Neuroprotective Actions of FK506 in Experimental Stroke: In Vivo Evidence against an Antiexcitotoxic Mechanism

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The cellular mechanisms underlying the neuroprotective action of the immunosuppressant FK506 in experimental stroke remain uncertain, although in vitro studies have implicated an antiexcitotoxic action involving nitric oxide and calcineurin. The present in vivo study demonstrates that intraperitoneal pretreatment with 1 and 10 mg/kg FK506, doses that reduced the volume of ischemic cortical damage by 56–58%, did not decrease excitotoxic damage induced by quinolinate, NMDA, and AMPA. Similarly, intravenous FK506 did not reduce the volume of striatal quinolinate lesions at a dose (1 mg/kg) that decreased ischemic cortical damage by 63%. The temporal window for FK506 neuroprotection was defined in studies demonstrating efficacy using intravenous administration at 120 min, but not 180 min, after middle cerebral artery occlusion. The noncompetitive NMDA receptor antagonist MK801 reduced both ischemic and excitotoxic damage. Histopathological data concerning striatal quinolinate lesions were replicated in neurochemical experiments. MK801, but not FK506, attenuated the loss of glutamate decarboxylase and choline acetyltransferase activity induced by intrastratial injection of quinolinate. The contrasting efficacy of FK506 in ischemic and excitotoxic lesion models cannot be explained by drug pharmacokinetics, because brain FK506 content rose rapidly using both treatment protocols and was sustained at a neuroprotective level for 3 d. Although these data indicate that an antiexcitotoxic mechanism is unlikely to mediate the neuroprotective action of FK506 in focal cerebral ischemia, the finding that intravenous cyclosporin A (20 mg/kg) reduced ischemic cortical damage is consistent with the proposed role of calcineurin.

Key words: FK506; tacrolimus; stroke; neuroprotection; excitotoxicity; ischemia; MK801; dizocilpine; cyclosporin A

The immunosuppressant FK506 (tacrolimus, Prograf) recently has been introduced into clinical use for the prevention of allograft rejection. Its immunosuppressive mechanism involves inhibition of calcineurin (protein phosphatase 2B) by a complex of FK506 and the 12 kDa immunophilin FKBP12 (Liu et al., 1991, 1992; Clipstone and Crabtree, 1992; Fruman et al., 1992a), resulting in an inability to assemble the active form of the transcription factor NFAT (Bierer et al., 1990; Flanagan et al., 1991) and subsequent attenuation of T lymphocyte gene transcription (Schreiber, 1991; Liu, 1993). The immunosuppressant cyclosporin A also inhibits calcineurin in a complex with cyclophilin, another member of the immunophilin protein family (Liu et al., 1991, 1992; Clipstone and Crabtree, 1992; Fruman et al., 1992a). In contrast, the immunosuppressive mechanism of rapamycin involves blockade of interleukin-2 receptor signal transduction (Schreiber, 1991; Liu, 1993) via an interaction of a rapamycin/FKBP12 complex with a novel protein termed RAPT or FRAP (Brown et al., 1994; Chiu et al., 1994; Sabatini et al., 1994); the precise details of this pathway still have to be elucidated.

Several lines of evidence indicate a role for immunophilins and/or calcineurin in brain function and development (Lyons et al., 1994; Mulkey et al., 1994; Nichols et al., 1994; Chang et al., 1995; Liu et al., 1995; Snyder and Sabatini, 1995; Tong et al., 1995). In addition, FK506 exerts a powerful neuroprotective action in experimental models of stroke (Sharkey and Butcher, 1994), suggesting a novel therapeutic application for this drug. Although the cellular mechanism underlying this effect remains uncertain, pharmacological data confirmed the importance of immunophilin binding and suggested a role for calcineurin (Sharkey and Butcher, 1994). The presence of FKBP12 in rat brain has been demonstrated using both in situ hybridization and Western blot analysis (Steiner et al., 1992; Dawson et al., 1994; Charters et al., 1995), and colocalization with calcineurin has been reported (Steiner et al., 1992; Dawson et al., 1994). FK506 also protects cortical cell cultures against excitotoxic neuronal death, suggesting a direct drug action on brain cells that may involve nitric oxide, because FK506 prevents the dephosphorylation of nitric oxide synthase (NOS) by calcineurin in vitro (Dawson et al., 1993). However, alternative mechanisms must be considered, especially in view of the proposed role for calcium ions in neurodegeneration (Choi, 1995). FKBP12 is an integral part of the ryanodine and IP3 receptor complexes, and functional effects of FK506 on the associated intracellular Ca2+ channels have been demonstrated (Timerman et al., 1993; Zhang et al., 1993; Brillantes et al., 1994; Chen et al., 1994; Cameron et al., 1995a,b). The involvement of reactive oxygen species in the neuroprotective mechanism is also possible because FK506 inhibits superoxide production in neutrophils (Nishinaka et al., 1993), and reactive oxygen species are reported to play a role in both apoptotic neuronal death and neurodegeneration resulting from cerebral ischemia (Kinouchi et al., 1991; Greenland et al., 1995). The present study examines the cellular mechanism underlying the
neuroprotective action of FK506, with particular reference to in vivo excitotoxicity and drug pharmacokinetics.

MATERIALS AND METHODS

Materials. Quinolinate (lot O-1375) was purchased from Sigma (Poole, UK), NMDA and AMPA from Tocris Chemicals (Bristol, UK), and MK801 (dizocilpine) from Research Biochemicals (St. Albans, UK). Excitotoxins were dissolved in sterile 50 mM PBS, pH-adjusted to 7.4 with NaOH. Endothelin-1 (Novo Biochem: lot A13210) was dissolved in sterile saline. FK506 (Fujisawa Pharmaceutical, Osaka, Japan) was dissolved in 10% ethanol in 50 mM PBS containing 1% Tween 80 for intraperitoneal studies and in 10% sodium carbonate in saline containing 400 mg/ml polyoxyl 60 hydrogenated castor oil for intravenous studies.

Excitotoxic lesions. Male Sprague Dawley rats (280–340 g; Charles River, Margate, UK) were anesthetized with either pentobarbitone (Sagittal; 60 mg/kg) for intraperitoneal studies or halothane (4% for induction; 1–2% for maintenance) in nitrous oxide/oxygen (80/20%; v/v) for intravenous studies. Normalthermia (37 ± 1°C) was maintained by using a thermostatically controlled heating blanket connected to a rectal thermometer. Excitotoxins were injected under stereotaxic guidance over 2 min into the striatum [anteroposterior (AP) ±0.5 mm; mediolateral (ML) ±3.0 mm; dorsoventral (DV) ±4.5 mm below dura], cortex (AP ±0.5 mm; ML ±2.5 mm; DV ±1.0 mm below dura), or hippocampus (AP ±4.0 mm; ML ±3.5 mm; DV ±3.0 mm below dura) in a volume of 1 µl (striatum 0.5 µl; hippocampus and cortex). The needle was left in place for a further 2 min before slowly being withdrawn. Animals were placed in an incubator to maintain normothermia until their recovery from anesthesia. Drugs were administered 30 min before excitotoxin injection in intraperitoneal studies and 1 min after excitotoxin injection in intravenous studies.

Induction of focal cerebral ischemia. Male Sprague Dawley rats (300–370 g; Charles River) were anesthetized with either pentobarbitone (Sagittal; 60 mg/kg) for intraperitoneal studies or halothane (4% for induction; 1–2% for maintenance) in nitrous oxide/oxygen (80/20%; v/v) for intravenous studies. Normalthermia (37 ± 1°C) was maintained by using a thermostatically controlled heating blanket connected to a rectal thermometer. Endothelin-1 (60 pmol in 3 µl) was injected via a 31-gauge cannula stereotaxically placed 0.5 mm above the middle cerebral artery (AP +0.2 mm; ML −5.9 mm; DV −7.0 mm below dura). The cannula was left in situ for 5 min before slowly being withdrawn over 2–3 min. Animals were placed in an incubator to maintain normothermia until their recovery from anesthesia. Drugs were administered 30 min before vessel occlusion in intraperitoneal studies and 1 min after vessel occlusion in intravenous studies (except when indicated).

Histopathological assessment of brain damage. Rats were reanesthetized with pentobarbitone (Sagittal; 60 mg/kg) 3 d after injection of excitotoxins or middle cerebral artery occlusion (MCAO) and were fixed by transcardiac perfusion first with 20 ml of heparinized saline (10 U/ml), followed by 200 ml of 4% paraformaldehyde in 50 mM PBS, pH 7.4. The brain was removed intact and immersed in fixative containing 10% sucrose in 0.2 M phosphate buffer, pH 7.0, for 48 hr before cryoprotection. Coronal sections (50 µm thick) were cut and stained with either cresyl violet or thionine. The volume of brain damage was determined as described previously (Park et al., 1989; Sharkey and Butcher, 1994). Briefly, the area of brain damage at eight predetermined brains was assessed using light microscopy by an observer who was unaware of the treatment groups. The volume of brain damage was calculated by integration of the cross-sectional area of damage at each stereotaxic level and the distances between the various levels (Park et al., 1989; Sharkey and Butcher, 1994).

Measurement of glutamate decarboxylase (GAD) and choline acetyltransferase (ChAT) activity. Rats were killed by cervical dislocation 3 d after intrastriatal injection of quinolinate injection. The brain was removed immediately, and injected and uninjected striata were dissected and homogenized in 20 vol of ice-cold water containing 1 mM EDTA and 0.1% Triton X-100, pH 7.4. Tissue GAD and ChAT activity was determined by using minor modifications of the methods of Kanazawa et al. (1976) and Fonnum (1975), respectively. Radioactivity was determined in a Packard 2500TR liquid scintillation counter using automatic quench correction. Enzyme activity was calculated after subtraction of zero time blanks. The protein content of striatal homogenates was determined according to the method of Bradford (1976) using bovine serum albumin as the standard. Protein determinations were made in a Packard 2500TR liquid scintillation counter using automatic quench correction. Enzyme activity was calculated after subtraction of zero time blanks. The protein content of striatal homogenates was determined according to the method of Bradford (1976) using bovine serum albumin as the standard. Protein determinations were made in a Packard 2500TR liquid scintillation counter using automatic quench correction. Enzyme activity was calculated after subtraction of zero time blanks. The protein content of striatal homogenates was determined according to the method of Bradford (1976) using bovine serum albumin as the standard.
Intravenous drug administration

MK801 (0.3 mg/kg) and FK506 (1 mg/kg) decreased the volume of ischemic brain damage in cortex after endothelin-induced MCAO by 34 and 58%, respectively (Fig. 2). In contrast to previous negative data obtained using 1 mg/kg cyclosporin A (Sharkey and Butcher, 1994), intravenous administration at 20 mg/kg (MK5) it reduced the volume of cortical damage induced by MCAO. FK506 (1 and 10 mg/kg; FK1 and FK10, respectively) inhibited ischemic damage, but it had no effect on excitotoxic damage. Neither drug reduced excitotoxic damage in striatum induced by AMPA (25 nmol). Data are the mean volume of brain damage (± SEM) for groups of 5–12 animals. Statistical comparisons between drug and vehicle (saline, S; FK506 vehicle, V) groups used unpaired t tests for excitotoxin data and ANOVA with post hoc Scheffé’s analysis for MCAO data (*p < 0.05; **p < 0.01; ***p < 0.001).

Figure 1. Neuroprotection studies used intraperitoneal drug administration 30 min before lesion induction. MK801 (MK3; 3 mg/kg) inhibited excitotoxic brain damage in striatum, hippocampus, and cortex induced by quinolinate (100 nmol, striatum; 50 nmol, hippocampus and cortex) and NMDA (100 nmol), and at 5 mg/kg (MK5) it reduced the volume of cortical damage induced by MCAO. FK506 (1 and 10 mg/kg; FK1 and FK10, respectively) inhibited ischemic damage, but it had no effect on excitotoxic damage. Neither drug reduced excitotoxic damage in striatum induced by AMPA (25 nmol). Data are the mean volume of brain damage (± SEM) for groups of 5–12 animals. Statistical comparisons between drug and vehicle (saline, S; FK506 vehicle, V) groups used unpaired t tests for excitotoxin data and ANOVA with post hoc Scheffé’s analysis for MCAO data (*p < 0.05; **p < 0.01; ***p < 0.001).
performed by using a paired *t* test (*p* < 0.05; **p** < 0.01).

**GAD and ChAT activity**

GAD activity, a marker for striatal GABAergic interneurons, was reduced by 44% (*p* < 0.05) from 4.37 ± 0.49 μmol/mg protein/hr in the contralateral striatum of vehicle-treated rats to 2.47 ± 0.27 μmol/mg protein/hr in the quinolinate-injected striatum (Fig. 3). GAD activity in the quinolinate-injected striatum of rats treated with FK506 using intraperitoneal (10 mg/kg; 30 min pretreatment) and intravenous (1 mg/kg; 1 min after excitotoxin injection) administration protocols was reduced by 44% (*p* < 0.05) and 46% (*p* < 0.05), respectively, as compared with the contralateral striatum (Fig. 3). In contrast, GAD activity was not reduced significantly in the quinolinate-injected striatum of MK801-treated rats using intraperitoneal (3 mg/kg) and intravenous (0.3 mg/mg) drug administration (Fig. 3). Similar data were obtained by using a ChAT assay to determine the survival of striatal cholinergic neurons. In this case, enzyme activity was reduced by 45% (*p* < 0.05) from 746 ± 94 nmol/mg protein/hr in the contralateral striatum of vehicle-treated rats to 413 ± 83 nmol/mg protein/hr in the quinolinate-injected striatum. ChAT activity in the quinolinate-injected striatum of rats treated with FK506 using intraperitoneal and intravenous administration protocols was reduced by 37% (*p* < 0.05) and 38% (*p* < 0.05), respectively, as compared with the contralateral striatum. ChAT activity was not decreased significantly in the quinolinate-injected striatum of MK801-treated rats; reductions of 9 and 25% were noted with intraperitoneal and intravenous drug administration, respectively, as compared with the contralateral striatum.

**Delayed intravenous administration of FK506**

The temporal window of therapeutic efficacy for FK506 with regard to its neuroprotective action was characterized in a separate group of animals. Although intravenous injection of FK506 (1 mg/kg) at 1, 60, and 120 min after endothelin-induced MCAO reduced the volume of cortical brain damage by 48, 46, and 57%, respectively, FK506 was ineffective when administered after 180 min (Fig. 4). FK506 did not reduce the volume of striatal damage at any time point studied (data not shown). A substantial increase in the volume of ischemic brain damage also was noted in vehicle-treated rats as the duration of anesthesia was extended (Sharkey and Butcher, 1995). Although this effect was not significant when comparing anesthetic durations of 5 and 60 min, further extension to 120 and 180 min after MCAO increased the volume of cortical ischemic damage by 88 and 82%, respectively (*p* < 0.05).

**Physiological variables**

MABP was monitored from 30 min before endothelin-induced MCAO until 180 min after vessel occlusion in animals treated intravenously with either FK506 (1 mg/kg) or FK506 vehicle; significant effects on MABP were not noted in either group (Fig. 5). Rectal and brain temperature similarly were unaffected after endothelin-induced MCAO in FK506 and vehicle-treated rats (Fig. 5).

**Brain and blood levels of FK506**

Brain and blood levels of FK506 were determined from 15 min until 72 hr after intraperitoneal (10 mg/kg) and intravenous (1 mg/kg) administration (Fig. 6). With the use of the intravenous administration route, a brain content of ~50 ng/gm tissue was detected throughout the monitoring period (Fig. 6). In contrast, the blood level of FK506 fell rapidly in an exponential manner from 163 ng/ml at 15 min postinjection to an undetectable level at 72 hr. A slightly different pattern was noted with the intraperitoneal administration route (Fig. 6). The brain content of drug rose to a maximum of ~400 ng/gm tissue at 12 hr after injection and thereafter fell slightly to 300 ng/gm tissue at 72 hr. It should be noted that a brain content of 135 ng/gm tissue was detected 30 min postinjection, the time at which the excitotoxic or ischemic
challenge was initiated using this route of drug administration. The blood level of drug was maximal 15–30 min after injection, with FK506 levels falling rapidly thereafter to a trough level of 2 ng/ml detected at 48–72 hr.

Cortical FK506 content also was measured following endothelin-induced MCAO with intravenous drug (1 mg/kg) administration 5 min after vessel occlusion (Fig. 6). Drug content in the contralateral nonischemic cortex was 36.8 ± 6.83 and 37.4 ± 4.54 ng/gm tissue at 1 and 3 hr, respectively, after vessel

Figure 5. Endothelin-induced MCAO in FK506 (filled circles) and vehicle-treated (open circles) rats does not affect the mean arterial blood pressure (MABP) nor rectal or intracerebral temperatures. Rats were anesthetized continuously with halothane with measurements made from 30 min before until 180 min after vessel occlusion. Data are mean values (± SEM) from four rats per group. P is the mean preocclusion value; endothelin was injected at time 0, and FK506 (1 mg/kg) was administered intravenously 1 min after vessel occlusion. Statistical comparisons were performed by using ANOVA with post hoc Scheffé’s analysis (p < 0.05).

Figure 6. Brain and blood levels of FK506 after intravenous (1 mg/kg) and intraperitoneal (10 mg/kg) dosing. Data are mean brain (filled circles) and blood (open circles) FK506 content (± SEM) from four animals per group. Histograms show FK506 content in cortical samples obtained 1 and 3 hr after endothelin-induced middle cerebral artery occlusion. Data are mean cortical FK506 content (± SEM) in the nonischemic (open bars) and ischemic (filled bars) hemisphere from four animals per group.
occlusion. FK506 content in the ischemic cortex was not significantly different: 28.6 ± 2.87 ng/gm tissue and 38.5 ± 7.7 ng/gm tissue at 1 and 3 hr, respectively. Blood levels of FK506 in these animals were 47.3 ± 3.58 and 24.1 ± 3.38 ng/ml, respectively.

Further neuroprotection experiments were performed to ascertain whether the FK506 detected in brain 24–72 hr after a single intravenous injection was bioavailable. FK506 (1 mg/kg, i.v.) was administered either 24 or 72 hr before endothelin-induced MCA occlusion, and the volume of ischemic brain damage was determined 72 hr after vessel occlusion. The volume of ischemic brain damage in cortex was reduced by 64% (p < 0.05) and 39% (p < 0.05) from 132 ± 20 mm³ in vehicle-treated rats (n = 11) to 47 ± 8 mm³ (n = 10) and 82 ± 10 mm³ (n = 11) in animals pretreated with FK506 for 24 and 72 hr, respectively.

**DISCUSSION**

The present study confirms that FK506 exhibits a powerful neuroprotective action in an experimental model of stroke (Sharkey and Butcher, 1994). Additional intravenous studies revealed that 1 mg/kg FK506 reduces ischemic brain damage in cortex when administered 120 min, but not 180 min, after MCAO, suggesting that a critical window of opportunity exists with regard to the neuroprotective effect. The ability of intravenous FK506 to reduce cortical brain damage induced by focal cerebral ischemia was mirrored in intraperitoneal pretreatment studies. The non-competitive NMDA receptor antagonist MK801, administered by intravenous and intraperitoneal routes as a positive control, also reduced ischemic brain damage in cortex. Neither FK506 nor MK801 prevented striatal damage after endothelin-induced MCAO in Sprague Dawley rats, presumably because of its vascular supply from the lenticulostriate artery; the lateral striatum represents end vessel territory that cannot be rescued by drug therapy (Park et al., 1989). In contrast to MK801, which reduced the volume of excitotoxic brain damage induced by NMDA receptor agonists, FK506 did not attenuate excitotoxic damage at doses that decreased ischemic brain damage. Histopathological data relevant to the striatal quinolinate lesion were replicated in separate neurochemical studies that quantified GAD and ChAT activity, markers for the viability of GABAergic and cholinergic neurons, respectively. MK801, but not FK506, attenuated the reduction in enzyme activity associated with intrastriatal injection of quinolinate. The lack of FK506 efficacy is unlikely to be attributable to regional selectivity, because negative histopathological results also were obtained using excitotoxic lesions in three structures: the hippocampus, cortex, and striatum. These data suggest that an antixcitotoxic mechanism is unlikely to underlie the neuroprotective action of FK506 in experimental stroke.

Pharmacokinetic findings suggest that differences in the time course of excitotoxic and ischemic damage cannot explain the contrasting efficacy of FK506. The brain content of FK506 rose rapidly after intravenous dosing and was maintained at a constant level from 15 min after injection until the experimental endpoint 72 hr later. The similar degree of neuroprotection afforded by FK506 pretreatment either 24 or 72 hr before MCAO and by drug treatment immediately after vessel occlusion confirmed the bioavailability of FK506 detected in pharmacokinetic studies. Although these findings rule out the possibility that excitotoxins exert an action in the brain that outlasts the half-life of the drug, an effective concentration of drug might be required immediately post insult to observe an antixcitotoxic effect. This is unlikely because intraperitoneal administration of FK506, at a dose that attenuated ischemic but not excitotoxic damage, resulted in a brain content of drug in excess of that required for ischemic neuroprotection using intravenous dosing, from the time of the excitotoxic challenge (30 min after intraperitoneal FK506 administration) until the experimental endpoint. The finding that FK506 content was similar in ischemic and nonischemic cortex was also of interest. These data indicate that the bioavailability of FK506 must be high because the drug penetrates readily into ischemic tissue and suggest that there is no gross perturbation of the blood–brain barrier in the endothelin model of focal cerebral ischemia.

Physiological data concerning MABP and rectal and brain temperature provided no clue to the neuroprotective mechanism of FK506 in experimental stroke. MABP was unaffected by FK506, and whereas body and brain temperature influence the severity of brain damage after focal cerebral ischemia (Morikawa et al., 1992; Xue et al., 1992), these variables were unaltered by endothelin-induced MCAO and/or FK506. These data indicate that neither a direct cardiovascular effect nor a drug-induced alteration in brain temperature mediates the neuroprotective effect of FK506. The possibility of a direct interaction between FK506 and the endothelin receptors mediating vasoconstriction in this model can be discounted because the drug, at concentrations up to 100 μM, failed to displace radiolabeled endothelin in a receptor binding assay (J. Sharkey and S. P. Butcher, unpublished data).

Further evidence to support the proposed role of calcineurin in the neuroprotective mechanism of FK506 was provided by the finding that 20 mg/kg cyclosporin A reduced ischemic brain damage. Subchronic pretreatment with equivalent doses of cyclosporin A previously had been reported to decrease brain edema after MCAO (Shiga et al., 1992). The lower potency of cyclosporin A, as compared with FK506, is presumably attributable to low blood–brain barrier permeability (Begley et al., 1990) and its lower affinity for its immunophilin binding site (Liu et al., 1992). The proposed role of calcineurin in the neuroprotective mechanism could involve a number of cellular processes. FKBP12 is associated with the ryanodine and IP₃ receptor complexes (Tim-erman et al., 1993; Zhang et al., 1993; Brallantines et al., 1994; Chen et al., 1994; Cameron et al., 1995a) in which it may function as an anchor for calcineurin (Cameron et al., 1995b). Both FK506 and rapamycin disrupt this complex (Cameron et al., 1995b) and interfere with the associated Ca²⁺ channel activity (Zhang et al., 1993; Brallantines et al., 1994; Chen et al., 1994; Cameron et al., 1995a,b). In contrast, rapamycin attenuates the neuroprotective action of FK506 (Dawson et al., 1993; Sharkey and Butcher, 1994), suggesting that a drug-induced alteration in ryanodine/IP₃ receptor channel activity is not involved in the neuroprotective mechanism. Alternatively, a role for NOS, an in vitro substrate for calcineurin, has been proposed on the basis of neuronal culture studies focusing on glutamate toxicity (Dawson et al., 1993). Nitric oxide-mediated toxicity is suggested to involve DNA damage with subsequent activation of poly(adenosine-5'-diphosphoribose) synthetase (PARS), ATP depletion, and cell death (Zhang et al., 1994, 1995). However, the role of nitric oxide in ischemic and excitotoxic neuronal death remains controversial, and the present findings demonstrate a clear discrepancy between in vitro and in vivo data concerning the antixcitotoxic effect of FK506. It also should be noted that, in contrast to the situation in vitro (Dawson et al., 1991), NOS inhibitors do not block excitotoxic damage in vivo (Globus et al., 1995; Mackenzie et al., 1995).

An alternative mechanism involving peroxynitrite, a neurotoxic free radical produced from nitric oxide and superoxide...
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Bonfoco E, Krainc D, Ankarcrona M, Nocitiera P, Lipton SA (1995) Peroxynitrite-induced cell death in primary neuronal cultures and a neuron-like cell line exhibits apoptotic characteristics (Bonfoco et al., 1995; Estevez et al., 1995), and evidence that apoptosis plays a key role in brain damage induced by focal cerebral ischemia has been reported (Li et al., 1995a,b; Linnik et al., 1995).

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