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# Immunosuppressant cytoprotection correlates with HMGB1 suppression in primary astrocyte cultures exposed to combined oxygen-glucose deprivation

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**Abstract:**
The protective potential of immunosuppressants has been reported in many experimental models of ischemia both *in vivo* and *in vitro*, suggesting a novel therapeutic application of these drugs. Because high-mobility group box 1 (HMGB1) protein has recently been reported to be involved in ischemic brain injury, the purpose of the present study was to determine whether treatment with immunosuppressants could decrease the expression and release of HMGB1 in astrocytes exposed to simulated ischemic conditions (combined oxygen-glucose deprivation, OGD). We also investigated whether immunosuppressive drugs could attenuate necrosis in astrocyte cultures exposed to OGD. Finally, we studied the influence of immunosuppressants on the expression of NFκB, inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2). Cells were treated with cyclosporine A, FK506 and rapamycin (all drugs at concentrations of 0.1, 1 and 10 μM). Our study provides evidence that immunosuppressants decrease the expression and release of HMGB1 in ischemic astrocytes. Our data suggest that HMGB1 release may be partly an active process triggered by oxidative stress because the antioxidant N-acetylcysteine (NAC) clearly attenuated HMGB1 expression and release. Furthermore, we show that the immunosuppressants, at the same concentrations that significantly suppressed HMGB1 expression and release, were also able to prevent the necrosis of ischemic astrocytes and inhibit the expression of inflammatory mediators (NFκB, iNOS and COX-2). These results provide further information about the cytoprotective mechanisms of immunosuppressants on ischemic astrocytes, especially in relation to the pathophysiology of ischemic brain injury. It appears that the protective effects of immunosuppressants can be mediated in part by the suppressing the expression and release of HMGB1 in astrocytes, which leads to the attenuation of ischemia-induced necrosis and neuroinflammation.

**Key words:**
HMGB1, cyclosporine A, FK506, rapamycin, astrocytes, ischemia, iNOS, COX-2

**Abbreviations:** CNS – central nervous system, COX-2 – cyclooxygenase-2, DIV – day *in vitro*, Erk– extracellular-signal regulated kinase, FBS – fetal bovine serum, FKBP12 – FK506 binding protein 12, HMGB1 – high mobility group box 1, iNOS – inducible NO synthase, MAPK – mitogen-activated protein kinase, MPTP – mitochondrial permeability transition pore, mTOR – mammalian target of rapamycin, NAC – N-acetylcysteine, OGD – oxygen-glucose deprivation, RAGE – receptor for advanced glycation end products, ROS – reactive oxygen species, SAPK – stress-activated protein kinase, STAT – signal transduction and activator of transcription, TLR – toll-like receptor, TSA – trichostatin A

## Introduction

High-mobility group box 1 (HMGB1) was originally identified as a non-histone chromatin-binding protein, a key regulator of the nucleosomal structure stabilization and a transcription factor of several genes [11]. In animal models of ischemic stroke, increased extracellular HMGB1 levels are thought to contribute to early and secondary ischemic brain injury [24]. Furthermore, a recent study by Goldstein et al. [18], showing that plasma levels of HMGB1 were elevated up to 10-fold in stroke patients, provides further support for this protein in the pathology of cerebral ischemia in humans. Numerous observations point to its proinflammatory cytokine-like properties and to the important role of extracellularly released HMGB1 in both infectious and sterile inflammation caused by peripheral tissue trauma [14], hemorrhagic shock [29], noninfectious hepatitis [40] and kidney ischemia/reperfusion [42].

In the central nervous system (CNS), HMGB1 is highly expressed in the nuclei of neurons and glial cells. It has been shown that within the ischemic brain, nuclear HMGB1 promptly translocates from the nucleus to the cytoplasm prior to extracellular release [15]. HMGB1 can be released into the extracellular space either passively or actively as a signal to trigger inflammation [34]. During necrotic cell death, the extracellular release of HMGB1 is passive and is not regulated by acetylation. In this case, HMGB1 is considered to be a critical factor that connects necrosis to inflammation [36]. While the secretion of HMGB1 from activated cells is an active process that does not involve cell death, it appears to involve HMGB1 hyperacetylation by nuclear acetyltransferase or may be regulated by reactive oxygen species (ROS) [32, 40]. However, HMGB1 is tightly associated with condensed chromatin in apoptosis because of generalized underacetylation of the histones [2]. For this reason, apoptotic cells failed to release HMGB1, even after undergoing secondary necrosis, and they do not mediate any inflammatory responses [1].

On the cell surface, released HMGB1 interacts with different receptors, such as the receptor for advanced glycation end products (RAGE) or toll-like receptor-2 and -4 (TLR-2 and -4) [15]. It has been demonstrated that both types of membrane receptors are functionally expressed in the brain [8, 25]. Mitogen activated protein kinases (MAPK), stress-activated protein kinase (SAPK)/JNK and transcription factors (NFκB and AP-1), in addition to the downstream molecules RAGE or TLR-2 and -4, are involved in the proinflammatory effect of HMGB1 [24].

The protective potential of immunosuppressants, such as cyclosporine A, FK506 and rapamycin, has been reported in many experimental models of ischemia both *in vivo* and *in vitro*, suggesting novel therapeutic application of these drugs [9, 38, 43]. These drugs exert their immunosuppressive actions through binding to small intracellular regulatory proteins called immunophilins [35]. Cyclosporine A binds to cyclophilin A, whereas FK506 binds to the FK506 binding protein 12 (FKBP12). Both types of drug-protein complexes block calcineurin ($Ca^{2+}$/calmodulin-dependent protein phosphatase), which upregulates numerous cytokines (e.g., IL-1β, TNF-α) and proinflammatory factors [5]. Rapamycin also binds to FKBP12, but the complex formed between rapamycin and FKBP12 binds to the mammalian target of rapamycin (mTOR), resulting in inhibition of p70 S6 kinase. Thus, unlike cyclosporine A and FK506 (which block the production of cytokines), rapamycin blocks cytokine signal transduction [10, 16]. Although the precise cellular processes underlying the neuroprotective effects of immunosuppressants in experimental models of stroke remain unclear, the current experimental data suggest that the mechanism of action of these drugs may involve inhibition of astroglial inflammatory responses [6, 44].

Because HMGB1 has recently been reported to be involved in ischemic brain injury [24], the purpose of the present study was to determine whether treatment with immunosuppressants could decrease HMGB1 expression and release in primary cultures of astrocytes exposed to simulated ischemic conditions (combined oxygen-glucose deprivation, OGD). Additionally, to determine if HMGB1 is secreted actively or passively from damaged astrocytes, we applied trichostatin A (TSA), which is a histone deacetylase inhibitor, and N-acetylcysteine (NAC), which is a potent antioxidant compound, in parallel experiments. We also investigated whether the immunosuppressive drugs could attenuate necrosis in astrocyte cultures exposed to OGD. Finally, we studied the influence of immunosuppressants on expression of NFκB, inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2).

## Materials and Methods

### Cell culture

Astrocytes were isolated from one-day-old Wistar rat pups and cultured essentially according to the method of Hertz et al. [21]. This study was approved by the Local Ethics Commission for the Animal Experimentation. Briefly, hemispheres of newborn Wistar rats were removed aseptically from the skulls, freed of the meninges, minced and mechanically disrupted by vortexing in DMEM containing penicillin (100 U/ml) and streptomycin (100 μg/ml). The suspension was filtered through sterile nylon screening cloth with pore sizes of 70 μm (first sieving) and 10 μm (second sieving). The cells were counted in a Coulter Z1 counter (Coulter Counter, Buckinghamshire, UK). The concentration of cells in suspension was adjusted to $1 \times 10^6$ cells/ml. The cells destined for analysis *via* western blot were sieved onto plastic dishes of 100 mm in diameter at a density of $1 \times 10^6$ cells/dish. Astrocytes for fluorescent studies (Live/Dead kit) were cultured on coverslips coated with poly-D-lysine. Each group of culture plates was repeated in three independent experiments. However, for LDH activity measurements, cells were seeded at $1 \times 10^4$ per well on 96-well plates. For these measurements, each experimental group was made up of 12 wells and repeated in three independent experiments. Subsequently, cultures were incubated at 37°C in 95% air and 5% $CO_2$ with 95% relative humidity ($CO_2$-Incubator, Kebo-Assab, Stockholm, Sweden). The culture medium initially contained 20% fetal bovine serum (FBS) and was replaced with medium containing 10% FBS after four days. The total volume of culture medium was changed twice a week. Cells were cultured for two weeks until confluence. On the 14$^{th}$ day *in vitro* (DIV), contaminating microglia and oligodendrocytes were removed by shaking at 200 rpm on an orbital shaker for 5 h and incubation with 5 mM L-leucine methyl ester [30, 37]. To identify astrocytes, cultures were stained immunocytochemically for glial fibrillary acidic protein (GFAP) (Sigma-Aldrich, St. Louis, MO, USA), a specific marker for astrocytes. Analysis of these cultures has shown that 90–95% of cells are GFAP-positive. About 1–2% of cultured cells reacted with *Ricinus Communis* Agglutinin-1, a lectin that binds to surface glycoproteins on microglia (Vector, Burlingame, CA, USA). No neurons, as confirmed by an immunocytochemical staining method using monoclonal antibodies against MAP-2 (Promega, Madison, WI, USA), were detected. All experiments were performed on 21-day-old cultures.

### Treatment of astrocyte cultures

Prior to the experiment, cells were incubated overnight with fresh medium. At the 21$^{st}$ DIV, cultures of astrocytes were placed in medium deprived of glucose and serum. Osmolarity of the medium was measured and adjusted to 319 mOsm with 20% mannitol (0.9 ml/100 ml), and cell cultures were incubated for 24 h in the simulated ischemic conditions (oxygen-glucose deprivation, OGD): 92% $N_2$, 5% $CO_2$ and 3% $O_2$ at 37°C ($CO_2$ incubator, Heraeus, Hanau, Germany). Cyclosporine A and rapamycin were purchased from Sigma-Aldrich (St. Louis, MO, USA), and FK506 was purchased from Calbiochem (Darmstadt, Germany). Cells were treated with cyclosporine A, FK506 and rapamycin (all drugs at concentrations of 0.1, 1 and 10 μM) during OGD. The drugs were added to the medium directly at the start of OGD. Drugs were dissolved in ethanol at an initial concentration of 1 mM (final concentration in the medium did not exceed 0.1%). Further dilutions were performed in the appropriate medium. Corresponding amounts of ethanol were added to the control cultures. Histone deacetylase inhibitor TSA (Sigma-Aldrich, St. Louis, MO, USA) was prepared as DMSO stock solutions at 1 mg/ml and added to the culture medium at the start of OGD at a final concentration of 200 ng/ml. In the same experiments, cultured astrocytes were treated with NAC (200 μM), a potentially therapeutic antioxidant compound.

### Western blot

Astrocyte cell cultures were washed with ice-cold PBS, and the proteins were extracted with 150 μl lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Igepal, 0.1% sodium dodecyl sulfate (SDS), 10 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 10 μg/ml of heat activated sodium orthovanadate]. After 20 min on ice, the cell lysates were centrifuged at 12,000 × g for 15 min at 4°C. Nuclear extracts containing NFκB were prepared from cells according to the method of Dignam et al. [7]. The protein concentrations in the samples were determined according to the Bradford method [4] with serum albumin as a standard. Samples con-

taining equal amounts of protein (20 μg) were boiled in protein loading buffer for 3 min, separated on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Nonspecific binding was inhibited by incubation in TBST [20 mM Tris-buffered saline (pH 7.5) with 0.1% Tween 20] containing 5% non-fat dry milk for 1 h at room temperature. Polyclonal antibodies against HMGB1 (Abcam, Cambridge, UK), NFκB p65, iNOS and COX-2 (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted (1:500) in TBST containing 5% skim milk. HMGB1 protein expression was measured by western blot in both cell lysates and culture media. Equal volumes of media were mixed with 20% ice cold trichloroacetic acid, kept on ice for 15 min and centrifuged at 10,000 × g for 10 min. Pellets were air-dried and resuspensed in $1/100^{th}$ of the original volume in 15 mM Tris HCl (pH 7.8). For loading controls, we used a mouse monoclonal antibody against β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a rabbit polyclonal antibody against lamin (Cell Signaling Technology, Beverly, MA, USA) for whole cell lysates or nuclear extracts, respectively (both at a 1:250 dilution).

Membranes were incubated with primary antibodies overnight at 4°C, washed with TBST and incubated at RT for 60 min with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Afterwards, membranes were washed again three times for 10 min with TBST. The chemiluminescence emitted from luminol and oxidized by HRP (ECL western blotting detection system; Amersham Biosciences, Piscataway, NJ, USA) was detected using Kodak XAR-5 film for autoradiography.

The molecular weights of HMGB1, NFκB p65, iNOS, COX-2, lamin and β-actin were estimated by electrophoresis of a pre-stained protein ladder (BenchMark™ Pre-Stained Protein Ladder, Invitrogen, Paisley, UK).

The integrated optical density (IOD) of the signals was semiquantified using Image Pro Plus software and expressed as the ratio of IOD from the tested proteins to IOD from β-actin or lamin. Experiments were repeated three times and the values of relative fluorescence were subjected to statistical analysis.

### Necrotic cell death

Necrotic cell death was determined by a fluorescent method with the use of a Live/Death Kit (Molecular Probes Inc., Eugene, OR, USA) containing calcein/AM and ethidium homodimer-1 (EthD-1). Calcein/AM is absorbed by living cells and subsequently converted by cytosolic esterases into a green fluorescent product (ex/em 495 nm/530 nm), whereas EthD-1 is known to only enter cells with compromised cell membrane permeability, and after being attached to nucleic acids, yields red fluorescence (ex/em 495/635 nm). After washing cells twice with PBS, both reagents were diluted in PBS and applied to the cell cultures immediately after exposure to OGD and drug treatment. Astrocytes were incubated with 2 μM of calcein/AM and 4 μM of EthD-1 for 30 min at 37°C. The number of live and dead cells was determined on at least six randomly selected areas from three coverslips of every experimental group, each containing approximately 200 cells. Cells were examined by fluorescence imaging using MiraCal Pro III workstation (Life Science Resources Ltd., UK), which is comprised of an inverted microscope Eclipse TE200 (Nikon, Japan) and a high-resolution cooled CCD camera (Photonic Science Ltd., UK). The results were expressed as a percentage of necrotic cells relative to the total number of cells.

The effect of immunosuppressive drugs on necrotic death was quantified by measuring the amount of LDH released from injured cells into culture media after 24 h of exposure of cell cultures to OGD. LDH activity was estimated by measuring the decrease in absorbance at 340 nm due to the conversion of enzyme co-factor NADH to $NAD^+$ (one enzyme activity unit = 0.001 ΔA/min). Briefly, 10-μl aliquots of culture media were collected and dissolved in 0.1 M pyruvate buffer (0.25 mg sodium pyruvate in PBS). NADH solution (0.25 mg in PBS) was then added to the samples. Cells were lysed by repeated freezing and thawing, and total released LDH was measured. Absorbance was measured immediately after the addition of NADH and 60 s after the initial reading. The data are presented as the percentage of the total releasable LDH.

### Statistical analysis

One-way analysis of variance (ANOVA) was used to compare mean responses among treatments. For each endpoint, treatment means were compared using the Bonferroni least significant difference procedure. In all analyses, p values $< 0.05$ were considered to be statistically significant. All data are expressed as the means ± SD.

## Results

To ensure that HMGB1 expression and release by immunosuppressant treatment is not caused by nonspecific protein release due to cell damage, toxicity of cyclosporine A, FK506 and rapamycin (all at concentrations of 0.1–10 μM) was quantified in normoxia by measurement of LDH release and the MTT conversion method. The drugs did not have any influence on cell viability at these concentrations after incubation for 24 h in normoxic conditions (data not shown).

### Effects of immunosuppressants on HMGB1 expression and release

To determine the influence of immunosuppressants on intracellular HMGB1 expression, we examined their effect on protein levels in cell lysates obtained from astrocyte cultures. As indicated in Figure 1A, astrocyte exposure to OGD for 24 h markedly increased the expression of HMGB1. This effect was attenuated by both cyclosporine A and rapamycin at a concentration of 0.1 and 10 μM FK506. In our experimental paradigm, we observed that increasing concentrations of cyclosporine A did not reduce HMGB1 expression, and TSA had no effect on its expression in ischemic astrocytes. In contrast, NAC strongly inhibited HMGB1 expression, and rapamycin inhibited its expression at all concentrations used (Fig. 1B).

To determine whether the differences in the influence of immunosuppressants on HMGB1 expression were also associated with their various effects on protein release, we examined the effects of these compounds on HMGB1 levels in electrophoresed media of astrocyte cultures. As indicated in Figure 1C, cellular exposure to OGD for 24 h markedly increased the release of HMGB1. This effect was attenuated by 0.1 μM cyclosporine A as well as 1 and 10 μM FK506. In addition, all concentrations of rapamycin significantly decreased HMGB1 release from OGD-injured astrocytes. NAC (200 μM) completely inhibited HMGB1 liberation, confirming the role of ROS produced during ischemia in HMGB1 release from astrocytes. TSA only partially attenuated HMGB1 levels in the medium of ischemic astrocytes, indicating that extracellular liberation of HMGB1 is mainly a passive process typical of necrosis.

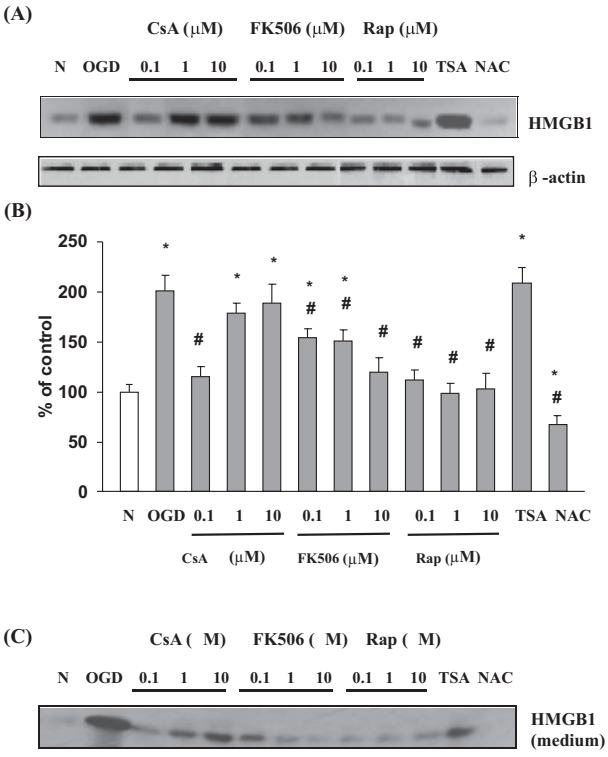


**Fig. 1.** (**A**) Western blot analysis of HMGB1 expression in astrocytes exposed to normoxia (N) or OGD and treated with cyclosporine A (CsA), FK506, rapamycin (Rap), trichostatin A (TSA) or N-acetylcysteine (NAC). Cell lysates (20 μg of protein) from experimental groups were analyzed by immunoblotting using an antibody against HMGB1. Detection of -actin ensured that equal amounts of proteins were loaded in the western blot analysis. Blots shown are representative of three independent experiments. (**B**) Semiquantification of western blots. The IOD ratio of HMGB1 signals to -actin signals was calculated. The ratio obtained from untreated cell cultures (normoxia) was normalized to 100%, and the data from three independent experiments are presented as the means ± SD of the percent change from control. * $p < 0.05$ *vs.* normoxia; # $p < 0.05$ *vs.* OGD. (**C**) Western blot analysis of HMGB1 expression in the media (20 μl of sample prepared as described in Materials and Methods) from astrocyte cultures exposed to normoxia or 24 h of OGD and treated with cyclosporine A (CsA), FK506, rapamycin (Rap), trichostatin A (TSA) or N-acetylcysteine (NAC)

### Effects of immunosuppressants on necrotic cell death

The Live/Dead and LDH release assays were used for determining the degree of necrosis in cultures exposed to OGD for 24 h. The percentage of dead cells stained with EthD-1 in comparison to cells stained with calcein in particular experimental groups is presented in Figure 2A. Treatment with OGD for 24 h significantly increased the number of dead astrocytes in the cultures as compared to normoxia. Cyclospor-

ine A, at a concentration of 0.1 μM, seems to be the most efficient drug in preventing necrosis of ischemic astrocytes. Interestingly, adding 1 or 10 μM FK506 to the culture medium in OGD for 24 h significantly reduced the number of dead cells; however, 0.1 μM FK506 did not cause statistically significant changes in the amount of dead astrocytes in simulated ischemic conditions. In contrast, all concentrations of rapamycin significantly decreased the number of dead cells, which might indicate that rapamycin shows distinct protective activity at these concentrations.

After 24 h of OGD, an increase in LDH activity in the medium occurred as a result of the loss of cell membrane integrity and enzyme leakage (Fig. 2B). In total, 0.1 μM cyclosporine A added during OGD reduced LDH activity. The amount of released LDH was also decreased in cultures exposed simultaneously to OGD and 1 or 10 μM FK506. Furthermore, all concentrations of rapamycin caused a decrease in LDH activity in the medium.

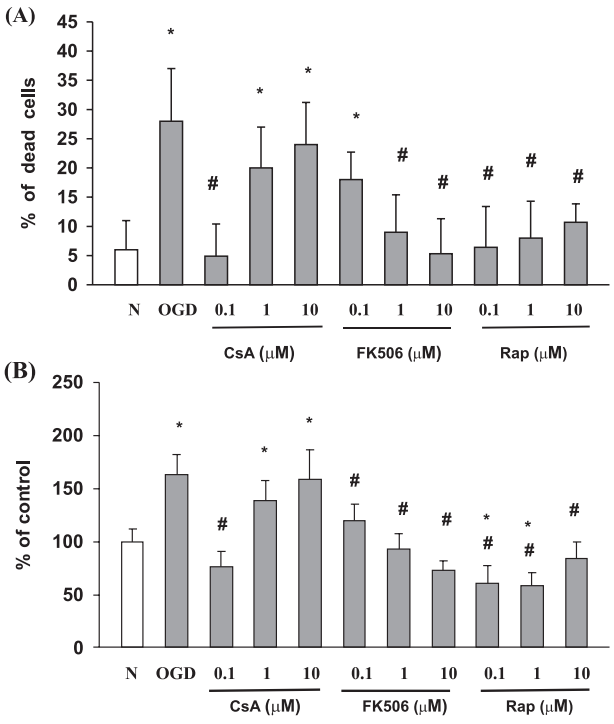


**Fig. 2.** Effect of immunosuppressants on OGD-induced necrotic death of astrocytes. (**A**) The effect of cyclosporine A (CsA), FK506 and rapamycin (Rap) on necrosis in astrocyte cultures exposed to normoxia or 24 h of OGD. The results are shown as a percentage of dead cells to the total amount of cells in the field and are the mean ± SD of six randomly selected areas from three culture dishes. * $p < 0.05$ *vs.* normoxia; # $p < 0.05$ *vs.* OGD. (**B**) The effect of cyclosporine A (CsA), FK506 and rapamycin (Rap) on LDH release in astrocyte cultures exposed to normoxia or 24 h of OGD. The results are presented as a percent change from the control value in normoxia. Each value is the mean ± SD of 12 wells in three separate experiments; * $p < 0.05$ *vs.* normoxia; # $p < 0.05$ *vs.* OGD

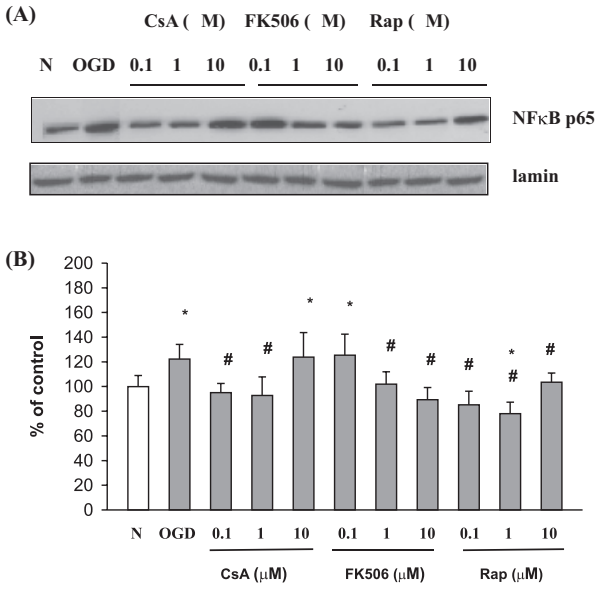


**Fig. 3.** (**A**) Western blot analysis of NF B p65 subunit levels in nuclear extracts from cultured astrocytes exposed to normoxia (N) or OGD and treated with cyclosporine A (CsA), FK506, or rapamycin (Rap). Nuclear extracts (20 μg of protein) from experimental groups were analyzed by immunoblotting using an antibody against NFκB p65. Detection of lamin ensured that equal amounts of nuclear extracts were loaded in western blot analysis. Blots shown are representative of three independent experiments. (**B**) Semiquantification of blots. The IOD ratio of NF B p65 signals in nuclear extracts to lamin signals was calculated. The ratio obtained from untreated cell cultures (normoxia) was normalized to 100%, and the data from three independent experiments are given as the means ± SD of the percent change from control; * $p < 0.05$ *vs.* normoxia; # $p < 0.05$ *vs.* OGD

### Effects of immunosuppressants on NFκB, iNOS and COX-2 expression

We examined whether treatment of ischemic astrocytes with immunosuppressants could lead to the stimulation of NF B, triggering the induction of COX-2 and iNOS. These molecules are activated downstream of RAGE or TLR-2 and -4 and are involved in the proinflammatory effect of HMGB1.

Experiments performed following 24 h of OGD treatment showed activation of NFκB in ischemic astrocytes (Fig. 3). The incubation of astrocytes during 24 h of OGD with 0.1 and 1.0 μM cyclosporine A caused a significant decrease in nuclear NFκB p65 subunit expression (Fig. 3). The level of nuclear NF B p65 subunits was also decreased after FK506 (1 and

10 μM) application to culture media during OGD in comparison to untreated ischemic cells. Furthermore, exposure of ischemic astrocytes to rapamycin at all concentrations used suppressed nuclear NFκB p65 protein expression in comparison to cells only exposed to OGD.

After a 24 h period of OGD, the expression of iNOS was significantly higher than in normoxic controls (Fig. 4). We have observed distinct decreases in the expression of iNOS in astrocytes treated with 0.1 or 1 μM CsA during OGD. Similar levels of iNOS expression were observed in ischemic cells exposed to 0.1 μM FK506. Treatment of astrocytes with 10 μM FK506 or rapamycin (at all concentrations used) during OGD significantly decreased the expression of iNOS in comparison to both normoxia and OGD groups.

As shown in Figure 5, simulated ischemia increased COX-2 expression in cultured astrocytes. COX-2 expression was suppressed after 0.1 and 1 μM cyclosporine A treatment in comparison to the OGD group. Incubation of astrocytes with 10 μM FK506 or rapamycin (0.1 and 1 μM) also distinctly blocked COX-2 expression. As illustrated (Fig. 5), under OGD conditions and treatment with 10 μM cyclosporine A, 0.1 or 1 μM FK506 or 10 μM rapamycin we observed an increase in COX-2 expression in comparison to untreated cells.

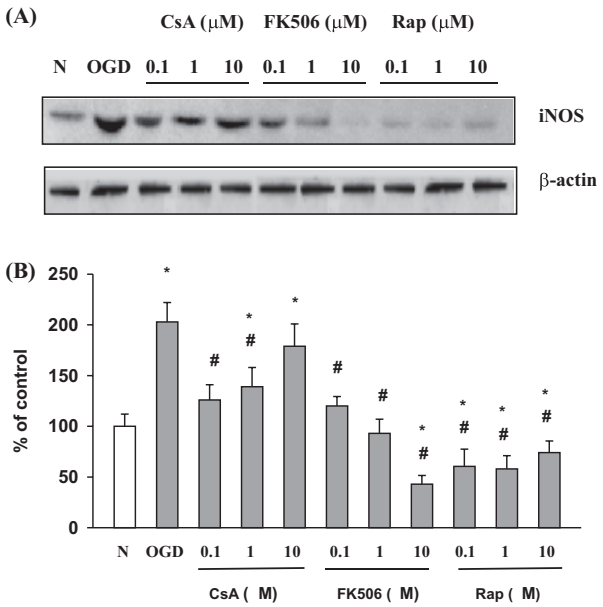


**Fig. 4.** (**A**) Western blot analysis of iNOS expression in astrocytes exposed to normoxia (N) or OGD and treated with cyclosporine A (CsA), FK506 or rapamycin (Rap). Cell lysates (20 μg of protein) from experimental groups were analyzed by immunoblotting using an antibody against iNOS. Detection of β-actin ensured that equal amounts of proteins were loaded in western blot analysis. Blots shown are representative of three independent experiments. (**B**) Semiquantification of blots. The IOD ratio of iNOS signals to β-actin signals was calculated. The ratio obtained from untreated cell cultures (normoxia) was normalized to 100%, and the data from three independent experiments are presented as the means ± SD of the percent change from control; * $p < 0.05$ *vs.* normoxia; # $p < 0.05$ *vs.* OGD

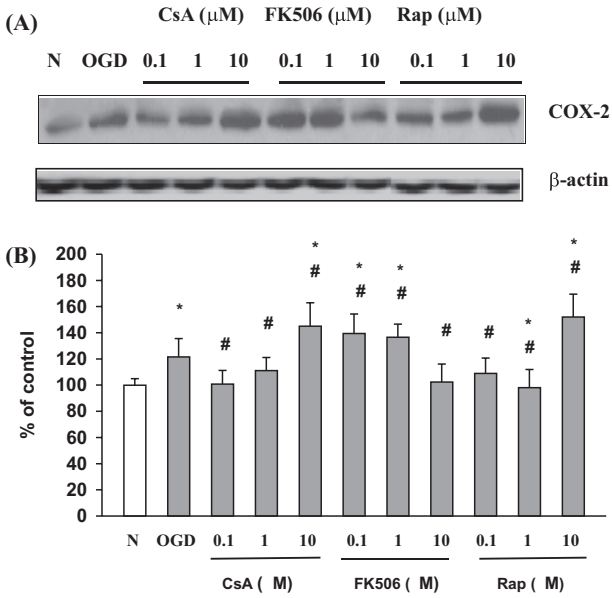


**Fig. 5.** (**A**) Western blot analysis of COX-2 expression in astrocytes exposed to normoxia or OGD and treated with cyclosporine A (CsA), FK506 or rapamycin (Rap). Cell lysates (20 μg of protein) from experimental groups were analyzed by immunoblotting using an antibody against COX-2. Blots shown are representative of three independent experiments. (**B**) Semiquantification of blots. The IOD ratio of COX-2 signals to β-actin signals was calculated. The ratio obtained from untreated cell cultures (normoxia) was normalized to 100%, and the data from three independent experiments are presented as the means ± SD of the percent change from control; * $p < 0.05$ *vs.* normoxia; # $p < 0.05$ *vs.* OGD

## Discussion

HMGB1 is highly expressed and released by both neurons and glial cells under ischemic conditions and stimulates the release of other cytokines to exacerbate brain injury [24]. This implies that HMGB1 may be an excellent target for the treatment of cerebral ischemia. Protection against transient brain ischemia by intravenous injection of neutralizing anti-HMGB1 mAb has already been documented [26]. It was also shown that minocycline, a semisynthetic tetracycline, inhib-

its activated microglia expressing HMGB1 and decreases neurological impairment induced by cerebral ischemia [20].

This study demonstrates that HMGB1 expression is significantly increased in ischemic astrocytes (Fig. 1). Additionally, western blotting of cell culture media showed that HMGB1 was present in the electrophoresed media of cultures exposed to OGD, suggesting that the protein is released extracellularly from astrocytes. It is not clear whether HMGB1 is secreted actively or passively from damaged astrocytes. The histone deacetylase inhibitor TSA, which inhibits HMGB1 binding to chromatin, only slightly affected the HMGB1 protein levels in media, indicating that extracellular release of HMGB1 is mainly a passive process typical of necrosis (Fig. 1C). However, it is possible that in our experimental paradigm, HMGB1 release may be partly an active process triggered by activation of glial cells or oxidative stress because the antioxidant compound NAC clearly attenuated HMGB1 expression and release. Our results are in accordance with results of Tsung et al. [40] who postulated that the initial HMGB1 release in ischemia is a partly active process triggered by redox signaling.

Our study provides evidence that immunosuppressants decrease the expression and release of HMGB1 in ischemic astrocytes. However, the effects of cyclosporine A, FK506 and rapamycin on HMGB1 expression were identical at the various concentrations used (0.1 μM for cyclosporine A and rapamycin and 10 μM for FK506). The fact that higher concentrations of cyclosporine A (1–10 μM) did not reduce HMGB1 expression and FK506 (0.1–1 μM) had lower efficacy suggests that the drugs cause toxicity through mechanisms separate from immune glia activation, for example, through suppression of cyclophilin D by cyclosporine A [19] or prevention of glutamate release and toxicity by FK506 [6]. *In vitro* studies demonstrated that FK506 protects primary cortical cell cultures against *N*-methyl-D-aspartate (NMDA)-induced neurotoxicity, which is well known to be mediated in part by nitric oxide (NO). We also observed strong inhibition of iNOS expression in ischemic astrocytes by FK506 (Fig. 4). Because ischemia-induced iNOS expression has been shown to be elevated in astrocytes in some brain regions for days after even brief periods of ischemia [12], it is likely that this effect could be involved in FK506-mediated protection in brain ischemia. In contrast to cyclosporine A and FK506, rapamycin inhibited the expression and release of HMGB1 at all concentrations used, although the observed effect did not appear to be dose-dependent. However, rapamycin is a specific inhibitor for signal transduction and an activator of transcription 3 (STAT3), and the JAK/STAT pathway plays a major role in the regulation of expression and inflammatory effects of HMGB1 [27]. Liu et al. [27] have also shown that rapamycin markedly suppressed HMGB1 expression in cultured macrophages after stimulation with LPS *in vitro*.

HMGB1 is one of the main diffusible signals of necrosis and it activates the inflammatory responses leading to necrosis, but not apoptosis [36]. In our study, immunosuppressants were also able to prevent the necrosis of ischemic astrocytes (Fig. 2). Cyclosporine A, at a concentration of 0.1 μM, appears to be the most efficient drug in preventing necrosis of ischemic astrocytes. The antinecrotic properties of cyclosporine A may depend on its interaction with cyclophilin D, a protein that induces mitochondrial permeability transition pore (MPTP) opening [19]. The results obtained by Nakagawa et al. [31] indicate that the cyclophilin D-dependent MPTP regulates some forms of necrotic, but not apoptotic, cell death. Measurements of LDH activity confirm that these drugs may inhibit passive HMGB1 release from cells. The involvement of ROS in the attenuation of HMGB1 release is also possible because we have previously demonstrated that immunosuppressants attenuate oxidative stress in ischemic astrocytes [17].

Once released into the extracellular milieu, HMGB1 can bind to cell-surface receptors (e.g., RAGE and TLR2 and -4) and activate inflammatory responses. Recent findings indicate that HMGB1 and its functional receptors are expressed in astrocytes in both murine brain and primary cultures [3, 33]. Furthermore, it was demonstrated that toll-like receptors (TLRs) and RAGE signaling are involved in ischemia/reperfusion-induced inflammation and injury [33, 40].

Classical TLR signaling initiated by ligand binding and dimerization of receptors allows TLR-specific recruitment of downstream signaling molecules, including the adaptor molecule MyD88, IL-1R-associated kinase (IRAK), TNFR-associated factor 6 and NF B-inducing kinase (IKK) [28]. The recruitment of these molecules triggers the stimulation of downstream kinases, including MAPKs, such as ERK1/2, p38 MAPK, and SAPK/JNK, and the activation of the transcriptional factor NFκB [28]. Moreover, in addition to TLR, HMGB1-induced intracellular signaling

through RAGE can activate two different cascades, one involving the small GTPases Rac and Cdc42, leading to cytoskeletal reorganization, and a second cascade that involves the Ras-MAP kinase pathway and subsequent NFκB activation [22]. Therefore, activation of both types of HMGB1 receptors results in the activation of NFκB, which leads to the induction of genes encoding cytokines (TNF-α, IL-1β), chemokines (IL-8, MIP-2, MCP-1) and other mediators, such as iNOS and COX-2, which contribute to neuroinflammation [23, 42]. Thus, key indications of the activation of these receptors upon OGD exposure include the subsequent downstream activation of the transcription factor NFκB and the up-regulation of iNOS and COX-2, which we noted after a 24-h OGD treatment (Figs. 3–5). In our study, immunosuppressants at the same concentrations that significantly decreased the expression and release of HMGB1 also inhibited NFκB, iNOS and COX-2. However, it should be emphasized that although it was demonstrated that receptors for HMGB1 are present in astrocytes [3, 17], the effect of immunosuppressants on their expression and activation requires additional investigation.

The relevance of HMGB1 to neuroinflammation was demonstrated in animal models of brain ischemia, showing that suppression of HMGB1 expression reduces the induction of proinflammatory cytokines and correlates with diminished infarct volumes in the rat [24, 33]. Also, a recent clinical study by Goldstein et al. [18] indicating that plasma levels of HMGB1 are elevated in stroke patients strengthens the possibility of a relationship between this protein and the pathogenesis of stroke in humans. These findings open new possibilities for treatment of postischemic brain injury *via* HMGB1 inhibition, which can be accomplished by various pharmacological strategies [20] in addition to anti-HMGB1 antibodies [26]. In brain ischemia, immunosuppressants appear to be especially therapeutic because the results of many experiments confirm their distinct protective influence [9, 41, 43]. For this reason, the potent neuroprotective actions of immunosuppressive drugs are of great interest for clinical treatment of brain ischemia. It should be noted that in the present study, astrocyte cell cultures were treated with immunosuppressants at low concentrations that do not have toxic effects and are commonly used in scientific studies on neuroprotection. FK506, cyclosporine A and rapamycin, at concentrations that most effectively down-regulated HMGB1 in ischemic astrocytes in our study, were also used by other researchers in *in vitro* experiments and that demonstrated notable protective effects. For example, the measured tissue concentration of the drug in the CA1 region of the hippocampus reached 0.292 μM in ischemic rats after the intravenous injection of 10 mg/kg of cyclosporine A [41]. Also, the concentrations of FK506 and rapamycin used (0.1–10 μM) are in a range necessary to produce the *in vivo* protective effects of the drug [13, 39].

In conclusion, the present results provide further information about the cytoprotective mechanisms of immunosuppressants on ischemic astrocytes in relation to the pathophysiology of ischemic brain injury. The protective effects of immunosuppressants may be mediated in part by suppression of HMGB1 expression and release in astrocytes, which leads to attenuation of ischemia-induced necrosis and neuroinflammation.

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