactive gliosis in ischemia

Resident non-neuronal brain cells respond rapidly to neuronal cell death and may have both deleterious and useful roles in neuronal damage. Few hours after ischemia, astrocytes become hypertrophic, while microglial cells retract their processes and assume an ameboid morphology typical for activated microglia [71]. There is increasing evidence that post-ischemic inflammation involving microglia contribute to ischemic damage. Microglia may participate in the induction of neuronal death in many different ways. Microglial cells activated *in vitro* produce toxic reactive oxygen radicals and NO, substances that may directly damage neurons. Activated microglia are also a source of many neurotoxic cytokines, including FasL, TNF-α, and proinflammatory cytokines such as: IL-1β, IL-6, INF-γ and numerous chemokines [72]. Cytokines produced by microglia can activate microglia in an autocrine manner and, further, may activate astrocytes that in turn become a source of successive cytokines of

potentially neurotoxic function. Growing evidence indicate that the inhibition of secretion or activity of IL-1β and TNF-α, with antibodies neutralizing cytokines or soluble cytokine receptors leads to decreased neuronal damage [72-74].

Reactive gliosis (hypertrophy and astrocyte proliferation) is a widespread response to damage of neurons. Expression of GFAP is the hallmark of activation process. Astrocytes also "switch on" transcription of genes coding for trophic factors and cytokines. A few neurotrophic substances were identified, which regulate viability of neurons that survived in lesion area and play an important role in supporting the "well-being" of neurons after ischemia [75]. In experiments *in vivo*, application of some trophic factors such as basic Fibroblast Growth Factor (bFGF), BDNF, instantly after or directly before ischemia, inhibits delayed neuronal death and decreases the severity of the brain lesion [76-79]. It is not yet known whether trophic factors cause delay of programmed death of neurons or rather are responsible for their prolonged protection. However, astrocytes are not only a source of trophic factors (NGF, bFGF), but also proinflammatory cytokines (IL-1β, IL-6) and cytotoxic cytokines (Fas L, TNF-α, TGF-β) [80-83]. Activated astrocytes also produce toxic molecules such as reactive oxygen species and NO [84-86].

A novel mechanism of neuroprotective action of immunosuppressants

The neuroprotective effect of both CsA and FK506 indicates that they share some neuroprotective pathways. A common target for both drugs is calcineurin. We have demonstrated that various cells of glial origin: rat C6 glioma cells, newborn and adult astrocytes express calcineurin regulatory subunit mRNA and this signalling pathway is probably connected with regulation of proliferation and hypertrophy of these cells [87]. It was found that both CsA and FK506 inhibit proliferation, and in high concentrations induce apoptotic death of reactive astrocytes [88]. Results from experiments carried out in our laboratory indicate that immunosuppressant drugs, both CsA and FK506, may inhibit activation of glial cells and cytokine expression *in vitro* [89].

In astrocytes treated with FK506 genes coding for proinflammatory/cytotoxic cytokines such as IL-1β and TNF-α were downregulated while expression of neuroprotective factor - BDNF was upregulated [89]. Increase in BDNF level could possibly have neuroprotective role, since injection of this cytokine was shown to be protective, leading to a decrease in the lesion grade, improvement in neurologic functions in models of global and focal ischemia. It has been shown that pro-survival pathway activated by BDNF and its receptor TrkA, may participate in neuroprotective effect of CsA in model of global ischemia [70].

Our findings suggested that not only neurons but also the glial cells could be targets for immunosuppressants. We used a transient middle cerebral artery occlusion (MCAo) induced with the intraluminal filament method in the rat. The grade of lesion caused by ischemia was estimated by observing neurological symptoms, extention of lesion (TTC staining and histological Nissl staining), level of microglia response and astrogliosis. Results obtained from our experiments *in vivo* confirmed neuroprotection in rat cortex with MCAo. A single injection of FK506 (1mg/kg, administered 1 hour after ischemia) inhibited a cell death in the cortex. The neuroprotective effect was maintained after 48 and 72 h. In animals treated with FK506 a necrotic death of neurons in the striatum was not inhibited, as previously demonstrated [15]. These data indicate that an antiexcitotoxic mechanism is unlikely to mediate the neuroprotective action of FK506 in focal cerebral ischemia, rather excluding a direct FK506 effect on excitotoxic neuronal death.

We found that administration of FK506 resulted in a decrease in the number of GFAP and lectin B4 positive cells in the ischemic cortex indicating that FK506 inhibits microglia and astrocyte activation [Zawadzka, Kaminska, submitted]. Similarly, Wakita and co-workers [90] have noticed an inhibition of microglia and astrocyte activation accompanied by a decrease in the lesion induced by global ischemia in animals treated with FK506 (0.2, 0.5, 1.0 mg/kg for 14 days, starting 1 day before operation).

Moreover, FK506 provoked attenuation of ischemia-induced increases in the levels of proinflammatory cytokines: IL-1β and TNFα mRNAs 24 hours after MCAo. Using a double staining we

demonstrated a significant downregulation of IL1β expression in astrocytes and microglia in the injured side of FK506-treated rats. Moreover, FK506 decreased the levels of mRNA encoding TNFα, and IL1β in astrocytes *in vitro*, while the levels of TGFβ1 and Interleukin-6 were constant. FK506 inhibits LPS-induced activation and cytokine expression in microglia *in vitro* in dose-dependent manner [Zawadzka, Kaminska, submitted]. Our findings suggest that astrocytes and microglia are targets of FK506 and modulating of glial response and inflammation is a possible mechanism of FK506-mediated neuroprotection in ischemia.

A variety of recent studies suggest a role for inflammatory cytokines, such as interleukin-1β, in ischemic brain injury. Reduced ischemic brain injury was observed in interleukin-1 beta converting enzyme-deficient mice [91]. In mice lacking either IL-1α or IL-1β alone ischemic damage was not significantly altered; however, mice lacking both IL-1 exhibited dramatically reduced ischemic infarct volumes compared with wild type [92]. Intracerebroventricular administration of recombinant IL-1 receptor antagonist significantly reduced infarct volume that confirms a major role this cytokine in ischemic brain injury [92, 93].

On the basis of obtained results and analysis of data from other laboratories, we hypothesised that administration of immunosuppressants within strictly specified interval after damaging factor allows for modulation of astrocytes and microglia activation, and furthermore, enables alterations of the level of cytokine expression and consequently of mode of glial cells response in area of moderate ischemia in the brain. These results indicate a new molecular mechanism underlying the neuroprotective action of immunosuppressants and can be of clinical value.

Summary

Stroke is one of the most frequent causes of death in highly developed countries. Many patients die directly or shortly after stroke, one-third of patients who survive the first month of this disease become handicapped in terms of basic life activities. One very attractive neuroprotective strategies can be neuroprotection in area of delayed neuronal death achieved through inhibition of proinflammatory response of microglia and astrocytes and modulation of neurotrophic factors and cytokine expression. Potential strategy of this type would also mean the ability of intervention and modulation of activity of different cell types engaged in response to ischemia. Administration of substances of neuroprotective action (non-steroid anti-inflammatory drugs, CsA, FK506) have neuroprotective effect that can not be merely explained by direct influence of these drugs on neurons, whereas their effects can be explained by inhibitory action on microglia and astrocyte activation. Recent studies demonstrate that when FK506 or tissue plasminogen activator (t-PA) was administered 2 hours after MCA occlusion, it reduced ischemic brain damage, however, combined treatment resulted in a significant reduction in ischemic brain damage. On administration 3 hours after MCA occlusion, FK506 alone showed no effect, and t-PA tended to worsen ischemic brain damage. However, the combined treatment with both drugs not only ameliorated the worsening trend seen with t-PA alone, but also tended to reduce ischemic brain damage [94]. It suggests that FK506 used in combination with t-PA, may augment therapeutic efficacy on brain damage associated with focal ischemia and extend the therapeutic time window compared to single-drug treatments.

Acknowledgments

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