Death-associated protein kinase (DAPK) is a calcium calmodulin-regulated serine/threonine protein kinase involved in ischemic neuronal death. In situ hybridization experiments show that DAPK mRNA expression is up-regulated in brain following a global ischemic insult and down-regulated in ischemic tissues after focal ischemia. DAPK is inactive in normal brain tissues, where it is found in its phosphorylated state and becomes rapidly and persistently dephosphorylated and activated in response to ischemia in vivo. A similar dephosphorylation pattern is detected in primary cortical neurons subjected to oxygen glucose deprivation or N-methyl-D-aspartate (NMDA)-induced toxicity. Both a calcineurin inhibitor, FK506, and a selective NMDA receptor antagonist, MK-801, inhibit the dephosphorylation of DAPK after in vitro ischemia. This indicates that DAPK could be activated by NMDA receptor-mediated calcium flux, activation of calcineurin, and subsequent DAPK dephosphorylation. Moreover, concomitantly to dephosphorylation, DAPK is proteolytically processed by cathepsin after ischemia. Furthermore, a selective DAPK inhibitor is neuroprotective in both in vitro and in vivo ischemic models. These results indicate that DAPK plays a key role in mediating ischemic neuronal injury.

Ischemic stroke is characterized by apoptotic and necrotic cell death leading to neuronal loss (1). To date, there is no effective neuroprotective drug in clinical use for the treatment of acute ischemic neuronal damage (2). Several biological processes have been proposed to contribute to ischemic cell death, including excitotoxicity, ionic imbalance, oxidative stress, and apoptosis (3). These pathological events in the brain trigger aberrant cell signaling (4) and subsequent gene expression (5), which have been studied to understand the pathophysiology of stroke. We have identified DAPK as a protein regulated by ischemic conditions both in vitro and in vivo.

DAPK is a Ca$^{2+}$/calmodulin-regulated serine/threonine kinase that acts as a positive mediator of apoptotic pathways, including those involved in neuronal cell death. It is involved in Fas- and tumor necrosis factor- (6), ceramide- (7), and p53-mediated apoptosis (8), as well as in the disruption of matrix survival signals and suppression of integrin-mediated cell adhesion (9). DAPK appears to function early in the apoptotic pathway prior to the commitment of the cells to apoptosis. DAPK has also been extensively implicated as a tumor suppressor whose inactivation predicts malignancy, as DAPK expression is frequently lost in tumors due to hypermethylation of the DAPK gene (10, 11).

DAPK has a unique multidomain structure containing a kinase domain, a calmodulin regulatory segment, eight ankyrin repeats, a cytoskeleton binding region, and a death domain (12). DAPK is negatively regulated by autophosphorylation on serine 308 in the Ca$^{2+}$/CaM regulatory domain. This autoregulation of DAPK serves as an inhibitory mechanism in the basal state (13). Dephosphorylation relieves autoinhibition and stimulates its pro-apoptotic activities (13). Overexpression of intact DAPK in HeLa cells (but not of the catalytically inactive kinase mutant) induces apoptotic cell death, suggesting that the cell death-inducing activity of DAPK depends on its intrinsic kinase activity (12).

In the developing and adult central nervous system, DAPK mRNA is widely expressed in proliferative regions within the cerebral cortex and hippocampus (14, 15). DAPK may have a role in both neuronal development as well as in recovery from injury, as an up-regulation of this enzyme has been reported in the affected hippocampal hemisphere 1 week after ischemic injury (16). The temporal and spatial regulation suggests an involvement in neuronal functions, including neuronal cell death. The expression of DAPK mRNA is increased prior to selective cell death induced by transient forebrain ischemia, indicating further a relationship between this enzyme and neuronal cell death (15). Moreover, inhibition of DAPK with a selective inhibitor attenuates hypoxia-ischemia-induced acute brain injury (17). Recently, DAPK activity has also been implicated in the molecular pathways activated during seizure-induced neuronal death (18, 19) as well as in tubular cell apoptosis in renal ischemia reperfusion injury (20).

We have characterized the mechanisms of DAPK activation during ischemia and have shown that DAPK is dephosphorylated and activated following ischemia in the brain. This activation can be inhibited by FK506, an inhibitor of the phosphatase calcineurin (21), or by MK-801, a selective NMDA receptor antagonist (22) in primary cortical neurons. These results suggest that DAPK plays an important role in the pathophysiology of ischemia. Calcineurin could be the candidate phosphatase responsible for DAPK dephosphorylation requiring NMDA receptor-mediated calcium flux for its activation. We demonstrated that a selective DAPK inhibitor is neuroprotective in primary cortical neurons subjected to oxygen glucose deprivation (OGD) and in vivo in the transient middle cerebral arterial occlusion (MCAO) model. Our results indicate the involvement of DAPK in delayed ischemic cell death and its relevance as a therapeutic target for ischemic brain injury.

MATERIALS AND METHODS

Global Ischemia—All experimental procedures were approved by AGY’s Animal Care and Use Committee and were in line with the guidelines of the National Institutes of Health. The global ischemia with or without preconditioning was done as described previously (23). Briefly, the two-vessel occlusion model of global cerebral ischemia was
Ischemia was induced by withdrawal of blood through the central venous catheter to a blood pressure of 50 mm Hg followed by clamping of the common carotid arteries with clips. Ischemia was terminated after 10 min. In the preconditioning protocol, the second ischemic event was induced in a similar manner at 2 days following a sublethal ischemia of 3 min duration. Sham-operated animals were treated similarly as those subjected to ischemia, except for the occlusion of the carotid arteries and the induction of hypotension.

MCAO—The transient MCAO was performed in male Wistar rats according to Memezawa et al. (25) with some minor modifications. Briefly, a small incision was made in the common carotid artery, and a nylon monofilament was inserted into the internal carotid artery through the common carotid artery. An occlusion time of 90 min or 2 h was allowed in all rats subjected to MCAO, and the filament was removed at the end of the 90 min or 2 h. The body temperature of the rats subjected to MCAO was maintained at 37°C for 6 h after the occlusion.

For the efficacy study with the DAPK-selective inhibitory compound, animals were subjected to 90 min of MCAO and treated with either vehicle (n = 6; 5% Me2SO/20% polyethylene glycol-400 in saline) or compound (n = 7; 5 mg/kg in vehicle) injected intraperitoneally 5 min prior to MCAO and 3.5 h post-occlusion.

2,3,5-Triphenyltetrazolium Chloride Staining and Brain Infarction Measurements—Rats subjected to 90 min of transient MCAO were decapitated after 24 h of reperfusion for determination of infarction volume. The isolated brains were quickly placed in cold saline for 20 min, sliced in seven coronal slices (2 mm thick), and stained in a 1.0% 2,3,5-triphenyltetrazolium chloride solution in saline at 37°C for 30 min (26). The same procedures were performed for sham-operated animals. The brain infarction was measured as described previously (27). Briefly, the brain slices were photographed with the use of a charge-coupled device camera, and the damaged unstained areas were defined as infarcted tissue. The corrected infarct volumes were calculated using an image analysis program (ImageProPlus).
In Situ Hybridization—Animals were terminated by decapitation at different reperfusion times, and the brains were removed, embedded, and frozen on dry ice. Rat brain coronal sections were cut into 14.5 mm thick sections and prehybridized by fixation in 4% paraformaldehyde followed by processing in 0.1 M triethylamine, 0.25% acetic anhydride/triethylamine, and 100% chloroform and alcohol dehydration. The DAPK RNA probe corresponding to nucleotides 2741–3178 on DAPK full-length cDNA (GenBank™ accession number gi34873589) was generated and labeled with \([\alpha^{32}P]UTP\) (PerkinElmer Life Sciences). The sections were then probed for 18 h at 55 °C followed by RNase treatment and stringent washes in standard saline citrate buffer. Finally, the sections were dehydrated and exposed to a phosphorimaging screen (Cyclone, Packard Instruments) for 5–7 days at room temperature. The specificity of the DAPK probe was confirmed in competition experiments using unlabeled probe at 100-fold the concentration of the labeled probe. No specific binding to the tissues was detected with the radiolabeled DAPK sense probe (data not shown).

Primary Cortical Neuronal Cultures—Rat primary cortical neurons were prepared from 17-day embryos. The brain cortices were dissected and the neurons dissociated, digested, and plated (0.6 × 10^5 cells/well for a 48-well plate, 9 × 10^5 cells/well for a 6-well plate). Three days later, 5-fluoro-2’-deoxyuridine (30 μM) was added to prevent glia cell proliferation. The cells were maintained for 12–14 days in Neurobasal medium supplemented with B27 and 2 mM glutamine in a humidified atmosphere at 37 °C with 5% CO₂.

OGD—Primary neuronal cultures were subjected to oxygen glucose deprivation for 120 min at 37 °C. The cultures were placed in an anaerobic chamber (Forma Scientific) and incubated with balanced salt solution (116 mM NaCl, 5.4 mM KCl, 1 mM NaH₂PO₄, 1.8 mM CaCl₂, 26.2 mM NaHCO₃, 0.01 mM glycine, pH 7.4) lacking glucose and aerated with an anaerobic gas mix (85% N₂/5%CO₂/10% H₂) to remove residual oxygen. Control cultures were kept in the original Neurobasal medium but were submitted to the anaerobic conditions. At the end of the OGD insult, the cells were removed from the anaerobic chamber, the OGD
medium was replaced with Neurobasal medium containing B27, and the cells were incubated for an additional 2 h. The test compounds were added to the neurons 2 h prior to the OGD induction and were also present during the OGD insult and the recovery period. Supernatants were used for cell viability measurements, and cells were lysed for Western blots.

**NMDA Toxicity**—Primary neuronal cultures were exposed to 50 μM NMDA for 1 h at 37 °C in control salt solution (25 mM Tris, pH 7.4, 120 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 15 mM D-glucose) containing 0.01 mM glycine. The cells were then washed and recovered for 20 h in Neurobasal medium containing B27. The test compounds were added to the neurons 2 h prior to the NMDA addition and were also present during the NMDA insult and the recovery period. Supernatants were used for cell viability measurements and cells were lysed for Western blots.

**Cell Viability**—Lactate dehydrogenase release to the medium was measured as an indicator of cell viability using a commercially available reagent (Cyto Tox, Promega). ATP content was also measured as an index for cell viability using a commercially available reagent (Celltiter Glo, Promega, Madison, WI) according to the manufacturer’s instructions. The data are presented as the induction of cell death (for lactate dehydrogenase) or as the percentage of cell viability (for ATP content) and represent three independent experiments performed in triplicates.

**Tissue Lysis and Western Blots**—Brain tissues were dissected on ice into ipsi (affected)- and contralateral (unaffected) hemispheres, and 0.5 cm thick sections were sampled from each hemisphere at the level of striatum and frozen in liquid nitrogen. Tissue from the frozen brain regions or neuronal cells were homogenized by sonication on ice in a buffer consisting of 50 mM MOPS, pH 7.6, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, 3 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 5 μg/ml aprotinin, 10 μg/ml pepstatin, 0.5 mM magnesium (CH₃COO)₂, and 0.32 M sucrose. Triton X-100 (0.2%) was added to the homogenates, and the protein concentration was determined. The samples (50 μg) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated at 4 °C overnight with anti-DAPK (Sigma catalog number D2178) or anti-phospho DAPK (Sigma catalog number D4941) antibodies, diluted 1:250 and 1:1000 respectively, and then incubated with a horse-radish peroxidase-conjugated goat anti-mouse antibody. The immunoreactivity was visualized by enhanced chemiluminescence using ECL Plus (Amersham Biosciences).

**Statistical Analysis**—Data are presented as means ± S.E. Comparisons among experimental groups were conducted by one way ANOVA followed with Bonferroni’s multiple comparison test and Student’s t test using GraphPad Prism, version 3.03, for Windows (GraphPad Software, San Diego, CA). The relative intensities for the bands in the Western blots experiments were normalized either to the average intensity of the contralateral (unaffected) hemisphere over the entire reperfusion time or to the control groups and presented as the percent of relative intensity.

**RESULTS**

**DAPK mRNA Expression following Global Cerebral Ischemia**—The mRNA expression of DAPK was analyzed in a model of global ischemia (Fig. 1). A brief period of ischemia (3 min) 2 days prior to the second ischemic episode (10 min) protects the hippocampus against ischemic neuronal injury, a biological phenomenon known as preconditioning or tolerance (23). Animals were subjected to 10 min of global ischemia with and without preconditioning followed by 12, 18, and 24 h of reperfusion. Ischemia without preconditioning induced a significant and persistent up-regulation of DAPK mRNA in the cortex (Fig. 1, *arrowhead*) as well as in the coru ammon 1/2 (CA1/CA2, *arrow*) layers of the hippocampus at all time points after reperfusion. In contrast, ischemia with preconditioning did not up-regulate DAPK mRNA. This indicates that DAPK mRNA is up-regulated in both vulnerable (hippocampus) and resistant (cortex) regions of the brain in response to detrimental global cerebral ischemia, and ischemic preconditioning suppresses this up-regulation in the brain.

**DAPK mRNA Expression following Focal Cerebral Ischemia**—DAPK mRNA levels were studied in transient focal ischemia using a 2-h occlusion of the middle cerebral artery followed by reperfusion. DAPK mRNA expression decreased significantly after MCAO in the ischemic core and the penumbra (Fig. 2, *arrow*). However, an up-regulation of the DAPK mRNA was detected in the medial striatum surrounding the ischemic core and ischemic penumbra at 3 and 6 h of reperfusion (Fig. 2, *arrowhead*). These data indicate that the immediate down-regulation of DAPK in the ischemic core precedes the development of acute ischemic...
neuronal damage in these tissues. On the contrary, an up-regulation of mRNA in the medial striatum could be the response of a compromised tissue that survives the sublethal ischemic insult.

**DAPK Protein Is Activated after Focal Cerebral Ischemia**—To determine the mechanisms of DAPK activation following focal ischemia, tissues from contralateral (unaffected) and ipsilateral (affected) brain regions were collected after 2 h of MCAO and various reperfusion times and analyzed for DAPK processing and phosphorylation. The levels of DAPK expression and phosphorylation were determined by Western blotting with an antibody that recognized the DAPK protein (160 kDa) and a phospho-antibody that recognized the phosphorylated form of DAPK at serine 308.

MCAO down-regulated the DAPK protein at 160 kDa in the affected ischemic hemispheres during the reperfusion period. (Fig. 3A, upper panel, and B). This effect was accompanied by the appearance of a degradation product of ~120 kDa. A proteolytic product at ~100 kDa has been previously described (28), although its significance is not clear. An additional degradation product of 60 kDa was also detected as previously reported in an epilepsy model (19). The death-promoting effects of DAPK depend on its catalytic activity, correct intracellular location, and the presence of the death domain (6, 12). The presence of the CaM-regulatory segment maintains DAPK in its inactive and autophosphorylated form. Activation of DAPK relieves this autoinhibition, and DAPK becomes dephosphorylated. Using a phosphospecific

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**FIGURE 5. DAPK activation and dephosphorylation following OGD.** Primary neuronal cultures were subjected to 2 h of OGD and 2 and 22 h of recovery time. Cells were lysed and proteins were analyzed by SDS-PAGE. A, neurons were sampled from control and at different times of recovery (2 h and 22 h) following the OGD insult and probed with a specific DAPK antibody (upper panel) or a phospho-DAPK antibody (middle panel). B, OGD-induced cell death in primary cortical neurons subjected to 2 h of OGD and 2 and 22 h of recovery. The data are expressed as the mean ± S.E. of at least three separate experiments. ** denotes the statistical significance between the control and the 22-h time point, with p < 0.01 (one-way ANOVA followed by Bonferroni’s multiple comparison test). C and D, densitometric analyses of A. Gels are representative of three independent experiments. Values represent the mean ± S.E. of three separate experiments. # and ##/*** denote statistically significant differences between the control and experimental groups, with p < 0.05 and p < 0.01, respectively (one-way ANOVA followed by Bonferroni’s multiple comparison test).
antibody that recognized the phosphorylated form of DAPK (serine 308), we demonstrated a dephosphorylation and activation of DAPK in the ipsilateral region of the brain immediately after MCAO and up to 24 h after reperfusion (Fig. 3A, middle panel, and C). These data indicate that DAPK is activated immediately after MCAO and remains persistently dephosphorylated and activated during the entire reperfusion.

**DAPK Is Activated during and after Oxygen-Glucose Deprivation—Oxygen-glucose deprivation of primary cortical cultures results in both hypoxia and energy deprivation and mimics in vitro the conditions of ischemic stroke. This results in an activation of NMDA receptors leading to the entry of toxic levels of calcium and sodium into the cells, causing a delayed cell death (29).** Primary cortical neurons were subjected to OGD and DAPK expression, and phosphorylation was measured at different intervals after the induction of OGD (Fig. 4A). DAPK was proteolytically cleaved (Fig. 4A, upper panel, and B) as indicated by the degradation of the 160-kDa band and formation of the 120- and 60-kDa bands after 2 h of OGD (Fig. 4B). When the same samples were analyzed for phosphorylation levels of DAPK, a significant dephosphorylation of DAPK was detected in cortical neurons subjected to 1.5–2 h of OGD (Fig. 4C, middle panel, and C). At these time points, neuronal cell death was not detected (data not shown).

We next examined the DAPK protein expression, phosphorylation levels, and cell viability in primary cortical neurons subjected to 2 h of OGD and recovery times of 2 and 22 h under normoxic conditions (Fig. 5). A significant increase in cell death was observed after 22 h compared with 2 h of recovery (Fig. 5B). However, a cleavage of the DAPK 160-kDa immunoreactive band was observed both at 2 and 22 h of recovery and was accompanied by the appearance of a breakdown product of ~120 kDa (Fig. 5A, upper panel, and C). These changes were similar to those observed in brain tissues after MCAO (Fig. 3). A robust dephosphorylation of DAPK coincided with the occurrence of degradation products after OGD. We have thus shown that DAPK becomes activated and dephosphorylated under ischemic conditions both in vitro (OGD) and in vivo (MCAO). Moreover, DAPK activation occurs immediately after the OGD insult and precedes the cell death that was detected 22 h later.

**DAPK Activation after OGD Is Inhibited by FK506 and MK-801—DAPK activation is calcium-dependent (30).** To elucidate the mechanism responsible for DAPK dephosphorylation and activation, primary cortical neurons were subjected to OGD in the presence of FK506, which inhibits the calcium-dependent phosphatase calcineurin (21), or MK-801, a non-competitive inhibitor of the NMDA receptor (22). Cells were pre-incubated with these compounds for 2 h before OGD, and the compounds were present during the OGD insult and the entire recovery time. DAPK was cleaved (Fig. 6A, upper panel, and C) and dephosphorylated (Fig. 6A, middle panel, and D) upon activation by OGD. Incubation with FK506 (10 μM) and MK-801 (10 μM) allowed DAPK to remain phosphorylated at the same level as the control (Fig. 6A, middle panel, and D). MK-801 protected the cells against OGD-induced cell death, whereas no significant protection was detected in the presence of FK506 (Fig. 6B), indicating that the inhibition of dephosphorylation by FK506 is not because of neuroprotection. The maintenance of the phosphorylated state of DAPK by FK506 suggests that calcineurin is the phosphatase responsible for DAPK dephosphorylation. Inhibition of DAPK activation by MK-801 indicates that the NMDA receptor ion
MK-801 neuronal cultures were incubated for 1 h with NMDA (50 
muM) prior to NMDA-induced toxicity. The cells were sampled from control and at 20 h of recovery following 1 h treatment with NMDA (50 
muM), probed with a specific DAPK antibody (upper panel) or a phospho-DAPK antibody (middle panel). We also found a degradation of the 160-kDa DAPK immunoreactivity under this condition, although the 120-kDa proteolytic fragments that were observed after OGD were not generated after NMDA treatment (Fig. 7A, middle panel, and C). Pre-incubation of the cells for 2 h with FK506 (1 
muM) or MK-801 (10 
muM) prevented the DAPK activation as indicated by the phosphorylation state of the enzyme (Fig. 7A, middle panel, and D). Cell viability measurements also indicated an increase in cell death 20 h after the NMDA insult (Fig. 7B, similar to what we observed after OGD, pretreatment with MK-801 was neuroprotective, whereas FK506 failed to protect the cells against cell death. Therefore, we have shown that both a calcineurin inhibitor (FK506) and an NMDA receptor antagonist (MK-801) inhibit DAPK activation. These results confirm our previous findings obtained with the OGD insult and strongly indicate that NMDA-mediated calcium fluxes are required to activate DAPK possibly via calcineurin-mediated dephosphorylation of the enzyme. However, it seems that the degradation of DAPK, which is observed during the OGD insult and following MCAO, is not mediated by calcium entry through the NMDA receptor.

This is confirmed by the fact that MK-801 does not prevent the DAPK proteolysis observed after OGD (Fig. 6).}

**A Selective DAPK Inhibitor Is Neuroprotective against OGD- and MCAO-induced Neuronal Damage**—An aminopyridazine-based small molecule inhibited DAPK in an *in vitro* biochemical assay and was neuroprotective in a model of hypoxia-induced brain injury (17). We tested whether this compound was also protective in *in vitro* and *in vivo* models of ischemia. Primary cortical neurons were pre-incubated for 2 h with different concentrations (0.1 
muM and 1 
muM) of this compound and then submitted to OGD insult for 2 h. This compound was protective, as indicated by the increase in cell survival after a 20-h recovery following the OGD treatment. At this time, the viability for the control cells was...
17 ± 5%, whereas the cells incubated with 0.1 and 1 μM DAPK inhibitor displayed a 44 ± 3% and 63 ± 5% survival, respectively (Fig. 9A) (p < 0.001, one-way ANOVA followed by Bonferroni’s multiple comparison test). We also tested the effects of this compound in animals subjected to 90 min of MCAO. The compound was administered in two intraperitoneal injections of 5 mg/kg each, given 5 min prior to MCAO and at 3.5 h post-MCAO. The brain infarction was measured by 2,3,5-triphenyltetrazolium chloride staining and presented as percent of total brain volume. A statistically significantly smaller brain infarction in the treatment group (6 ± 1.3%) compared with the vehicle group (12 ± 1.6%, p = 0.05 unpaired Student’s t test) was detected in these animals (Fig. 9B). We have therefore shown that a DAPK small molecule inhibitor is able to protect in vitro and in vivo against ischemic damage in a stroke model. This is in agreement with previously reported findings of this molecule at attenuating hypoxia-ischemia-induced acute brain injury (17).

DISCUSSION

DAPK has been implicated in the molecular response to ischemic brain injury and apoptosis-mediated cell death in neurons (7, 15, 16). We determined the expression changes of DAPK after focal and global ischemia and showed that ischemia activates DAPK by calcineurin-mediated dephosphorylation. Disruption of calcium homeostasis is a well known trigger for neurodegeneration (36), and we have also shown that DAPK dephosphorylation and activation is enhanced in response to NMDA-mediated calcium influx. Our results show that DAPK mRNA expression is induced in both vulnerable and resistant regions of the brain in response to global ischemia. This suggests that DAPK up-regulation may be a general response of brain tissue to ischemic conditions. We have shown an up-regulation of DAPK mRNA following the ischemic insult in the vulnerable regions of the hippocampus, which could correlate to cell death observed in these areas. However, a similar up-regulation was also detected in the cortex, which is compromised but survives the ischemic episode. This indicates that DAPK mRNA up-regulation does not exclusively correlate with cell death and does not reflect the vulnerability or immunity of the neurons to such an insult. When animals were preconditioned, there was no induction of DAPK mRNA in these regions. This could correlate with the tolerance to ischemic neuronal injury observed in this model. In animals subjected to transient MCAO, a persistent down-regulation of DAPK mRNA expression was observed in the ischemic core and the penumbra, which undergo a rapid and delayed cell death, respectively. On the contrary, an up-regulation was detected in the medial striatum, which survives. The differences between the regulation of DAPK after focal and global ischemia could be due to different molecular mechanisms leading to cell death (37). Our data indicate that up- or down-regulation of DAPK mRNA after the ischemic insult could occur in both vulnerable and resistant tissues and that there is no direct correlation between DAPK mRNA up-regulation and tissue vulnerability to the ischemic insults.

To investigate mechanisms of DAPK activation in focal ischemia, we studied the phosphorylation state of this enzyme as an indirect indicator of enzymatic activity. Our data show that DAPK is dephosphorylated at serine 308 after ischemic injury, both in vitro, after transient MCAO, and...
DAP Kinase and Ischemic Brain Injury

FIGURE 9. A selective DAPK inhibitor is neuroprotective against OGD. A, neurons were pre-incubated with a selective DAPK inhibitor for 2 h before OGD insult and cell viability was determined. Results are presented as the percentage of viability of control (100%) of at least four independent experiments performed in triplicates. Data are presented as mean ± S.E. *** denotes the statistically significant difference between treatment and vehicle groups, with p < 0.001 (one-way ANOVA followed by Bonferroni’s multiple comparison test). B, animals were subjected to 90 min of MCAO and treated 5 min prior to and 3.5 h post-occlusion by intraperitoneal injection with either vehicle (n = 6) or 5 mg/kg DAPK inhibitor (n = 7). The brain infarction was assessed by 2,3,5-triphenyltetrazolium chloride staining (inset) and is presented as the percentage of total brain volume. Data are presented as mean ± S.E. * denotes statistically significant differences between treatment and vehicle groups, with p < 0.05 (Student’s t test).

in vitro, during OGD and NMDA-induced neurotoxicity. DAPK dephosphorylation could be observed in the ipsilateral hemisphere immediately after MCAO and during reperfusion. We confirmed this finding in two in vitro models, OGD- and NMDA-induced toxicity in primary cortical neurons. In both models, calcium entry via the NMDA receptors initiated a series of events that culminated in a delayed cell death at 16–24 h post-insult. A time course of DAPK phosphorylation during the 2-h OGD indicates that DAPK is dephosphorylated and activated immediately after an OGD insult and remains both dephosphorylated and activated up to 20 h later. Therefore, DAPK remains activated for an extended period of time, and its activation precedes cell death.

These effects on the phosphorylation state induced by transient MCAO coincide with a marked processing of the DAPK protein, as indicated by the appearance of a 120-kDa band. We identified cathepsin D as a possible enzyme responsible for the DAPK processing. The involvement of different proteases in neuronal cell death is well known. Cathepsins, calpains, and caspases have been associated with the induction of cell death in neuronal tissues (34, 35). Similarly, Araki et al. (19) have reported a 60-kDa band after seizure-induced brain injury that correlates with cathepsin activation and DAPK degradation. Jin et al. (28) have also described a proteolytic breakdown product at 100 kDa in mouse tissues. However, other authors have not commented on the appearance of a cleavage fragment after an apoptotic stimulus (6–8).

The activation of cathepsins may occur under conditions of ischemic or seizure-induced cell death, and proteolytic processing may not always be necessary for dephosphorylation and activation of the enzyme. DAPK is proteolytically processed after OGD and MCAO but not after an NMDA insult. During MCAO and OGD, oxygen and glucose deprivation occur with concomitant loss of ATP. Although energy loss is not a feature of NMDA toxicity, both processes have in common calcium influx into the cells.

We have shown that FK506, a calcineurin inhibitor, and MK-801, a selective NMDA receptor antagonist, keep DAPK in its phosphorylated and inactive form after OGD and NMDA insults. We have thus identified calcineurin as the putative phosphatase that releases DAPK from its autoinhibitory phosphorylation. Moreover, we have shown that calcium entry via the NMDA receptor is a key factor in this process. We propose that calcium flux through NMDA receptors activates calcineurin, which dephosphorylates and activates DAPK. Inhibiting either calcineurin with FK506 or the NMDA receptor activation with MK-801 is enough to prevent DAPK activation.

Further demonstrating the relevance of ischemia-mediated activation of DAPK, we could show that a selective DAPK inhibitor, previously shown to be neuroprotective in a brain hypoxic injury model during an extensive time window (17), is also neuroprotective in in vitro and in vivo ischemic models. This compound prevented the ischemic injury in primary neuronal cultures as well as in the brain following OGD and MCAO, respectively. In line with these findings, it has been shown that FK506 (38) and MK-801 (39) reverse neuronal damage induced by focal ischemia in the brain, and DAPK has been implicated in the neuronal response to injury caused by seizures (19). Recent data have further illustrated the contribution of DAPK activity to apoptosis in renal ischemia reperfusion (20, 40).

Our data suggest that DAPK plays an important role in ischemic injuries and suggests that calcineurin could be a candidate phosphatase responsible for DAPK dephosphorylation and activation. DAPK may be a good therapeutic target for treating acute ischemic stroke.

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