Significant Neuroprotection against Ischemic Brain Injury by Inhibition of the MEK1 Protein Kinase in Mice: Exploration of Potential Mechanism Associated with Apoptosis

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ABSTRACT

MEK1/2 is a serine/threonine protein kinase that phosphorylates and activates extracellular signal-responsive kinase (ERK)1/2. In the present study we explored the role of MEK1/2 in ischemic brain injury using a selective MEK1/2 inhibitor, SL327, in mice. C57BL/6 mice were subjected to a 30-min occlusion of the middle cerebral artery (MCAO) followed by reperfusion. Western blot analysis demonstrated the immediate activation of MEK/ERK after reperfusion (within the first 10 min) in the ischemic brain; this activation was dose-dependently blocked by SL327 (10–100 mg/kg, i.p.). A single dose of SL327 (100 mg/kg) administered 15 min before or 25 min after the onset of ischemia resulted in 63.6% (n = 18, p < 0.001) and 50.7% (n = 18, p < 0.01) reduction in infarct size, respectively, compared with vehicle-treated mice. Similarly, SL327 significantly reduced neurological deficits 1 to 3 days after reperfusion (n = 12, p < 0.01). The salutary effect of SL327-induced neuroprotection was independent of mitochondrial cytochrome c release or caspase-8-mediated apoptosis; however, SL327 markedly suppressed the levels of active caspase-3 and DNA fragmentation (as a measure of apoptosis) after ischemia/reperfusion. Our data suggest that the inhibition of MEK1/2 results in neuroprotection from reperfusion injury and that this protection may be associated with the reduction in apoptosis.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases involved in many cellular programs such as cell proliferation, differentiation, and death (Garrington and Johnson, 1999). MAPK signaling cascades operate by phosphorylating/activating downstream kinases or specific substrates in response to cell surface receptor or external stimuli. MAPK/extracellular signal-regulated kinase (ERK) kinase 1 (MEK1) is a dual-specific kinase that phosphorylates and activates ERK1 and ERK2. The MEK/ERK pathway has been associated with neuronal development, growth, and survival (Seger and Krebs, 1995; Skaper and Walsh, 1998). The exact role of MEK/ERK pathway in the brain appears to be complicated because both neuroprotection and injury were demonstrated for this pathway (Murray et al., 1998; Runden et al., 1998; Anderson and Tolkovsky, 1999; Singer et al., 1999; Han and Holtzman, 2000). In addition to the MEK/ERK pathway, p38 MAPK and c-Jun N-terminal kinase (JNK) pathways have been demonstrated to play an important role in cell survival and apoptosis in response to various stimuli (Xia et al., 1995). The balance between ERK and JNK-p38 MAPK has been suggested to mediate cell survival or death (Xia et al., 1995).

Cerebral ischemia is a pathophysiological condition caused by decrease in blood supply to the brain, and hence the deprivation of oxygen and glucose in the ischemic brain eventually leads to cell death (necrosis and apoptosis), inflammation, and tissue repair (Wang and Feuerstein, 2000). The concomitant activation of ERK, JNK, and p38 MAPK has been reported in both gerbil and rat transient brain ischemia (Irving et al., 2000; Sugino et al., 2000). The activation of ERK1/2 was also demonstrated in humans after acute ischemic stroke (Slevin et al., 2000). Although the inhibition of ERK1/2 by a selective MEK1 inhibitor PD98059 failed to protect ischemic cell death in the CA1 region in the gerbil (Sugino et al., 2000), the same compound revealed significant neuroprotection after transient cerebral ischemia in mice by means of intracerebroventricular (i.c.v.) administration.

ABBREVIATIONS: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; ECA, external common carotid; ICA, internal common carotid; MCA, middle cerebral artery; DMSO, dimethyl sulfoxide; CBF, cerebral blood flow; MCAO, occlusion of the middle cerebral artery; TTC, 2,3,5-triphenyltetrazolium chloride; ELISA, enzyme-linked immunoassay; COX, cytochrome oxidase.
(Alessandrini et al., 1999). Furthermore, the inhibition of MEK1/2 by a specific inhibitor U0126 was shown to be neuroprotective against forebrain ischemia and focal cerebral ischemia in gerbil (Namura et al., 2001).

In the present study, we have applied a novel class of MEK1/2 inhibitor, SL327 (Scherle et al., 2000), which was demonstrated to be able to selectively inhibit ERK activation in the brain following systematic administration and disrupt learning and memory (Atkins et al., 1998;), to investigate its role in neuroprotection after ischemic brain injury using a transient cerebral ischemia model in mice. As demonstrated previously by means of other MEK1 inhibitors (Alessandrini et al., 1999; Namura et al., 2001), our study has shown that administration of SL327 significantly reduced infarct size and improved neurological function following ischemic injury. Furthermore, our present study has further explored the potential molecular mechanisms involved in the protective effect of MEK1/2 inhibition. In particular, the regulation of various aspects involved in intrinsic pathway in apoptosis (including cytochrome c, caspase-3, and DNA fragmentation) have been investigated in association with brain functional and structural outcomes following focal stroke.

Materials and Methods

Focal Brain Ischemia. Adult male C57BL/6 mice (18–22 g) were used for the present study. Animals were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals. Procedures using laboratory animals were approved by the Institutional Animal Care and Use Committee of Bristol-Myers Squibb Company.

Mice were anesthetized with gas inhalation comprised of 30% oxygen (0.3 l/min) to 70% nitrous oxide (0.7 l/min) mixture. The gas was passed through an isoflurane vaporizer set (VetEquip Inc., Pleasanton, CA) to deliver 3 to 4% isoflurane during initial induction and 1.5 to 2% during surgery. After shaving the neck and swabbing the surgical site with Betadine, an incision of the skin was made directly on top of the right common carotid artery region. The fascia was then blunt dissected until the bifurcation of the external common carotid (ECA) and internal common carotid (ICA) was isolated. A small incision was made on the ECA, and a 5-0 monofilament suture (9- to 11-mm long with a round tip) was threaded into the ICA via the ECA. The suture was advanced toward the middle cerebral artery (MCA) to create focal ischemia. For our ischemia-reperfusion via the ECA. The suture was advanced toward the middle cerebral artery (MCA) to create focal ischemia. For our ischemia-reperfusion model, the suture was removed after 30 min, and the wound was closed. Sham operation was performed using the same procedure except that no suture was inserted into the carotid artery. Consistent temperature was maintained (37°C) and monitored during the experimental procedure. Mice were anesthetized with gas inhalation and forebrains were removed at various times following reperfusion or sham surgery as indicated in each figure legend. SL327 (0, 10, 30, and 100 mg/ml dissolved in 100% DMSO) or vehicle were administered into mice through i.p. 15 min before or 25 and 60 min after ischemia using a Hamilton syringe. For RNA and biochemical analysis, the entire ipsilateral and contralateral hemispheres were dissected and immediately frozen in liquid nitrogen and stored at −80°C for later use.

Physiological Parameters. In randomly selected animals, regional cerebral blood flow (CBF) was measured with a Laser Doppler Perfusion Monitor (Moor Instruments Inc., Wilmington, DE). After anesthesia, a small incision was made at the midpoint between the right orbit and the external auditory canal. The temporals muscle was retracted and the underlying fascia cleared. The laser Doppler probe was placed on the skull 1.5 mm posterior and 3.5 mm lateral to the bregma on the ipsilateral hemisphere. CBF was carefully monitored (to avoid any large vessel) before, during (15 min), and after (30 min) MCAO in SL327- or vehicle-treated animals.

The arterial blood pressure and heart rate were measured by connecting a tubing through femoral artery using an MP100 Workstation and analyzed using an AimKnowledge software (BIOPAC Systems, Inc., Santa Barbara, CA) according to the manufacturer’s specification. Femoral arterial blood samples were analyzed for pH, oxygen (pO2), and carbon dioxide (pCO2) by direct collection through a polyethylene-50 tubing into an i-STAT G3+ cartridge and processed with a portable clinical analyzer (Abbott Laboratories, Abbott Park, IL).

Infarct Volume. To measure the infarct volume, brains were removed at 24 h after MCAO and evaluated using 2,3,5-triphenyltetrazolium chloride (TTC) staining of 2-mm thick brain slices. The stained brain tissue was fixed in 10% formalin in phosphate-buffered saline phosphate-buffered saline. The image was captured using a Microtek ScanMaker 4 DUO Scanner (MicroWarehouse, Lakewood, NJ) and quantitated using Image Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD). Total ischemic lesion was determined by direct measurement of the infarct areas against the total areas of the sequential forebrain slices and illustrated as a percentage of ischemic lesion.

In addition, indirect technique (Swanson et al., 1990) was modified to determine infarct size by measuring the non-ischemic areas in the ipsilateral hemisphere to normalize potential errors introduced by edema. Specifically, the infarct volumes of the lesioned structure were expressed as a percentage of the 2× volume of the structures in the contralateral hemispheres. Thus, total ischemic lesion for each animal was determined: %I = 100 × (Vc − Vi)/(2 × Vi), where %I = total ischemic lesion (%), Vi = total volume of the control hemisphere, and Vc = total volume of nonischemic area in the ipsilateral hemisphere.

Neurological Deficits and Rota-Rod Test. Neurological deficits were examined at days 1 and 3 after MCAO (n = 10) using a 5-point scale adapted and modified from Zhang et al. (1997). Specifically, no neurological deficit = 0; right Horner’s syndrome counts 1 point; failure to extend left forelimb and hindlimb, 1 point each; turning to left, 1 point; and circling to left, 1 point.

The same groups of animals were monitored for performance in the rota-rod test using an Accelerating Speed treadmill (Stoelting, Wood Dale, IL). Each mouse was given four trials and the mean values were collected for group data analysis.

Western Blot Analysis. Western blot analysis was used to evaluate the levels of ERK, p38, and JNK phosphorylation, and the active form of caspase-3 expression. The protocol for ERK, p38, and JNK phosphorylation analysis in the brain was adapted with minor modification from Favata et al. (1998). Briefly, frozen hemispheric brain tissue was thawed on ice and homogenized with a Polytron modification from Favata et al. (1998). Briefly, frozen hemispheric brain tissue was thawed on ice and homogenized with a Polytron. The insoluble component of the tissue lysate was removed by centrifugation at 3,000g for 10 min. Protein concentration was determined using a Bio-Rad protein assay kit (Hercules, CA). Western blot (100 μg of protein/lane) was carried out as described in detail previously (Wang et al., 2001b) using a rabbit polyclonal anti-phosphospecific p44/42 MAP kinase (ERK) antibody (1:2000 dilution; New England Biolabs, Beverly, MA). The blot was stripped and re-probed using rabbit polyclonal anti-p44/42 MAP kinase antibody (New England Biolabs), or antibodies against p38, JNK, and actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

To evaluate the genes that are differentially regulated by MEKI inhibition, mice were subjected to ischemia/reperfusion in the presence or absence of SL327 (n = 6). The ipsilateral and contralateral hemispheric brain tissues were pulverized under liquid nitrogen using a mortar and pestle. The tissue powders were stored in −80°C
and used for Western blotting, ELISA, or apoptosis assay (described in the following section).

To measure the active forms of caspase-1, -3, and -8 in the ischemic brain tissues, the brain powders were incubated in 1 × cell lysis buffer (EnzChet caspase-3 assay kit; Molecular Probes, Eugene, OR) for 30 min on ice. The tissue was further processed (as described above) for Western analysis using a mouse monoclonal IgG against caspase-3 (sc-7272), rabbit polyclonal antibody against caspase-8 (sc-7890), and goat polyclonal anti-actin (sc-1616) (Santa Cruz Biotechnology, Inc.).

**Analysis of Cytochrome c.** To evaluate the levels of cytochrome c, mice (n = 4 for each group) were subjected to 30 min MCAO followed by 4 h of reperfusion for the peak release of cytochrome c from mitochondria (Noshita et al., 2001) or sham operation, and the mitochondrial and cytosolic fractions were prepared from approximately 60 mg of left striatum and adjacent cortex. Tissue was gently homogenized by douncing 35 times in a glass tissue grinder (Wheaton, Millville, NJ) in 7 volumes of cold suspension buffer [20 mM HEPES-KOH (pH 7.5), 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and protease inhibitor cocktail (0.7%; Sigma-Aldrich, St. Louis, MO)]. The homogenates were centrifuged at 750g at 4°C, and then at 8,000g for 20 min at 4°C. The 8,000g pellets were used to obtain the mitochondrial fraction. The supernatant was further centrifuged at 100,000g for 60 min at 4°C. Protein concentrations were determined by the Bradford method (Bio-Rad), and 4 μg of protein from the cytosolic fraction and 2 μg from the mitochondrial fraction were loaded per lane. The primary antibodies were either against cytochrome c (1:1,000; BD PharMingen, San Diego, CA), cytochrome oxidase (COX) subunit IV (1 μg/ml; Molecular Probes), or β-actin (Sigma-Aldrich). Western blots were performed as described above. The Western blot signal was scanned by GS-700 imaging densitometer (Bio-Rad), and the results were quantified using Multi-Analyst software (Bio-Rad). The amount of cytochrome c was compared using the ratio of cytochrome c/β-actin for cytosolic fraction and cytochrome c/COX for mitochondrial fraction.

**Apoptosis Analysis.** Apoptosis was measured by quantitating the DNA fragmentation in the ipsilateral and contralateral hemispheric brain tissue at 24 h after MCAO/reperfusion in both SL327- and vehicle-treated mice (n = 6) using a Cell Death detection ELISA kit (Roche Diagnostics, Indianapolis, IN). This sandwich-enzyme immunoassay provides a quantitative determination of histone- and DNA-associated D NA fragments (mono- and oligonucleosomes) based on a photometric reaction using monoclonal antibodies directed against both DNA and histones. Frozen, pulverized brain tissue was lysed using the lysis buffer provided by the kit (30 min at room temperature) and pelleted (200g), from which an aliquot of the supernatant was used in the assay according to the manufacturer’s protocol.

**Statistical Analysis.** Data are presented as mean ± S.E., and the number of animals (n) used for each group are indicated in each figure legend. Statistical comparisons were made by analysis of variance (analysis of variance; Fisher’s protected least-squares difference) and values were considered to be significant when p < 0.05.

**Results**

**Increase in ERK Phosphorylation after MCAO/Reperfusion Injury in Mice.** Western blot analysis showed constitutive expressions of ERK and phospho-ERK in normal brain tissues; the levels of phospho-ERK1/2, however, were markedly increased immediately following reperfusion in the brain (Fig. 1A), showing a very strong induction within the first 10 min after reperfusion and then diminished toward a baseline. No change was observed in the contralateral brain tissues after reperfusion (data not shown). To exclude a possibility that phospho-ERK induction might be due to the stress condition associated with tissue collection, we directly compared brain tissues collected using a routine dissecting procedure with those immediately frozen (with the head) in liquid nitrogen. No difference in phospho-ERK levels was observed in both methods (data not shown). Thus, the relative high basal levels of phospho-ERK in the brain may reflect its constitutive expression under normal physiological condition.

**Systemic Administration of SL327 Specifically Inhibits ERK Phosphorylation in Normal and Ischemic Brain Tissues.** SL327 is a highly selective MEK1/2 inhibitor, with IC50 = 0.18 and 0.22 μM to MEK1 and MEK2, respectively (Scherle et al., 2000). Systemic administration of SL327 (100 mg/kg, i.p.) specifically blocked ERK, but not...
JNK and p38, phosphorylation in both sham-operated and ischemic brain tissues (Figs. 1B and 2A). As shown in Fig. 2A, SL327 resulted in 67% and 45% reduction in phospho-ERK signals in sham-operated \( (p < 0.001, n = 6) \) and ischemic (1 min after reperfusion, \( p < 0.05, n = 5 \)) brain tissues, respectively, compared with vehicle treatment.

A concentration-dependent inhibition of SL327 in ERK phosphorylation in ischemic brain tissues \( (n = 5) \) was demonstrated using Western blot analysis, showing 22, 59 \( (p < 0.01) \), and 74% \( (p < 0.001) \) reduction over controls for 10, 30, and 100 mg/kg SL327 administration, respectively (Fig. 2B).

**Neuroprotective Effect of SL327 on Mouse Brain after Focal Cerebral Ischemia.** Figure 3 shows significant reduction in infarct size (by direct measurement of the infarct areas) in mice dosed with 100 mg/kg SL327 15 min before (63.6% over controls, \( n = 23, p < 0.001 \)) and 25 min after (50.7%, \( n = 18, p < 0.01 \)) MCAO. No significant effect was observed when SL327 was given at 60 min after MCAO (or 30 min after reperfusion) (20.0% reduction, \( n = 11, p = 0.51 \)). The reduction in infarct was observed in both cortical and subcortical regions (the breakdown data not shown). The reduction in infarct size was 63.0, 51.1, and 20.5% for the SL327-treated groups over controls for dosing at 15 min before MCAO and 25 and 60 min after MCAO, respectively, using indirect measurement to correct potential errors of edema. No obvious difference was observed for the effect of drugs using the two techniques of infarct measurement in the present study.

The neuroprotection by SL327 was also assessed based on neurological deficits and motor function (rota-rod) after MCAO (Fig. 4). The neurological deficits were significantly reduced in the SL327-treated animals \( (n = 17) \) compared with vehicle treatment at day 1 (62% improvement, \( p < 0.001, n = 17 \)) and day 3 (61% improvement, \( p < 0.001, n = 14 \)) after MCAO (Fig. 4A). Similarly, functional recovery was observed in rota-rod tests for the same groups of animals.
Although no significant difference was observed between SL327- and vehicle-treated mice one day after MCAO, SL327-treated mice demonstrated a marked improvement in rota-rod performance ability over vehicle at day 3 (163% improvement, \( p < 0.05, n = 14 \)) after MCAO. The rota-rod score was significantly lower (56% less, \( p < 0.01 \)) in the SL327-treated group (\( n = 17 \)) at day 1 compared with that of sham operation (\( n = 12 \)), but there was no statistically significant difference (35% less in SL327-treated group, \( p = 0.16 \)) between these two groups at day 3. It should be pointed out that the accelerating rota-rod test may evaluate not only motor function but also the improvement in learning and adaptation since all the experimental groups including sham operation showed the improvement in the test score (Fig. 4).

**Effect of SL327 on Physiology after Cerebral Ischemia.** Table 1 shows the physiological parameters (including cerebral blood flow, heart rate, arterial blood pressure, pH, blood oxygen, and carbon dioxide) measured before, during (15 min), or 60 min after MCAO in both SL327- and vehicle-treated mice. As expected, the relative levels of regional CBF were markedly reduced after MCAO (approximately 20% and 18% of arbitrary units for SL327- and vehicle-treated, respectively, compared with those of before MCAO) and significantly resumed after reperfusion (approximately 85% and 86% for SL327- and vehicle-treated, respectively). However, no significant difference was noted between SL327- and vehicle-treated groups, and therefore it is likely to have no physiological consequence in the SL327-treated animals.

**Effect of SL327 on Ischemia/Reperfusion-Induced Apoptosis and Cytochrome c Release from Mitochondria.** Because MAPK has been implicated in cell survival as well as apoptosis following cerebral ischemic injury, we evaluated the effect of SL327 on both intrinsic and extrinsic pathways of apoptosis including cytochrome c release, caspase-8, caspase-3 activation (assessed for the expression of active caspase-3), and DNA fragmentation. As shown in Fig. 5A, cytochrome c immunoreactivity was evident as a single band of molecular mass 14-kDa cytosolic fraction in the ischemic brain 4 h after ischemia/reperfusion (peak expression of cytochrome c in this model), whereas it was barely detected in the sham-operated animals. The amount of cytosolic cytochrome c was significantly increased in the vehicle- and SL327-treated brain tissues (with the mean ratio of cytochrome c to \( \beta \)-actin to be 1.56 and 1.34, respectively) compared with the sham operation (0.11, \( p < 0.001, n = 4 \)). No statistical difference was observed between vehicle- and SL327-treated groups. The mitochondrial fraction of cytochrome c was also examined (Fig. 5B). The amount of mitochondrial cytochrome c was significantly less in vehicle- and SL327-treated ischemic brain tissue (mean ratio of cytochrome c versus COX, 0.96 and 1.07, respectively) compared with sham-operated animals (mean ratio 1.46, \( p < 0.05, n = 4 \)). Again, no statistical difference was observed between vehicle- and SL327-treated groups.

Western analysis was used to detect the expression of active caspase-8 (p20) and caspase-3 (p20) in the brain after MCAO. The increase in p20 caspase-3 was evident in the ischemic brain tissues at 8 and 24 h after MCAO/reperfusion (with a mean ratio of 2.2 and 2.3, respectively, \( n > 5 \); Fig. 6). No effect was observed for SL327 on the elevation of p20 caspase-3 early (8 h) after reperfusion; however, the levels of active caspase-3 in the ischemic brain was markedly reduced 24 h after MCAO/reperfusion (with 46% reduction in the ratio of ipsilateral to contralateral p20 caspase-3 compared with the vehicle-treated animals, \( p < 0.05, n > 9 \)). Comparatively, there was no obvious difference for active caspase-8 in the same samples (Fig. 6A).

The effect of SL327 on apoptosis after cerebral ischemia was also evaluated by monitoring DNA fragmentation using an ELISA method. Similar to the change of p20 caspase-3, the levels of apoptosis were significantly reduced in the

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CBF %</th>
<th>HR ( \text{min}^{-1} )</th>
<th>MABP mm Hg</th>
<th>( \text{pCO}_2 ) mm Hg</th>
<th>( \text{pO}_2 ) mm Hg</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>12</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Before</td>
<td>100</td>
<td>299 ± 38</td>
<td>84 ± 4</td>
<td>31 ± 2</td>
<td>190 ± 15</td>
<td>7.32 ± 0.04</td>
</tr>
<tr>
<td>During</td>
<td>18 ± 1</td>
<td>313 ± 24</td>
<td>74 ± 13</td>
<td>40 ± 3</td>
<td>198 ± 9</td>
<td>7.30 ± 0.04</td>
</tr>
<tr>
<td>After</td>
<td>86 ± 5</td>
<td>348 ± 40</td>
<td>87 ± 4</td>
<td>37 ± 1</td>
<td>204 ± 8</td>
<td>7.39 ± 0.02</td>
</tr>
<tr>
<td>SL327</td>
<td>12</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Before</td>
<td>100</td>
<td>296 ± 20</td>
<td>82 ± 5</td>
<td>32 ± 2</td>
<td>192 ± 16</td>
<td>7.33 ± 0.02</td>
</tr>
<tr>
<td>During</td>
<td>20 ± 1</td>
<td>291 ± 14</td>
<td>75 ± 7</td>
<td>45 ± 2</td>
<td>183 ± 27</td>
<td>7.31 ± 0.05</td>
</tr>
<tr>
<td>After</td>
<td>85 ± 4</td>
<td>315 ± 27</td>
<td>78 ± 7</td>
<td>37 ± 3</td>
<td>226 ± 13</td>
<td>7.28 ± 0.06</td>
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HR, heart rate (per minute); MABP, mean arterial blood pressure (mm Hg).
Inhibition of MEK1 Protects Ischemic Injury and Apoptosis

In the present study, we demonstrated that systemic administration of a selective MEK1/2 inhibitor, SL327, significantly protected the brain from ischemic injury as evidenced by the reduction in infarct size and improvement in neurological function. These data are consistent with previous reports using other MEK1 inhibitors, PD98059 in mice (Alessandrini et al., 1999) and U0126 in gerbil (Namura et al., 2001). The novel finding of the present report was to provide potential evidence for the molecular mechanism involved in the protective effect of MEK1/2 inhibition. In particular, inhibition of MEK1/2 has been associated with the down-regulation of caspase-3 activation and DNA fragmentation but independent of cytochrome c release from mitochondria following focal stroke.

Several lines of evidence provided in this work further support the role of MEK1/2 inhibition in ischemic brain injury. As reported previously in a mouse model of cerebral ischemia (Alessandrini et al., 1999), our present study also showed the acute and transient increase in ERK phosphorylation immediately after reperfusion. The significance of this temporal induction in phospho-ERK was demonstrated by lack of neuroprotection when SL327 was administered 30 min after reperfusion. In addition, the systemic administration of SL327 significantly blocked ERK phosphorylation but not p38 and JNK in the brain.

Of the three MAPK pathways, only the MEK/ERK pathway has been associated with neuroprotection (Seger and Krebs, 1995; Xia et al., 1995; Skaper and Walsh, 1998; Anderson and Tolkovsky, 1999; Singer et al., 1999; Han and Holtzman, 2000). For example, withdrawal of nerve growth factor from cultured PC-12 pheochromocytoma cells (to induce apoptosis) led to sustained activation of JNK and p38 MAPK but inhibition of ERKs (Xia et al., 1995). Likewise, MEKI inhibitor, PD98059, resulted in a significant increase in apoptosis induced by the nucleotide analog cytosine arabinoside (a potent antineoplastic agent induces apoptosis in postmitotic neurons) in cultured rat sympathetic neurons (Anderson and Tolkovsky, 1999), or abolished the effect of estrogen-induced neuroprotection in cultured primary cortical neurons (Singer et al., 1999), suggesting that MEK1/2 promotes neuronal survival. On the other hand, inhibition of MEK1 (by PD98059) was shown to be neuroprotective in okadaic acid (a serine/threonine protein phosphatase inhibitor)-induced cell death of hippocampal slice cultures (Runden et al., 1998). Similarly, inhibition of MEK1 reduced neuronal death in a cell culture model of seizure activity (Murray et al., 1998). Our present study, along with two previous reports (Alessandrini et al., 1999; Namura et al., 2001), provides additional evidence for a neuroprotective role of MEK inhibition in vivo as manifested by reduction of infarct size and improvement of functional outcome following transient ischemia. Taking together, a growing body of evidence has suggested that the MEK/ERK pathway could be involved in both neuroprotection and injury.

While the precise mechanism of this MEK/ERK pathway in neuroprotection and injury remains to be elucidated, it is the critical signaling pathway in response to nerve growth factors for cell survival, possibly by counteracting p38- and JNK-mediated cell death as shown in PC-12 cells (Xia et al., 1995). Less is known about the mechanism of neuroprotection induced by MEK inhibition and in particular following brain ischemia. Therefore, in the present work we investigated several key components involved in apoptosis since emerging biochemical and pharmacological evidence suggests that apoptosis may play an important role in ischemic brain injury (Graham and Chen, 2001). Two major distinct apoptotic pathways of caspases have been demonstrated for...

Fig. 6. Western analysis of active caspase-3 (p20) expression in the ischemic brain after SL327 treatment. Mice were administered 100 mg/kg SL327 or vehicle 15 min before 30 min MCAO followed by 24 h of reperfusion. A, representative Western blots for expression of active caspase-3 (p20) and active caspase-8 (p20) in the SL327- or vehicle-treated ischemic (Ipsilat.) and non-ischemic (Contral.) brain tissues. B, quantitative data to show the expression of p20 caspase-3 at 8 and 24 h after reperfusion (n = 8). Data were illustrated as the ratio of ipsilateral/contralateral brain tissues for mean ± S.E. after normalizing with the housekeeping gene, actin. *, p < 0.05, compared with the vehicle-treated group.

Fig. 7. Bar graph illustrates the DNA fragmentation (apoptosis) in the ischemic brain after SL327 or DMSO administration. DNA fragmentation was measured using an ELISA method, as described under Materials and Methods to represent the level of apoptosis in the brain after SL327 or vehicle (DMSO) treatment. *, p < 0.05, compared with the ipsilateral hemisphere of vehicle-treated group.
their involvement in ischemia/reperfusion injury (Namura et al., 1998; Velier et al., 1999; Noshita et al., 2001). One involves the activation of caspase-8 from cell surface receptors linked via death domains to caspase cascade activation and cell death; another is cytochrome c-dependent mitochondrial pathway of apoptosis (Budihardjo et al., 1999). Both pathways lead to the activation of caspase-3 and finally result in apoptosis (Budihardjo et al., 1999). Since MEK1/2 inhibitor SL327 had no effect on caspase-8 activation (Fig. 6), it is likely that SL327-mediated neuroprotection might not involve the caspase-8-dependent pathway of apoptosis or that it is acting downstream. Similarly, our data do not suggest the possibility of cytochrome c-dependent pathway since SL327 did not affect cytochrome c release from mitochondria (Fig. 5). The failure of SL327 to suppress p20 caspase-3 expression early (8 h) after reperfusion (Fig. 6) further supported this notion. However, it is evident that the levels of active caspase-3 was significantly decreased in the SL327-treated ischemic brain 24 h after reperfusion, and so also apoptosis (as measured by histone-associated DNA fragmentation). These data are in agreement with the significant reduction in infarct size and improvement in motor function in SL327-treated animals 24 h after the insult. Therefore, neuroprotection mediated by MEK1/2 inhibition may be associated with a potential novel apoptotic pathway, independent of cytochrome c and possibly caspase-8, to suppress caspase-3 activation and apoptosis.

In addition to its association with apoptosis, SL327 may involve the regulation of inflammatory reaction, another mechanism that has been demonstrated to play a crucial role in ischemic brain injury (del Zoppo et al., 2000). We have previously demonstrated that the expression of interleukin-1β was significantly down-regulated by SL327 after brain ischemia (Wang et al., 2001a). These data were further supported by the direct evidence of a neuroprotective role using antagonizing interleukin-1 (using interleukin-1 receptor antagonist) in ischemic brain injury (Lodddick and Rothwell, 1996).

In conclusion, our study demonstrated that the inhibition of MEK/ERK pathway results in protection of the brain tissue from ischemia/reperfusion injury. Our data suggest that the neuroprotective effect of MEK inhibition may be involved in reduction in caspase-3 activation and apoptosis (likely a novel mechanism independent of caspase-8 activation and cytochrome c release), as well as inflammatory reaction after focal stroke.

References