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Effect of Cyclosporin A on Immediate Early Gene in Rat Global Ischemia and Its Neuroprotection

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Abstract. The expressions of the immediate early genes, c-fos and c-jun, and their product proteins C-FOS, C-JUN, and P-JUN were examined in the hippocampal CA1 subfield after global ischemia and reperfusion in rats treated with cyclosporin A. More than 90% neuronal cell death was seen in hippocampal CA1 7 days after global ischemia in control animals, but only 5% cell death after ischemia was seen in the CsA-treated animals. The expressions of c-fos and c-jun mRNA in the control animals were detected with an increase from 1 to 48 h after ischemia. On the other hand, they showed significant suppression in the CsA-treated animals. Increased expressions of C-FOS were found 1, 24, and 48 h after reperfusion in the control animals. In the CsA-treated animals C-FOS expression was found to increase, but the expression level reduced to a statistically insignificant level within 48 h after the ischemia. C-JUN and P-JUN expressions increased in control animals, but were almost completely suppressed in the CsA-treated animals. The present study demonstrated that the suppressant effects of CsA on IEGs and their products might have causal relationship to the dramatic protecting effect of the drug against delayed neuronal cell death.

Keywords: immediate early gene, ischemia, immunosuppressant, hippocampus, delayed neuronal death

Introduction

It has been suggested that gene expression after cerebral ischemia plays an important role in determining the fate of neuronal cells. The genes in the immediate early gene (IEG) group respond to a wide range of weak and strong stimulants and are involved in expression of a variety of genes through transcription factors (1). They are known to regulate physiological responses of cells by activating the expression of secondary genes in response to stimulants (2, 3). In particular, there have been many reports on the changes after cerebral ischemia of C-FOS or C-JUN whose expression are induced by responses through the activation of ionotropic glutamate receptors for *N*-methyl-D-aspartate (NMDA) and AMPA (4 – 6). On the other hand, it has been debated whether C-FOS or C-JUN play a role in determining cell death or cell protection (7 – 9).

The neuro-protection by FK506, an immume-suppressant, during focal cerebral ischemia had been reported as an immunophillin-mediated effect (10). We also have found the dramatic anti-ischemic effects of cyclosporin A (CsA), an immune-suppressant, by regulating calcineurin and mitochondrial cyclophilin D (11 – 13). In the present study, we examined the expression of those IEGs during development of neuronal cell death after the ischemia in the rats with or without the treatment of CsA, which will provide us important clues to rule out the causal relationship between expression of those IEGs and neuronal cell death due to ischemia.

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Materials and Methods

Animal experiments were performed in accordance with "Guiding Principles for the Care and Use of Laboratory Animals", which were approved by the Animal Care Committee of Tokyo Medical University.

Rat forebrain ischemia model

Ninety-eight male Wistar rats, 8 weeks of age (body weight 280 – 300 g), were used for this study, according to the method of Smith et al. (14). After fasting overnight, the rats were intubated on a respirator (2.7 ml per an exchange, 80 exchanges per min) under anesthesia by oxygen, N_2O gas, and 3% Isoflurane. Anesthesia was maintained with oxygen, N_2O gas, and 1% – 2% Isoflurane in the peroperative period. Blood pressure was monitored through a P_{10} polyethylene tube inserted in the tail artery. A transverse incision was made on the skin in the neck, followed by removal of the subcutaneous tissue. A vertical incision was made in the muscle surrounding the trachea to expose the right and left common carotid arteries and to isolate them with a 3-0 silk thread. In addition, a silicon tube was inserted in the external jugular vein for blood drainage. A temperature probe was inserted in the temporal muscle for monitoring cerebral temperature. Body temperature was also monitored through a temperature probe inserted in the rectum. Throughout the experiment, cerebral and rectal temperatures were maintained at 37°C by heating with an incandescent lamp appropriately.

After blood circulation is stabilized, the blood pressure was reduced to 50 mmHg or lower by drainage of 7 ml of blood from the external jugular vein. Forebrain ischemia was induced by affixing an aneurysm clip to each of the common carotid arteries and obliterating the blood flow 10 min. Blood pressure was maintained at 50 mmHg or lower by further drainage, when necessary, from the external jugular vein. After 10 min of ischemia, circulation was restored and the aneurysm clips were removed when the blood pressure was restored to 80 mmHg, whereupon 0.5 ml sodium bicarbonate solution was added. The incision was closed following removal of all tubes. Then, with anesthesia off and oxygen on and after spontaneous respiration had returned, the intubation tube was removed. After rats were returned to their cages, the body temperature was maintained at 37°C by the use of the incandescent lamp when necessary.

Rats were assigned into one of the two administration groups: the vehicle administration group or the CsA (Novartis Pharma, Basel, Switzerland) administration group. Immediately after completion of the 10-min forebrain ischemia treatment, vehicle (the same volume of the cremaphore used for CsA solution, i.v.) or CsA (10 mg/kg, i.v.) was administered, followed by intraperitoneal administration at the same dose once a day for 7 days.

Histopathological analysis

After 1 week, brain tissue was dissected following perfusion of 4% formaldehyde (n = 10 for each experimental group), and paraffin sections of 8 μm were prepared for hematoxilin-eosin (HE) staining. Delayed neuronal cell death was examined by counting the total alive cells of the dorsal hippocampal CA1 level at bregma −3.8 mm according to the Paxinos-Watson rat brain map.

In situ hybridization

To examine mRNA expression in the hippocampal CA1 after ischemia, in situ hybridization analysis was performed according to the method of Kamme et al. (15). Forebrain ischemia was induced in 35 rats (16 rats each in the vehicle and CsA administration groups and 3 for sham operation); and following reperfusion for 1, 6, 24, and 48 h (4 rats for each time point), the rats were decapitated, and frozen sections of 20 μm were prepared from frozen brain tissues.

Oligonucleotides, 48 nucleotides in length, were used. The probe for labeling c-fos was made as described by Curran et al. (16), using the oligonucleotides complementary to the sequence at positions 544 – 591 nt of the rat c-fos RNA. The probe for labeling c-jun was made according to the method of Sakai et al. (17) using the oligonuecleotides complementary to the 485 – 542 nt sequence of the rat c-jun RNA. These oligonucleotides were shown to hybridize to RNAs in neuronal cells by Kamme et al. (15). An 80-ng sample of each oligonucleotide was labeled at the 3' terminus by terminal deoxynecleotidyl transferase (TdT) using α-[^{35}S]dATP by incubation at 37°C for 2.5 h in a total volume of 25.5 μl including 0.11 nmol α-[^{35}S]dATP (1400 Ci /mmol), 27.5 U TdT, and Mg^{2+} buffer (IBI® + DEPC). Following termination of the reaction by addition of 500 μl of 0.1 M Tris buffer, pH 8, labeled probes were obtained by using a NENSORB 20 Nucleic Acid Purification Cartridge (NEN, Boston, MA, USA), followed by the addition of DTT (Sigma, St. Louis, MO, USA) to the final concentration of 10 mM. Labeling of probes for use at about 500,000 cpm/μl was confirmed with the β-counter (LS 2800; Beckmann, Tokyo) (13).

After drying, brain sections were fixed with 4% paraformaldehyde for 30 min, followed by washes with 0.1 M phosphate-buffered saline (pH 7.2, PBS) a total of three times for 5 min and dehydration with ethanol (70% once for 2 min and 95% once for 2 min). Each

section was covered with 250 μl of the hybridization solution containing 1×10^7 cpm/ml, 40 μl/ml MDTT, 50% formamide, 4 × SCC, 1 × Denhardt's solution, 1% sarcosyl (*N*-lauryl sarcosine, Sigma), 0.02 M phosphate buffer, 10% dextran sulphate (Amersham Bioscience Corp., Piscataway, NJ, USA), and 500 μl/ml sheared salmon sperm DNA (Sigma) and then sealed with parafilm. Following hybridization at 42°C for 18 h, the sections were washed with 1 × SSC 4 times for 15 min at 55°C and DEPC water twice for 2 min. Finally, the slides were dehydrated with ethanol (70% once for 2 min and 95% once for 2 min), dried, and exposed to Hyperfilm-βMax (Amersham Bioscience Corp.) for 5 to 7 days. From the analysis of developed films with the Image Analyzer (Dr. Wayne Rasband, NIH, Bethesda, MD, USA), optical densities (OD) were obtained for the hippocampal CA1 using internal standards. Statistical analysis was performed with mean values. Optical Densities in each ischemic group were compared to OD at the same site of the non-ischemic model taken as 1. All values are shown as the mean ± S.D. and analyzed by One-factor analysis of variance (ANOVA) followed by the Bonferroni/Dunn post-hoc test with a significance level of $P<0.05$.

Western blotting analysis

Male Wister rats (43 animals: 20 rats each in the vehicle and CsA administration groups and 3 for sham operation) were used. Rats were sacrificed in accordance with recovery times [1, 6, 12, 24, and 48 h (4 rats for each time point)] after forebrain ischemic treatment. Hippocampal CA1 sections were dissected in ice-cold homogenization buffer (15 mM Tris-HCl, pH 7.6, 1 mM DTT, 0.25 M sucrose, 1 mM $MgCl_2$, 1.25 μg/ml pepstatin A, 10 μg/ml leupeptin, 2.5 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 1 mM EGTA, 0.1 M Na_3VO_4, 50 mM NaF, 2 mM sodium pyrophosphate, and 0.1% Triton X-100). After homogenizing in 10 times volumes of the homogenization buffer, brain tissue was centrifuged at 15,000 rpm to obtain the supernatant. Protein concentration was determined with the DC Protein Assay Kit (Bio-Rad Lab, Tokyo).

For Western blotting, 20 μg protein was subjected to electrophoresis using an 8% polyacrylamide gel, followed by transfer to PVDF membrane (Immobilon P; Cosmobio, Tokyo). Membranes were incubated at 4°C overnight with C-FOS (Calbiochem, San Diego, CA, USA), C-JUN polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phosphorylated C-JUN antibody [Cell Signaling Tech solutions (1:1000); Cell Signaling Tech, Danvers, MA, USA], followed by labeling with the HRP-binding secondary antibody (antirat-rabit). Specific proteins were identified by exposure of hyperfilm to chemiluminescence (ECL Amersham Bioscience Corp.). Quantification of Optical densities were calculated as a ratio between the densitmetric score for C-FOS, C-JUN, and β-actin by an NIH image analyzer. The expression of phosphorylated protein was similarly calculated as a ratio between the densitmetric score for phosphorylated C-JUN versus unphosphorylated C-JUN. All values were shown as the mean ± S.D. and analyzed by One-factor ANOVA, with a significance level of $P<0.05$. Mann-Whitney's *U*-test was applied for pathohistological analysis with a significance level of $P<0.01$.

Results

Protective effects of CsA on the neuronal cell death after global ischemia and reperfusion

To confirm the protective effects of CsA on the ischemic neuronal cell death. We administered CsA (10 mg/kg, i.v.) immediately after the completion of the 10-min forebrain ischemia, and then administered the drug (10 mg/kg, i.p.) for 7 days once a day. As shown in Fig. 1, 93.5 ± 0.5% of neuronal cells in the hippocampal

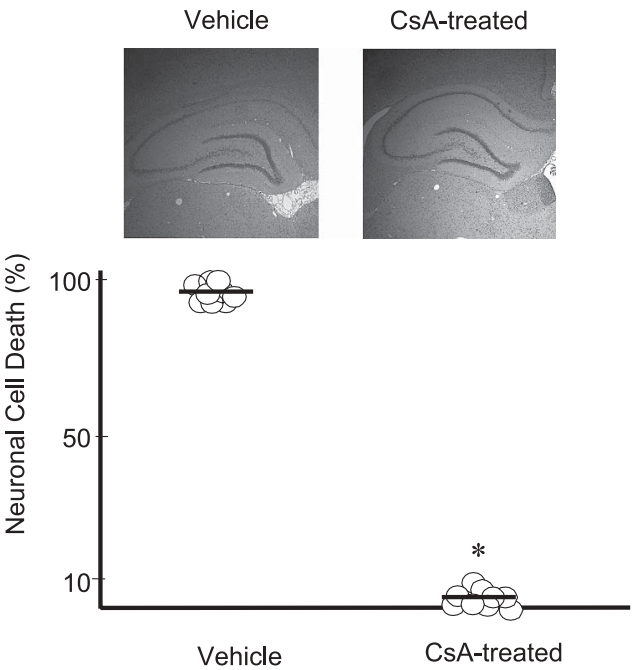

Fig. 1. Delayed neuronal cell death due to global ischemia and reperfusion and its protection by cyclosporin A treatment. The numbers of alive hippocampal CA1 pyramidal neurons were counted 7 days after 10-min global ischemia and reperfusion. In the preparations treated with vehicle, 93.5 ± 0.8% (n = 9) of the cells was lost, but only 5.4 ± 0.1% (n = 9) was lost in the CsA-treated preparation. *Statistically significant ($P<0.01$) in Mann-Whitney's *U*-test.

CA1 region disappeared after 7 days in the animals treated with only vehicle. However, the neuronal cells in the animals treated with CsA were almost completely protected (only 5.4 ± 0.1% of the cells died). The present results confirmed the marked anti-ischemic effect of CsA and our further studies on the effects of the drug on IEGs expression deserve attention.

In situ hybridization analysis of time course change in IEG

Time course of change in c-fos mRNA expression: No expression of c-fos mRNA was detected at each area of the hippocampus under non-ischemic conditions (OD: 1.0 ± 0.14). A significant increase of c-fos mRNA expression was observed in the hippocampal CA1 area at 1 h after ischemia treatment in the vehicle-only administration group (Fig. 2A: left row; OD: 10.15 ± 3.54, Fig. 2B). After 6 h, c-fos mRNA expression decreased in the hippocampal CA1 area (Fig. 2A: left row; OD: 1.89 ± 0.56, Fig. 2B), followed by a significant re-elevation in the hippocampal CA1 after 24 and 48 h (Fig. 2A: left row; OD: 2.11 ± 0.56, 2.30 ± 0.43, Fig. 2B)); the re-elevation was observed in CA3 and DG.

A significant increase of c-fos mRNA expression was detected in the hippocampal CA1 area of CsA-treated animals at 1 h after ischemia treatment (Fig. 2A: right row; OD: 1.23 ± 0.23, Fig. 2B), although the extent of increase was smaller than in the vehicle-only administration group. In addition, in the hippocampal CA1 area of the CsA-treated animals, there were no significant changes in c-fos mRNA expression between 6 and 48 h after ischemia treatment (Fig. 2A: right row; OD: 2.5 ± 1.6, 1.50 ± 0.62, Fig. 2B).

Time course changes of c-jun mRNA expression: The expression of c-jun mRNA was observed at each area of the hippocampus under non-ischemic conditions (OD: 1 ± 0.22). In the vehicle-only administration group, a significant increase was observed in the hippocampal CA1 area at 1 h after ischemia treatment (Fig. 3A: left row; OD: 8.34 ± 2.67, Fig. 3B). Although the expression in the hippocampal CA1 area decreased after 6 h, it increased again after 24 and 48 h (Fig. 3A: left row; OD: 5.44 ± 2.11 and 7.13 ± 1.89, Fig. 3B). In the CsA administration group, c-jun mRNA significantly increased between 6 and 24 h after ischemia treatment, (P<0.05), although the expression level was lower than in the vehicle-only administration group

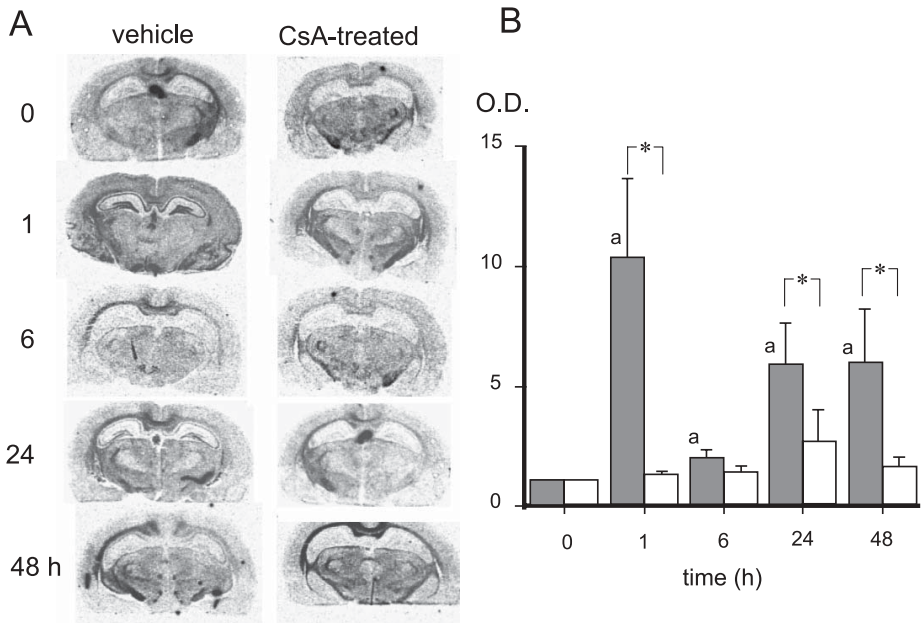

Fig. 2. Comparison of c-fos mRNA by in situ hybridization in CsA-treated and -untreated animals. A: In situ hybridization of c-fos mRNA in the brain section at bregma –3.8 mm according to the Paxinos-Watson brain map. In the vehicle-treated group, significant increase of c-fos expression was seen 1 h after reperfusion in the hippocampal area and it returned to the baseline level by 6 h after reperfusion. The c-fos expression increased again 24 h after reperfusion and persisted until 48 h. The CsA-treated group did not show any significant increase during each reperfusion. B: Comparison of c-fos mRNA expression by optical density: In the vehicle-treated group (filled column), c-fos mRNA expression showed the maximal increase 1 h after reperfusion and decreased until 6 h of reperfusion. It showed the increase again 24 h after reperfusion and persisted until 48 h after reperfusion. In the CsA-treated group (open column), no significant changes in the c-for mRNA level in hippocampal region. [a]Statistically significant increase compared to 0 time (vehicle-treated group) (ANOVA, P<0.05). *Statistically significant change between vehicle- and CsA-treated group (ANOVA, P<0.05).

(Fig. 3A: right row; OD: 1.56 ± 0.24, 2.11 ± 0.56, and 2.30 ± 0.43 after 1, 6, and 24 h, respectively, Fig. 3B). The expression of c-jun mRNA at CA3 and DG recovered to the control level.

The expression of c-jun mRNA in the CsA-treated animal was not significant in the hippocampal CA1 area at 48 h after ischemia treatment (Fig. 3A: right row; OD: 1.34 ± 0.33, Fig. 3B).

Western blot analysis of time course in C-FOS, C-JUN, and P-JUN protein expression

Time course of C-FOS protein expression: C-FOS protein expression increased from 1 h after ischemia treatment in the vehicle-only administration group compared to the non-ischemic condition, with a continuous significant increase between 24 and 48 h after ischemia treatment (Fig. 4: A and B). In the CsA administration group, a statistically significant increase of C-FOS protein expression was observed between 1 and 6 h after ischemia treatment (Fig. 4: A and B), followed by a decrease after 12 h and a return to the level before ischemia treatment after 48 h.

Time course in C-JUN protein expression: In the vehicle-only administration group, the C-JUN protein expression increased from 1 h after ischemia treatment compared to the level in the non-ischemic condition, followed by a continuously significant increase in the late period after ischemia (24 and 48 h later) (Fig. 5: A and B). In the vehicle-only administration group, the C-JUN protein expression was significantly higher between 6 and 48 h after ischemia treatment than that in the CsA administration group. Although there was an apparent time-dependent increase in C-JUN protein expression in the CsA administration group, there were significant differences in the increase observed between the expressions at 6 and 48 h after ischemia treatment (Fig. 5: A and B).

Time course in phosphorylated C-JUN protein expression: In the vehicle-only administration group, the expression of phosphorylated C-JUN protein increased from 1 h after ischemia treatment compared to the level in the non-ischemic condition, followed by a continuous significant increase in the late period after ischemia treatment (24 and 48 h later) (Fig. 5: C and D). In addition, the expression of phosphorylated C-JUN protein was significantly higher between 1 and 48 h after ischemia treatment in the vehicle-only administration group than in the CsA administration group (Fig. 5: C and D). In the CsA-treated animals, there was no significant difference in phosphorylated C-JUN protein

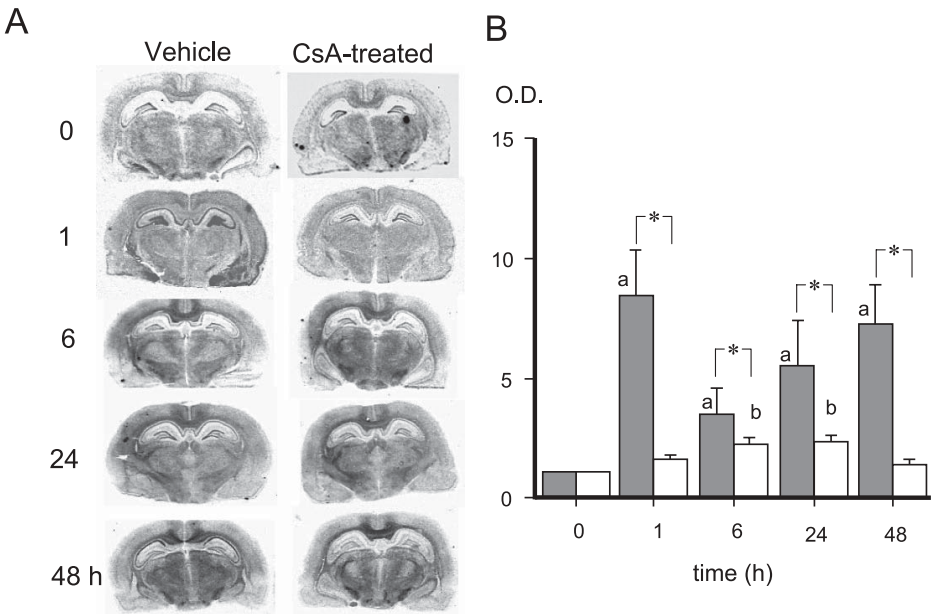

Fig. 3. Comparison of c-jun mRNA by in situ hybridization. A: In the vehicle-treated group, significant increase of c-jun expression was seen 1 h after reperfusion in hippocampal area and it showed slight decrease until 6 h after reperfusion. The expression of c-jun mRNA increased again 24 h after reperfusion and persisted until 48 h after reperfusion. The CsA-treated group did not show any significant increase during each reperfusion. B: In the vehicle-treated group (filled column), the expression of c-jun mRNA showed the maximal increase 1 h after reperfusion and transient decrease at 6 h of reperfusion, and increased again 24 h after reperfusion that persisted until 48 h after reperfusion (P<0.05). The CsA-treated group (open column) did not show significant increase at 1 h, but showed significant increase at 6 and 24 h (P<0.05). [a]Statistically significant increase compared to 0 time (vehicle-treated group) (ANOVA, P<0.05). [b]Statistically significant increase compared to 0 time (CsA-treated group). (ANOVA, P<0.05). *Statistically significant change between vehicle- and CsA-treated group (ANOVA, P<0.05).

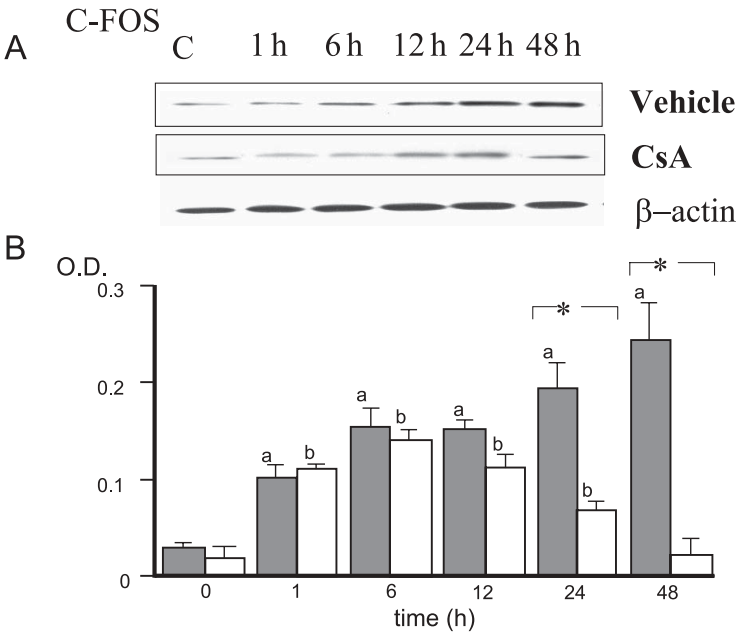

Fig. 4. Expression of C-FOS in CA1 sector detected by Western blot analysis. A: Examples of C-FOS expression detected by Western blot analysis of the vehicle-treated group and CsA-treated group. The expression of *β*-action was examined as a standard. B: Comparison of C-FOS expression by optical density. In the vehicle-treated group (filled column), C-FOS expression showed an increase 1 h after reperfusion that persisted until 48 h after reperfusion. The CsA-treated group (open column) also showed the increase of C-FOS expression from 1 h until 24 h after reperfusion. C-FOS expression in the vehicle-treated group showed significant increase compared to the CsA-treated group. [a]Statistically significant increase compared to 0 time (vehicle-treated group) (ANOVA, $P<0.05$). [b]Statistically significant increase compared to 0 time (CsA-treated group) (ANOVA, $P<0.05$). *Statistically significant change between the vehicle- and CsA-treated group (ANOVA, $P<0.05$).

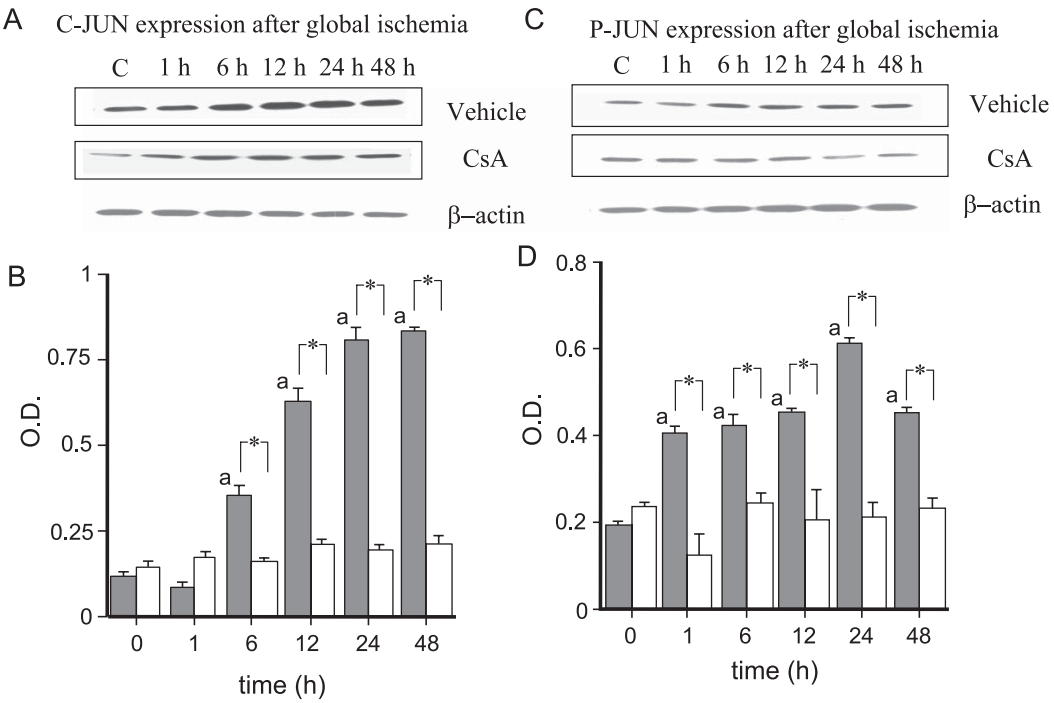

Fig. 5. Comparison of C-JUN and P-JUN expression in CA1 subfield by Western blot analysis. A: Examples of C-JUN expression detected by Western blot analysis in the vehicle-treated group and CsA-treated group. The expression of *β*-action was examined as a standard. B: C-JUN expression determined by optical density: In the vehicle-treated group (filled column), C-JUN expression showed an increase from 1 h until 48 h after reperfusion. The CsA-treated group (open column) did not show any significant increase during reperfusion. The vehicle-treated group also showed significant increase in C-JUN expression compared to the CsA-treated group. C: Examples of P-JUN expression in the CA1 subfield by Western blot analysis in the vehicle-treated group and CsA-treated group. The expression of *β*-action was examined as a standard. D: P-JUN expression determined by optical density. In the vehicle-treated group (filled column), P-JUN expression also showed an increase from 1 h until 48 h after reperfusion ($P<0.05$). The CsA-treated group (open column) did not show any significant increase during reperfusion. The vehicle-treated group also showed significant increase in P-JUN expression compared to the CsA-treated group. [a]Statistically significant increase compared to 0 time (vehicle-treated group) (ANOVA, $P<0.05$). *Statistically significant change between vehicle- and CsA-treated group (ANOVA, $P<0.05$).

expression in the early and late periods after ischemia treatment (1 and 48 h later) (Fig. 5: C and D).

Discussions

In the present study using the rat forebrain ischemia model, in which delayed neuronal cell death is selectively induced in the hippocampal CA1 subfield, the expression of immediate early genes (IEGs), c-FOS and c-JUN, were compared and analyzed at the levels of mRNA and protein in the two groups: vehicle-only administration group and CsA administration group, which strongly suppressed delayed neuronal cell death.

Although delayed neuronal cell death following cerebral ischemia is a phenomenon that has been reported in a variety of animal species (18), the mechanism for how it occurs has not been well understood. It has been suggested that the activation of the glutamate receptor, which accompanies glutamate release upon cerebral ischemia, plays an important role in neuronal cell death, and its regulation, therefore, is the key for the protection of brain function. In addition, it has been shown that glutamate release induces IEG expression during cerebral ischemia (19, 20). C-fos was reported to induce neuronal cell death through the NMDA receptor (21) and c-jun to contribute to neuronal cell death through the activation of NMDA and AMPA receptors (22). Gerlach et al. (1) showed that Ca^{2+} influx by the activation of glutamate receptors induced c-fos and c-jun expression and that IEG expression was inhibited by those receptor antagonists, MK801 or NBQX, showing a close correlation between Ca^{2+} influx and IEG expression through the glutamate receptor.

Among IEGs, which function as so-called third messengers in cellular signal transduction (23), zinc-finger IEGs, including c-fos and c-jun, are induced by a variety of stimulants including ischemia (7). Normally, C-FOS and C-JUN proteins form homo- or hetero dimmers and bind to the AP-1 site on DNA to regulate transcription of the gene expression involved in differentiation, proliferation, and survival of neuronal cells. There have been many reports that show the correlation of c-fos and c-jun expression and neuronal cell death (9, 24, 25). On the other hand, it has been suggested that the expression of C-FOS in an early phase of ischemia is associated with protection of neurons (23).

The present study showed that the expression of c-fos and c-jun mRNAs were strongly induced in the hippocampal CA1 region at 1 h after cerebral ischemia treatment, along with an increased expression of C-FOS and C-JUN proteins, showing an ischemia-associated expression of c-fos and c-jun mRNA, as well as their product proteins. Although protein expression was apparently at lower levels, it appeared to be due to suppression of protein synthesis associated with ischemia. C-FOS and C-JUN protein expression increased with time and continued for 48 h. Wu and Liu reported that sustained expression of C-FOS and C-JUN proteins greatly contributed to cell death (22). The ischemia model used in the present study, called the Maj-Lis model, is characterized by ischemia in the forebrain only, rather than in the midbrain (14). In this model, delayed neuronal cell death is induced in the cerebral cortex, CA1 region of the hippocampus, and corpus striatum from 24 – 48 h after ischemia onwards. A close correlation between sustained expression of IEG and delayed neuronal cell death is suggested from the results of the present study. The expression of c-fos and c-jun mRNA and their product proteins was obviously inhibited by administration of CsA, an immunosuppressant.

CsA is an circular undecapeptide extracted from *hypocladium inflatum gam* and is widely used as an immunosupressant for suppression of the immunological rejection following organ transplantation. Inhibition of calcineurin activity of dephosphorylated oxidase is known to underlie its mechanism of action. Liu et al. reported that calcineurin is the target of the immunosuppressants, CsA or FK506 (26), while immunophillin (cyclophilin D and FKBP), which binds to CsA or FK506, plays an important role in mediating their effects. It is known that immunophillin, with a prolyl*cis/trans* isomerase activity, forms a complex with CsA or FK506 and inhibits calcineurin activity by inducing structural changes in catalytic sites. Calcineurin inhibits Ca^{2+} influx by dephosphorylation of the NMDA receptor, IP3 receptor, and ryanodine receptor under normal physiological conditions. Morioka et al. have suggested that calcineurin plays a role as Ca^{2+}-buffering protein (27). It has been also reported that calcineurin protects neuronal cells by inducing SOD expression through NFκB after cerebral ischemia (28). On the other hand, there are contradicting reports on the function of NFκB, in that it has been shown to cause injury of neuronal cells by induction of iNOS (inducible NO synthetase), while it is thought to protect them by induction of SOD, showing a complexity of NFκB effect. Since it is suggested that calcineurin may be involved in induction of delayed neuronal cell death in the hippocampus following ischemia by elevating production of highly damaging NO by dephosphorylating NOS, the function of calcinurin remains unclear.

It is also known that another effect of CsA is to preserve mitochondrial function by inhibiting the opening of mitochondrial permeability transition (MPT) pores

through binding to cyclophilin D, which is specifically expressed in the mitochondrial matrix. The results showed that CsA may dramatically prevent neuronal cell death through mitochondrial function, in addition to suppression of calcineurin activity, and thus greatly contributed to the understanding of the mechanism for induction of ischemic neuronal cell death by CsA. A variety of anti-apoptosis effects have been reported for CsA, including suppression of Ca^{2+}-induced swelling of isolated hippocampal mitochondria, suppression of cerebral neuronal cell death in the hypoglycemic cerebral injury model, and protection of mitochondrial function (29), although the relationship between calcineurin and cyclophilin D is not clear.

Since the increase in cellular Ca^{2+} by activation of glutamate receptors plays an important role in IEG expression, the decrease in mRNA and protein levels for c-fos and c-jun, observed in the present study, could be a result of suppression of Ca^{2+} influx by CsA directly or the action of calcineurin on the glutamate receptor. C-JUN protein, when phosphorylated by C-JUN N-terminal kinase (JNK), activates transcription of a variety of genes. It was shown, using cerebellar granular cells, that apoptosis was induced by serine 63 or 73 phosphorylation of C-JUN protein under the condition where survival signals were blocked (30). In addition, it has been reported that sustained phosphorylation of C-JUN protein by JNK activation following neuronal injury may contribute to cell death (31). In the present study, the level of phosphorylated C-JUN protein decreased at 1 h after cerebral ischema treatment, compared to the level before treatment in the vehicle-only administration group, followed by a continuous increase after 6 h, suggesting a time course increase. These results were consistent with previous reports (31, 32). On the other hand, no significant increase of phosphorylated C-JUN protein was observed between 1 and 48 h after ischemia treatment in the CsA administration group, suggesting that calcineurin, a dephosphorylated oxidase, is involved in the expression of phosphorylation of C-JUN protein. Consistent with these results, it has been reported that JNK is activated by calcineurin (33) and that CsA inhibits signal transduction by JNK and p38 (34). In addition, when calcineurin activity was measured with time after cerebral ischemia in the CA1 area of the hippocampus, it increased 3-fold in the early phase after cerebral ischemia (after 1 h), compared to the level before ischemia, and continued to increase (up to 6-fold) for 24 h, following a transient decrease after 6 h, whereas it was repressed through all stages in the CsA administration group (35). Therefore, the present results support the mechanism that intracerebral calcineurin activation after cerebral ischemia regulates the expression of phosphorylation of C-JUN protein through its effect on JNK activation.

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