Calcineurin Inhibitors FK506 and SDZ ASM 981 Alleviate the Outcome of Focal Cerebral Ischemic/Reperfusion Injury

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ABSTRACT
The neuroprotective properties of drugs binding to FKBP12, with and without subsequent inhibition of calcineurin, were investigated in rat models of ischemic embolic stroke. Drug effects on brain infarct volumes evoked by transient middle cerebral artery occlusion (MCAO) and by permanent MCAO were determined in vivo by T2-weighted magnetic resonance imaging and post mortem by triphenyltetrazolium chloride staining and histology. Drugs binding to FKBP12 and inhibiting calcineurin, such as FK506 and SDZ ASM 981, dose dependently reduced the infarct volumes, determined 48 h after MCAO by both magnetic resonance imaging and triphenyltetrazolium chloride staining but only in the transient MCAO model. In vivo potencies to reduce brain infarcts paralleled the in vitro potencies to inhibit calcineurin. Histological staining after 6 days of survival showed that the neuroprotective effects were permanent. Rapamycin, known to bind with similar affinity to FKBP12 but not to inhibit calcineurin, was not neuroprotective but abolished the neuroprotective effects of FK506 when coadministered. In the permanent MCAO models, FK506 showed no effect when injected before and little effect when injected after MCAO. Measurements of core temperatures after MCAO in controls and drug-treated rats do not support hypothermia being the mechanism responsible for neuroprotection. We conclude that drugs inhibiting calcineurin activity are neuroprotective in focal cerebral ischemia/reperfusion but not in permanent ischemia models, possibly by preventing reperfusion injury.

The immunosuppressive drugs FK506 and cyclosporin A are currently used in organ transplantation to prevent allograft rejection. In addition to their immunosuppressive properties, these drugs have been shown to have neuroprotective properties. Cyclosporin A and FK506 protect neurons from necrosis induced by global cerebral ischemia (Uchino et al., 1995; Yagita et al., 1996; Li et al., 1997). In models of focal brain ischemia induced by middle cerebral artery occlusion (MCAO), FK506 and cyclosporin A alleviate the outcome of the ischemic injury. Sharkey and Butcher (1994) have shown that low doses of FK506 reduce the infarct volume after MCAO, performed by means of stereotaxic injection of endothelin-1. FK506 not only reduces the infarct size but also alleviates the neurological deficit (Sharkey et al., 1996). Cyclosporin A has been shown to decrease the infarct size and the edema when administrated orally over a period of 1 week before a transient MCAO (Shiga et al., 1992).

The mechanisms underlying these neuroprotective properties of immunosuppressants are not fully understood, although the molecular effects of FK506 and cyclosporin A are well documented. They bind to a class of endogenous receptors denominated immunophilins: cyclophilins are specific for cyclosporin A and FK506 binding proteins (FKBPs) for FK506 (Bram et al., 1993). Immunophilins are prominent in the brain, where their level is 10- to 100-fold higher than in the immune system (Steiner et al., 1992; Dawson et al., 1994). Distribution patterns of cyclophilins and FKBPs in the brain are similar and their localization is predominantly neuronal (Dawson et al., 1994). Immunophilins have prolyl isomerase (rotamase) activity that is inhibited by the binding with cyclosporin A and FK506, respectively (for a review, see Snyder and Sabatini, 1995). Although this mechanism might be implicated in neuroprotection in certain models of neurodegeneration (Steiner et al., 1997), other mechanisms probably account for the neuroprotective properties of cyclosporin A and FK506 in models of cerebral ischemia. One of the major hypothesis is the inhibition of the calcium-calmodulin dependent phosphatase (calcineurin; Bram et al., 1993) by immunosuppressive drugs. In vitro, calcineurin inhibition by FK506 decreases nitric oxide synthase (NOS) dephosphorylation, thus inactivating the enzyme and protecting cultured neurons against glutamate neurotoxicity (Dawson et al., 1994). Furthermore, calcineurin is present at high levels in the brain and is colocalized with cyclophilins and FKBPs (Dawson et al., 1994).

ABBREVIATIONS: MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; ANOVA, analysis of variance; ICA, internal carotid artery; MABP, mean arterial blood pressure; ECA, external carotid artery; MRI, magnetic resonance imaging; TTC, triphenyltetrazolium chloride; NOS, nitric oxide synthase; FKBPs, FK506 binding protein.
Permanent MCAO is a widely used model of focal cerebral ischemia; nevertheless, because in the past years therapeutic efforts have focused on recirculation either by surgical or by pharmacological means (e.g., recombinant tissue plasminogen activator), several models of transient MCAO emerged in the literature. Although reperfusion when performed in time salvage penumbral tissue (Aronowski et al., 1994), a growing body of evidence suggests that restoration of the blood flow to the ischemic tissue may provoke additional damage. Proposed mechanisms for this reperfusion injury are free radical generation, neutrophil infiltration, and microvascular plugging (Zhang et al., 1994; Matsuo et al., 1995). We have used the intraluminal thread method described by Koi- zumi et al. (1986) to occlude transiently or permanently the MCA through a peripheral approach. This technique has the advantage to preserve the integrity of the cranial and thus to limit the trauma. Compared with other methods (e.g., MCA ligation, clamping, or endothelin-1 application), the vessel is occluded by an intravascular device and therefore mimics the clinical situation of the occlusion of a major brain vessel by an embolus.

We tested the effect on the outcome of MCAO/reperfusion injury of immunosuppressive drugs that have different affinities for FKBP's and calcineurin inhibitory potencies. The ascomycin derivatives FK506 and SDZ ASM 981 (Mein-gassner et al., 1997) and rapamycin bind to FKBP's and inhibit prolyl isomerase (rotamase) activity, but only FK506 and SDZ ASM 981 inhibit calcineurin activity. SDZ ASM 981 has an approximately 3-fold lower affinity to FKBP's and consequently about a 3-fold lower calcineurin inhibiting potency (A. Enz, personal communication). The comparison of the neuroprotective efficacy of these molecules should shed light on the implication of calcineurin inhibition in neuroprotection.

Materials and Methods

All procedures described in the present article were performed in strict accordance with the Swiss animal health care rules.

MCAO. Male adult spontaneously hypertensive rats (SHR) from BRL (Switzerland), with 220 to 250 g body weight and free access to standard rat chow and tap water, were used. Anesthesia was induced with isoflurane in a glass jar and maintained in the spontaneously standard rat chow and tap water, were used. Anesthesia was induced strictly accordance with the Swiss animal health care rules.

Transient/Permanent MCAO with Intraluminal Thread. Animals were placed in a supine position on a heated operating table, and the body temperature was monitored by a rectal probe and maintained at 37.5°C during the surgical procedure, which lasted approximately 20 min. The right common carotid artery (CCA) was isolated and ligated with a 3-0 silk suture. The CCA was followed rostrally until the bifurcation of the internal carotid artery (ICA) and external carotid artery (ECA) was placed in view. The ECA was then ligated proximally to the bifurcation of the superior thyroid artery and distally to the occipital artery and divided. The occipital artery, which arises from the proximal ECA and courses across the proximal ICA, was isolated at its origin and divided using a bipolar microcoagulator. A 3-0 silk suture was tied loosely around the ICA, and a microvascular clip was placed distally across the ICA. A puncture was performed at the ICA-ECA bifurcation and a 4-0 polyester monofilament thread (Miralene, Braun, Switzerland), with its end rounded by heating, was introduced into the ICA. The suture around the ICA (and the polyester thread) was tightened, and the microvascular clip was removed. The polyester thread was then gently advanced into the ICA lumen on a length of 18 mm. Recirculation required reexploration of the wound to remove the occluding device. Under isoflurane anesthesia lasting no longer than 5 min, the bifurcation of ICA-ECA was reexposed, and the thread was pulled back until the tip reached the suture around the ICA. After the wound had been closed, the animals were allowed to recover from anesthesia before they were returned to their home cages.

Permanent MCAO by electrocoagulation was performed as previously described (Sauter and Rudin, 1986) with minor modifications. Briefly, under isoflurane anesthesia (as above), a craniotomy was performed using a dental drill; the dura was opened; and the exposed MCA was electrocoagulated with a small bipolar.

Drug Administration. FK506 (tacrolimus), SDZ ASM 981 (33-epi-chloro-33-desoxy-ascomycin), and rapamycin were dissolved in ethanol/polyethylene glycol 200 1:2 (v/v); 2 ml/kg drug solution or vehicle were injected i.v. as a bolus in the tail vein. For the rapamycin study, where animals received two injections of vehicle or drug solution, the total amount of injected ethanol/polyethylene glycol 200 (1:2) (v/v) was the same as in the other experiments; for this, it was diluted 1:1 with 0.9% NaCl.

Body Temperature Monitoring. During surgery, an electrical temperature probe was inserted 5 cm into the rectum to control core temperature, which was maintained at 37.5 ± 0.5°C. Rectal temperature was also measured in awake rats with a temperature probe after MCAO and after drug administration at the indicated time points.

Blood Pressure Measurement. To measure the mean arterial blood pressure (MABP) the femoral artery was cannulated. MABP was measured in awake, freely moving rats during at least 5 min before the surgery to establish the baseline and then 45 min after MCAO (i.e., 15 min after drug administration) and again 15 min after reperfusion (i.e., 45 min after drug administration).

Infarct Volume Determination. Infarct volume was assessed by T2-weighted magnetic resonance imagery (MRI) and triphenyltetrazolium chloride (TTC) and toluidine blue staining.

MRI was performed as described previously (Sauter and Rudin, 1986). Briefly, a Biospec 47/15 spectrometer (Bruker, Germany) with imaging facilities was used. Eight coronal sections were taken using a spin echo SE(1365/80) sequence. The ischemic tissue appeared in these T2-weighted images as hyperintense areas. The infarct area in each section was determined using a semiautomatic segmentation procedure based on intensity thresholding. The total infarct volume was calculated by adding up the area of each slice and multiplying by the slice thickness.

Infarct volume was assessed with the vital TTC staining. Animals were sacrificed by decapitation, the brains dissected out, and 2-mm-thick coronal slices were cut using a brain slicer. The slices were immersed in 1% TTC in 0.25 M phosphate buffer (pH 8.5) for 20 min in the dark; tissue sections were stored in a 10% formaldehyde solution. A subset of animals was sacrificed by decapitation after 6 days of survival, and brains were rapidly dissected out and frozen on dry ice. Consecutive slices of 20-μm thickness were cut and stained with toluidine blue. For both staining methods, the infarct areas were assessed with a computerized image analyzer, and the infarct volume was calculated.

Data Analysis. Data are expressed as mean of n values ± S.E.M. and were compared using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (FLSD) test (SSS).
somatosensory, and temporal), caudate putamen, and globus pallidus, as visualized by MRI (Fig. 1, A and B). Treatment with FK506 at the high dose of 1 mg/kg, injected i.v. 30 min after MCAO, markedly reduced the extent of infarction as assessed by MRI 48 h later. Reduction in T2-weighted signal intensity by FK506 was most prominent in the upper frontoparietal cortex (Fig. 1, C and D) and parietal cortex (data not shown), whereas little effect was seen in the striatum.

The dose-response study shown in Fig. 2 demonstrated that FK506 was fully neuroprotective at a dose as low as 0.1 mg/kg (42% reduction). Higher doses (1 and 5 mg/kg) gave a similar protection (38% and 45%, respectively, of infarct reduction). The drug SDZ ASM 981, an ascomycin macrolactam derivative (Meingassner et al., 1997) with approximately 3-fold lower affinity to FKBP12 and corresponding lower calcineurin inhibiting potency (A. Enz, personal communication) but slightly more lipophilic than FK506, was also evaluated for its in vivo efficiency to reduce the infarct volume in the rat MCAO/reperfusion model. As shown in Fig. 2, SDZ ASM 981, although being as efficacious as FK506 at higher doses (37% and 44% reduction of the infarct volume for 1 and 5 mg/kg, respectively), appeared to be slightly less potent than FK506 at lower doses (20% versus 42% reduction at 0.1 mg/kg). These results suggest that binding to FKBPs alone and/or subsequent inhibition of calcineurin is involved in neuroprotection after transient MCAO.

Possible effects of FK506 on the infarct size after permanent MCAO performed either by the intraluminal thread method or by electrocoagulation were further investigated. In vehicle-treated animals, infarct volumes were 322 ± 11 and 410 ± 21 mm³ when MCAO was performed by electrocoagulation and intraluminal thread, respectively. This difference in infarct volume was due to the more distal occlusion site of the MCA with the electrocoagulation technique. No reduction in the infarct size was obtained with either method of permanent MCAO when FK506 (up to 5 mg/kg) was administered before MCAO (Fig. 3). When treatment with FK506 was initiated 30 min after permanent MCAO with the thread method, the infarct volumes were slightly reduced (16%), although these results were statistically not significant (Fig. 3).

Because contrast in T2-weighted MRI depends on tissue water distribution, areas showing increased signal intensity

Fig. 1. T2-weighted MR images taken 48 h after a 1-h MCAO; two consecutive coronal sections comprising the caudate putamen and frontoparietal somatosensory cortex are shown. Slices were 1.0 mm thick, and the center was approximately at the level of interaural 10.2 mm (A and C) and 8.7 mm (B and D). The necrotic tissue appears as a hyperintense, white area due an increase in T2-weighted signal that is a consequence of the edema. Animals received a single i.v. injection of vehicle (A and B) or 1 mg/kg FK506 (C and D) at 30 min after MCAO.

Fig. 2. Infarct volumes were assessed by MRI 48 h after a 1-h MCAO. Animals received i.v. injections of the indicated dose (mg/kg) of FK506 (■) or SDZ ASM 981 (○) at 30 min after MCAO (30 min before reperfusion). Results are expressed as the mean of n values ± S.E.M. *p < .05, **p < .01, ***p < .001, ANOVA test followed by FLSD test versus vehicle-treated group.

Fig. 3. Infarct volumes were assessed by MRI 48 h after permanent MCAO either with the intraluminal thread method (■, ●) or electrocoagulation (○). Animals were treated with FK506 either before MCAO (■, ○) or 30 after MCAO (●). For comparison, the curve obtained in the transient MCAO model (dashed line, same data as in Fig. 2) is also shown. Infarct volumes are expressed as percent of vehicle-treated animals and represent the mean of n values ± S.E.M.
consequent to ischemia merely reflect edema and not necessarily irreversible tissue damage. Hence, infarcts in a subset of vehicle and drug-treated rats were assessed by both MRI and TTC staining 48 h after 1-h MCAO. Necrotic brain areas as revealed by vital TTC staining matched reasonably well with those seen by T2-weighted MRI (data not shown). Figure 4, top, shows that both FK506 and SDZ ASM 981 at a dose of 5 mg/kg reduced the infarcted area to the same extent at every considered level of the brain. Statistically significant reductions were obtained in all slices, except the two most caudal, in which the lesions were already small in control rats. Infarct volumes assessed by TTC did correlate reasonably well ($r = 0.91, p < .001$) with those obtained by MRI (see Fig. 4, bottom), although the volumes obtained by vital staining were larger by approximately 20%.

To determine whether the protective effect of FK506 is permanent, histological staining was also performed after 6 days of survival. The location of the infarcted tissue (Fig. 5) matched quite well with the edematous areas as visualized by T2-weighted MRI (compare with Fig. 1), although the edema appeared to be more outspread than the necrotic tissue. The infarcted tissue appeared to be densely covered with heavily stained cells, probably reflecting glial reaction and macrophage infiltration (Clark et al., 1994). The infarct volumes obtained in the same subset of vehicle-treated animals were markedly smaller than those measured by MRI 48 h after MCAO ($171 \pm 6$ versus $303 \pm 8$ mm$^3$). Animals treated with 1 mg/kg FK506 had infarcts determined by histology 6 days after MCAO that were reduced by 36% ($109 \pm 7$ versus $171 \pm 6$ mm$^3$, $n = 8$, $p < .001$). These results were consistent with a 30% reduction in infarct volumes ($303 \pm 8$ versus $213 \pm 19$ mm$^3$, $n = 8$, $p < .001$) observed in this subset of rats by MRI 48 h after MCAO.

To exclude unspecific mechanisms known to lead to cytoprotection, relevant physiological parameters, such as body temperature and MABP, were closely monitored during ischemia, after drug application, and after reperfusion. Figure 6 shows the temperature evolution in vehicle or drug-treated rats after either a transient (A) or a permanent (B) MCAO by the thread method. In vehicle-treated animals, the body temperature rose transiently from 38.4°C to 39°C between 120 and 240 min after transient MCAO, returning to baseline values at 300 min (Fig. 5A). In rats treated with FK506 (5 mg/kg), the body temperature was 38°C at the time of reperfusion (i.e., 30 min after drug application) and remained

Fig. 4. Infarcted tissue areas (A), assessed at different brain levels after 1-h MCAO and 48-h reperfusion by vital TTC staining in vehicle ($n = 8$, $\bullet$), 5 mg/kg FK506- ($n = 10$, $\square$) or 5 mg/kg SDZ ASM 981- ($n = 12$, $\circ$) treated animals. The correlation ($r = 0.91, P < .001$) between infarct volumes measured by MRI and TTC staining is shown in B. Mean infarct volumes assessed by MRI and TTC for all three groups are also plotted. Results are the mean of $n$ values ± S.E.M. *$P < .05$, **$P < .01$, ***$P < .001$, ANOVA test followed by FLSD test versus vehicle-treated group.
Fig. 6. The body temperature was measured in awake freely moving rats with a rectal temperature probe at the indicated time points after either transient 1-h MCAO (A) or permanent MCAO (B). A, animals were treated 30 min after MCAO with vehicle \((n = 6, \bullet)\), 5 mg/kg FK506 \((n = 5, \square)\), or 5 mg/kg SDZ ASM 981 \((n = 5, \lozenge)\). B, animals were treated 30 min after MCAO with vehicle \((n = 6, \bullet)\), FK506 1 mg/kg \((n = 6, \lozenge)\), or 5 mg/kg \((n = 6, \square)\). Results are the mean of \(n\) values ± S.E.M. *\(P < .05\), **\(P < .01\), ***\(P < .001\), ANOVA test followed by FLSD test versus vehicle-treated group.

constant during the period of observation (Fig. 5A). In SDZ ASM 981-treated rats (5 mg/kg), the body temperature also remained constant, although at a slightly higher level (38.5°C). In the three groups of permanent MCAO after 30 min, the body temperature was 39.2°C. However, in contrast to the transient MCAO, the temperature increase in vehicle-treated animals was long lasting (Fig. 5B) and still observable at 24 h (data not shown). Treatment with FK506 after permanent MCAO resulted in a dose-dependent temperature reduction, which remained close to normal values (38.5°C and 38°C for 1 and 5 mg/kg, respectively) for up to 24 h.

Before surgery, MABP was similar in all three groups (144 ± 6, 143 ± 6, and 139 ± 8 mm Hg), as shown in Table 1. At 45 min after surgery (15 min after vehicle or drug injection), when the rats had recovered from anesthesia, MABP was slightly increased in all three groups (Table 1). At 15 min after reperfusion (45 min after vehicle or drug injection), the MABP decreased to values not different from preischemia values. The differences in MABP between vehicle and drug-treated rats were statistically not significant at any time point.

To clarify whether inhibition of rotamase or calcineurin activity is the mechanism responsible for cytoprotection, rapamycin was administrated to rats, alone or in combination with FK506. The immunosuppressant rapamycin, like FK506, is a macrolide with high affinity to the immunophilin FKBP12, resulting in inhibition of its rotamase activity. However, the FKBP12/rapamycin complex, in contrast to the FKBP12/FK506 complex, does not inhibit calcineurin. In this set of experiment (Fig. 7) the mean infarct volume in vehicle-treated rats \((n = 18)\), assessed by TTC staining 48 h after a 1-h MCAO, was 404 ± 57 mm³. Treatment with FK506 (1 mg/kg, 30 min after MCAO) reduced the infarct volumes by 42% \((n = 9)\), confirming the results obtained in the previous experiment (see Fig. 2). Treatment with rapamycin (2 mg/kg, 20 min before MCAO) had no significant effect on the infarct volume (95 ± 10% of controls, \(n = 9\); indicating that inhibition of rotamase activity does not lead to cytoprotection in this model. Combined treatment with rapamycin (2 mg/kg, 20 min before MCAO) and FK506 (1 mg/kg, 30 min after MCAO) did not lead to infarct reduction (108 ± 11% of control, \(n = 7\)) compared with vehicle-treated animals, suggesting that rapamycin competes with FK506 for binding to FKBP12 and consequently abolishes the protective effect of FK506.

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Baseline</th>
<th>45 min after MCAO</th>
<th>15 min after reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle, (n = 4)</td>
<td>144 ± 6</td>
<td>149 ± 10</td>
<td>142 ± 8</td>
</tr>
<tr>
<td>FK506, (n = 4)</td>
<td>143 ± 6</td>
<td>160 ± 8</td>
<td>148 ± 4</td>
</tr>
<tr>
<td>ASM-981, (n = 4)</td>
<td>139 ± 8</td>
<td>159 ± 3</td>
<td>147 ± 11</td>
</tr>
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Effect of MCAO and drug treatment on blood pressure

The MABP was assessed in awake freely moving rats, over a period of 5 min, before surgery (baseline), 45 min after MCAO (15 min after drug application), and 15 min after reperfusion. Animals were treated with vehicle, 5 mg/kg FK506, or 5 mg/kg ASM-981. Results are the mean of \(n\) values ± S.E.M.

Fig. 7. Infarct volumes assessed after 1-h MCAO and 48-h reperfusion by vital TTC staining in animals that received injections of vehicle \((n = 18)\), 1 mg/kg FK506 \((n = 9, 30\) min after MCAO), 2 mg/kg rapamycin \((n = 9, \text{ rapam.}, 20\) min before MCAO), or both rapamycin \((20\) min before MCAO) and FK506 \((n = 7, 30\) min after MCAO). Results are expressed as percent of controls that were vehicle-treated animals and are the mean of \(n\) values ± S.E.M. *\(P < .05\), ANOVA test followed by FLSD test versus vehicle-treated group. ††\(P < .01\), ANOVA test followed by FLSD test versus FK506-treated group.
Discussion

It has repeatedly been demonstrated that FK506 reduces the infarct size in rat brain after occlusion of the MCA (Sharkey and Butcher, 1994; Sharkey et al., 1996). In all of these studies, however, the artery has been occluded by stereotactic injection of endothelin in the vicinity of the MCA, with the exact extent and duration of occlusion remaining uncertain. In addition, it cannot be excluded that drugs leading to an infarct size reduction in this model do so by interference with the mechanism causing artery occlusion. We have therefore used a different method of MCAO in the rat that allows precise control of the occlusion time and that is hardly affected directly by pharmacological interventions. Blood flow through the MCA was interrupted by an intraluminal thread that can be left in place permanently or retracted at anytime. In contrast to other methods of precisely controlled transient or permanent MCAO, like direct ligation or clamping of the MCA, the thread method is less invasive in that craniotomy and opening of the dura are not required.

Extending beyond previous work with FK506 (Sharkey and Butcher, 1994), infarct sizes were determined in vivo by T2-weighted MRI 48 h after MCAO and confirmed post mortem by the TTC and histological staining methods 48 h and 6 days after MCAO, respectively. Although T2-weighted MRI is based on water distribution and essentially detects brain edema, an excellent linear correlation with TTC staining, which biochemically differentiates normal and dead tissue, was obtained. Infarct volumes determined by the TTC method were approximately 20% larger than those measured by MRI. This discrepancy can be explained by the ipsilateral brain hemisphere becoming distended on removal from the skull without previous perfusion-fixation. Drug effects relative to vehicle-treated controls, however, were identical whether infarct volumes were measured by MRI or TTC staining 48 h after MCAO, or even histology 4 days later.

Similar effects in terms of potency and efficacy on the infarct size were obtained with FK506 in the thread model as those reported using the endothelin method but only when the MCA was occluded transiently (i.e., for 1 h, followed by reperfusion). Permanent MCAO, either with the thread method or by electrocoagulation, resulted in brain infarcts that were hardly affected by FK506. The brain damage produced by cerebral ischemia is a dynamic, time-dependent phenomenon (Aronowski et al., 1994). In the core region (caudate putamen and lower frontoparietal somatosensory cortex), where the reduction of blood flow is most severe, energy failure occurs rapidly, followed by neuronal death. In surrounding at-risk areas, mainly frontal and parietal cortex, neurons remain viable and may be salvaged by restoration of blood flow. The neuroprotective effects of FK506 and related drugs, such as SDZ ASM 981, were most prominent in these cortical areas surrounding the core region. Taken together, these results may suggest, in analogy with observations in the heart (Nishinaka et al., 1993), that FK506 and related drugs protect brain tissue mainly against “reperfusion injury.” Important mechanisms that are believed to account for the detrimental effects of reperfusion are free radical generation (Matsuo et al., 1995) and microvessel plugging by infiltrated neutrophils (Zhang et al., 1994). Interestingly, in the heart, FK506 apparently protects the myocardial tissue against ischemia/reperfusion injury by scavenging free radicals produced by neutrophils (Nishinaka et al., 1993). Moreover, calcineurin inhibitors block in vitro the integrin-mediated adhesion of neutrophils (Hendey et al., 1992).

Unspecific mechanism, known to lead to infarct size reductions, such as hypothermia (Chen et al., 1992) can most likely be rejected for FK506 and SDZ ASM 981, although both compounds dose dependently normalized the hyperthermia observed after transient MCAO. However, the difference between drug-treated animals and controls was small, even with the highest dose used (1°C at 5 mg/kg), and not related to the dose-dependent reduction of infarct size, which had started by 0.1 mg/kg. In the case of permanent MCAO with the intraluminal suture, the body temperature was increased to more than 39°C all over the considered period in vehicle-treated animals. Although FK506 reduced the body temperature in a dose-dependent way after permanent MCAO, hardly any neuroprotective effect has been detected. These results suggest that the reduction of body temperature induced by immunosuppressive drugs cannot account for their neuroprotective property.

The involvement of calcineurin inhibition in neuroprotection is strongly supported by our observation that FK506, which binds with a 3× higher affinity to FKBP1s and consequently is about a 3× more potent calcineurin inhibitor in vitro than SDZ ASM 981, also is by a factor of approximately 3 more potent in vivo in reducing brain infarcts. Moreover, binding to FKBP12 alone does not seem to be sufficient for neuroprotection in this ischemia/reperfusion model because rapamycin, which binds to FKBP12 with similar affinity as FK506 but does not inhibit calcineurin, has no effect on the infarct size. However, the neuroprotective effects of FK506, which binds to FKBP12 and subsequently inhibits calcineurin, can be completely blocked by rapamycin, supporting the notion that binding to FKBP12 and subsequent inhibition of calcineurin are necessary for the neuroprotection observed in this model. There are several ways in which calcineurin inhibition may mediate neuroprotection, such as glutamate-mediated neurotoxicity results from its interaction with the N-methyl-D-aspartate receptor and subsequent intracellular Ca2+ level increase, which in turn leads to the activation of the neuronal isoform of NOS (Dawson et al., 1993), which is dephosphorylated by calcineurin, resulting in increased synthesis of NO. Thus, FK506 has been shown to protect cultured neurons against N-methyl-D-aspartate-induced neurotoxicity (Dawson et al., 1993). In vivo, nonspecific inhibition of NOS has variable effects on the outcome of focal cerebral ischemia (Hamada et al., 1995; Quast et al., 1995). Nevertheless, specific inhibition of the neuronal isoform of NOS is neuroprotective (Dalkara et al., 1994).

Calcineurin also is implicated in the signaling pathways that lead to apoptotic death of lymphocyte B cell because this pathway is blocked by FK506 and Bel-2 (Wolvetang et al., 1997). The apoptotic repressor Bel-2 sequestrates calcineurin in the membrane and separates it from its cytoplasmic substrates, thus resulting in apparent calcineurin inhibition (Shibasaki et al., 1997). Interestingly, Bel-2 transfection also protects neurons against ischemia-induced cell death (Linnik et al., 1995).

It is interesting to note that FK506 treatment produces toxic effects in both experimental animals and humans, not only in peripheral organs but also in the brain (Menegaux et al., 1994; Mueller et al., 1994; Wilson et al., 1994). The
etiology of the brain lesions is currently unknown; direct effects on the central nervous system or changes secondary to effects on hemodynamic functions may be possible explanations. Toxicological studies in the rat suggest, however, that the histopathological findings of acute necrosis and vacuolation in the brain observed after oral administration of FK506 for 4 weeks are not directly linked to calcineurin inhibition because SDZ ASM 981 at a 10× higher dose (30 mg/kg/day) given for 4 weeks and producing approximately 10× higher peak blood levels (C_{max}) and exposure (AUC_{0–24 h}) is clearly not neurotoxic. Thus SDZ ASM 981 is at least 10× less toxic than FK506, and the therapeutic index for neuroprotection seems to be more favorable for SDZ ASM 981 than for FK506.

Our results strongly suggest that inhibition of calcineurin leads to the permanent reduction in the cerebral infarct size resulting from transient occlusion of the MCA, followed by reperfusion. Whether calcineurin has to be inhibited in the brain alone and/or in the periphery cannot be answered at present because no brain-specific calcineurin inhibitor is available yet.

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