Regional Selective Neuronal Degeneration after Protein Phosphatase Inhibition in Hippocampal Slice Cultures: Evidence for a MAP Kinase-Dependent Mechanism

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The regional selectivity and mechanisms underlying the toxicity of the serine/threonine protein phosphatase inhibitor okadaic acid (OA) were investigated in hippocampal slice cultures. Image analysis of propidium iodide-labeled cultures revealed that okadaic acid caused a dose- and time-dependent injury to hippocampal neurons. Pyramidal cells in the CA3 region and granule cells in the dentate gyrus were much more sensitive to okadaic acid than the pyramidal cells in the CA1 region. Electron microscopy revealed ultrastructural changes in the pyramidal cells that were not consistent with an apoptotic process. Treatment with okadaic acid led to a rapid and sustained tyrosine phosphorylation of the mitogen-activated protein kinases ERK1 and ERK2 (p44/42^{mapk}). The phosphorylation was markedly reduced after treatment of the cultures with the microbial alkaloid K-252a (a nonselective protein kinase inhibitor) or the MAP kinase kinase (MEK1/2) inhibitor PD98059. K-252a

and PD98059 also ameliorated the okadaic acid-induced cell death. Inhibitors of protein kinase C, Ca²⁺/calmodulin-dependent protein kinase II, or tyrosine kinase were ineffective. These results indicate that sustained activation of the MAP kinase pathway, as seen after e.g., ischemia, may selectively harm specific subsets of neurons. The susceptibility to MAP kinase activation of the CA3 pyramidal cells and dentate granule cells may provide insight into the observed relationship between cerebral ischemia and dementia in Alzheimer's disease.

Key words: okadaic acid; K-252a; PD98059; KT5926; H7; KN-62; KN-04; KN-92; KN-93; naringin; staurosporine; genistein; MAP kinase; p44/42 MAP kinase; ERK1/2; MEK1/2; CA3; propidium iodide; fluorescence microscopy; nonapoptotic cell death; apoptosis; cytoskeleton; electron microscopy; image analysis

Many attempts have been made to identify the signal transduction cascades that mediate nerve cell damage in the CNS (Wieloch et al., 1996; Billingsley and Kincaid, 1997). Excitotoxic injury is associated with alterations in multiple signaling systems, including the protein kinase C cascades (Cardell and Wieloch, 1993), the MAP kinase pathways (Campos-Gonzalez and Kindy, 1992; Kindy, 1993; Hu and Wieloch, 1994; Takagi et al., 1997), the Ca²⁺/calmodulin-dependent protein kinase cascade (Cardell and Wieloch, 1993; Hu and Wieloch, 1995; Hu et al., 1995; Wieloch et al., 1996), and the nitric oxide signaling system (Garthwaite and Boulton, 1995; Strijbos et al., 1996).

In Alzheimer's disease (AD), a histological hallmark is the neurofibrillary tangle resulting from an aggregation of paired helical filaments (PHFs) and unpaired straight filaments that consist mainly of the microtubule-associated protein tau in a hyperphosphorylated form. Several protein kinases have been implicated (Billingsley and Kincaid, 1997). Protein phosphatases restore the biological activity of abnormally phosphorylated tau *in vitro* (Wang et al., 1996), and abnormalities in phosphatase activ-

ity may therefore be involved in AD pathogenesis. Protein phosphatases may also be involved in excitotoxic damage (Ankarcrona et al., 1996; Drake et al., 1996). The importance of protein phosphorylation in the regulation of apoptosis is also well documented (Datta et al., 1997; Ito et al., 1997; Jacobson, 1997; Yang et al., 1997).

Induction of sustained hyperphosphorylation with protein phosphatase inhibitors is one way to investigate the role of protein phosphorylation in cellular degenerative processes. Inhibition of protein phosphatases 1 and 2A by the algal toxin okadaic acid (OA) (first isolated from the marine sponge *Halichondria okadaei*) leads to disruption of the cytoskeleton and cell death in several cell culture systems (Holen et al., 1992; Blankson et al., 1995; Benito et al., 1997; Rossini et al., 1997; Yan et al., 1997). In neurons, OA has been reported to cause hyperphosphorylation of tau, modification of synapse structure, destruction of stable microtubules, and apoptosis (Harris et al., 1993; Mawal-Dewan et al., 1994; Garver et al., 1995; Saito et al., 1995; Burack and Halpain, 1996; Fernández-Sánchez et al., 1996; Garver et al., 1996; Malchiodi-Albedi et al., 1997; Merrick et al., 1997).

Here we present evidence for a selective vulnerability of CA3 hippocampal neurons to hyperphosphorylation induced by OA. We also show that such inhibition of serine- and threonine-directed protein phosphatases leads to a rapid and persistent tyrosine phosphorylation of the mitogen-activated protein (MAP) kinases ERK1 and ERK2 (p44/42^{mapk}) that was markedly reduced after inhibition of the MAP kinase kinase MEK1/2 with

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the specific inhibitor PD98059 (Alessi et al., 1995) and after treatment of the cultures with the microbial alkaloid K-252a. These drugs also protected the cultures against the OA-induced cell death. The findings demonstrate that specific subsets of neurons are vulnerable to sustained MAP kinase activation. Therapy directed at the untoward consequences of elevated MAP kinase activity may emerge as an adjunct to other neuroprotective strategies.

MATERIALS AND METHODS

Materials. Culture media were from Life Technologies (Gaithersburg, MD). Okadaic acid was from Alexis Company (Läufelfingen, Switzerland). KN-04, KN-62, KN-93, and KN-92 were from Seikagaku Corporation (Tokyo, Japan), K-252a was from Kamiya Biomedical Company (Tokyo, Japan), KT5926 was from Biomol Research (Plymouth Meeting, PA), H7 was from Sigma (St. Louis, MO), Genistein was from Life Technologies, and PD98059 was from New England Biolabs (Beverly, MA). Antibodies to ERK1/2 and phosphorylated MAP kinase were purchased from New England Biolabs. Phosphotyrosine antibodies were from Transduction Laboratories (Lexington, KY). All other chemicals used were from Sigma unless indicated otherwise.

Slice cultures. Organotypic slice cultures from hippocampus were prepared according to the technique described by Gähwiler (1988) [also see Laake et al. (1995)]. Male Wistar rat pups [postnatal days 4–7 (P4–P7)] (Møllegaard) were decapitated, and the brains were removed and placed in Gey's balanced salts solution (Life Technologies) to which glucose (5 mg/ml) was added. The hippocampi of both sides were removed and cut into transverse slices of 400 μ m thickness on a McIlwain tissue chopper. The slices were carefully separated and placed in a drop of 20 μ l of chicken plasma on coverslips of glass (12 $\times$ 24 mm, Kindler GmbH, Freiburg, Germany) or thermax plastic (10 $\times$ 22 mm, Nunc, Roskilde, Denmark). Twenty microliters of thrombin (from bovine plasma; Merck KGA, Darmstadt, Germany) were then added. The slices were left for 30–60 min at room temperature to let the plasma and thrombin form a clot surrounding the slices. The coverslips were then transferred to flat-sided tissue culture tubes (Nunc) with 750 μ l culture medium consisting of 50% Basal medium Eagle (BME) (with HBSS; Life Technologies), 25% heat-inactivated horse serum (Life Technologies), 25% HBSS (Life Technologies), 100 U/ml penicillin G, and 100 μ g/ml streptomycin (BioWhittaker, Walkersville, MD), 1 mM L-glutamine, and glucose (33 mM). The culture tubes were placed in a roller drum on a rotator (Bellco) tilted at an angle of 5° and rotating at $\sim$ 10 rph in an incubator at 35–36°C. The medium was changed after 1 week, and the cultures were used after 13–14 d *in vitro* (DIV) when they were thin enough to allow identification of the cells in the pyramidal fields and in the dentate gyrus, and when most of the debris on the surface of the cultures had disappeared.

Induction of cell death. Cell death was induced by adding okadaic acid (0–300 nM, Alexis Co.) to the cultures at 13 DIV. OA inhibits serine and threonine phosphatases and thereby induces a hyperphosphorylation, which has previously been shown to induce neuronal as well as non-neuronal cell death (Candeo et al., 1992; Davis et al., 1996; Tergau et al., 1997; Yan et al., 1997). Before incubation, slice cultures were washed in serum-free medium containing 75% BME, 25% HBSS, 100 U/ml penicillin G, 100 μ g/ml streptomycin, 1 mM L-glutamine, and 33 mM glucose. This medium was also used for incubation. Propidium iodide (PI) (5 μ g/ml) in DMSO was used as a fluorescent indicator of dead cells. Drugs used in an attempt to block OA-induced hyperphosphorylation injury included the K-252a (1 nM–10 μ M) (Kase et al., 1987; Bird et al., 1992; MacKintosh and MacKintosh, 1994), KT5926 (100 nM–300 μ M) (Nakanishi et al., 1990), KN-62 (10–40 μ M), KN-04 (10–40 μ M), KN-93 (10–40 μ M), KN-92 (10–40 μ M), H7 (10–100 μ M) (Hidaka et al., 1984; Quick et al., 1992), staurosporin (1–100 nM), genistein (10–100 μ M) (Hidaka et al., 1984; Tremblay et al., 1992; MacKintosh and MacKintosh, 1994; Wang et al., 1997), PD98059 (5–50 μ M) (Alessi et al., 1995), and naringin (10–100 μ M) (Gordon et al., 1995). The final concentration of the vehicle (DMSO) was always 0.8%.

Quantitation of cell death. Initially, photographs of the PI-labeled cultures were taken at 0, 24, and 48 hr after drug treatment using a rhodamine filter set in an inverted Olympus IMT2 fluorescence microscope equipped with a 100 W mercury lamp. If necessary, the excitation light intensity was attenuated with a gray filter. Cell death was evaluated by visually comparing the regional level of fluorescence in diapositives of

the cultures, and no attempt was made to quantify the results at this stage.

Later, quantitative data were obtained using a Hamamatsu C4880-96 cooled CCD camera with a resolution of 1280 $\times$ 1024 pixels and 12-bit pixel depth. The camera was mounted on the IMT2. All images were recorded with a 4 $\times$ objective and 1.67 $\times$ ocular in the C-mount adapter. At this magnification one image will hold an entire culture. The camera was connected to a computer with HiPic image processing software provided by the manufacturer (Hamamatsu).

In each experiment the cultures were divided into experimental and control groups from which images were obtained and saved at 0, 24, and 48 hr. Illumination and exposure (camera gain and exposure time) were kept constant throughout each series of recordings and were nominally reproduced across the time points of an experiment. One series of recordings comprised all the images from a certain time point of one experiment. Preliminary tests were performed on maximally fluorescing cultures to determine the exposure parameters (camera gain and exposure time) that would exploit nearly the full intensity range of the total imaging system without saturating it.

Using a constant exposure period, the images were obtained at 0, 24 and 48 hr and saved. The pictures were then recalled to the monitor for analysis of the regional gray-level intensity using the AnalySIS software (Soft Imaging Software GmbH, Münster, Germany) (Laake et al., 1995). Using an interactive drawing tool, the dentate gyrus and CA3 and CA1 fields of each culture were outlined as regions of interest (ROIs), and the mean gray-level intensity of each ROI was then calculated by the program.

Before each series of recordings, but after the temperature of the camera was stabilized, a dark-current image and a shading correction image were recorded. The dark-current image was obtained with the chosen exposure settings, but with the light path from the microscope closed. The shading correction image was recorded from a preparation of PI dissolved in DMSO (2.5 mg/ml) that was filled in a shallow groove in a transparent slide made from Perspex and mounted on the stage of the microscope.

The shading correction (or “flat-fielding”) procedure was performed to correct for the spatially nonuniform sensitivity of the complete imaging system. Reasons for the spatial nonuniformity include uneven illumination from the mercury lamp, lens shading, and nonuniform sensitivity of the CCD chip. The illumination will always be strongest in the middle and gradually weaker at the periphery of the image. Shading correction performs the following calculation: $C(x,y) = D(x,y) \times K/S(x,y)$, in which C represents the corrected data (final image), D represents the uncorrected data (uncorrected image), and S represents the shading data (D and S having been corrected by subtraction of the dark-current image). The software automatically assigned the highest gray level in the image as the constant K in the calculation above.

To compare images obtained at different time points in a single experiment (0, 24, and 48 hr), it was necessary to take into account the fact that the imaging system performance may vary over time. The factor that affects the quantitative data most is the shading correction performed for each image series, because new shading images were used each time. The mean gray values of each ROI were therefore adjusted using the shading correction constant K to calculate the factor by which each value was multiplied. The final values were thus obtained by the following calculation: $F(x,y) = C(x,y) \times K_1/K_2$ in which F represents the final data, C the final image as calculated above, and K_1 and K_2 the shading correction constant of the image series at two different time points.

Autofluorescence and PI-emission caused by unspecific accumulation of PI in the tissue was adjusted for by subtracting the gray values obtained from the images taken immediately after starting the experiment (0 hr) from the gray values of images of the same cultures obtained after 24 and 48 hr.

Despite the corrections made, we point out that the *absolute* gray-scale values found in separate experiments should not be compared directly. Inevitable alterations in the general fluorescence intensity were caused by change of light source, adjustments to the microscope, etc. There was also an attenuation of the potency of okadaic acid over time that contributed to the interexperimental variability.

The relationship of cell death and fluorescence intensity was assessed by counting the number of dead cells per field of view at high magnification (100 $\times$). Linear regression analysis (data not shown) indicated that the values correlated well (Pearson's $r^2 = 0.77, 0.91, \text{ and } 0.79$ for dentate gyrus, CA3, and CA1, respectively; $p < 0.01$).

Figure 1. Okadaic acid causes selective neuronal degeneration. *A*, False color-coded images of PI-labeled hippocampal slice cultures. Images were obtained at the start of the experiment and at 24 and 48 hr. The *top left image* is from a control culture at the start of the experiment (0 nM okadaic acid, 0 hr). It shows the outline of the different subregions of the

Light and electron microscopy. Cultures were fixed in 2.5% glutaraldehyde and 1% formaldehyde, treated with 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4, dehydrated in a graded series of ethanol and propylene oxide, and flat-embedded in an epoxy resin (Durcupan ACM, Fluka, Neu-Ulm, Germany). Semithin sections were stained with toluidine blue, and ultrathin sections were stained with 1% uranyl acetate for 20 min and 1% lead citrate for 2 min.

Light microscopy was performed with an upright Leitz DM R microscope (Leica). Electron microscopic images were obtained with a Philips CM10 transmission electron microscope.

Immunoblotting. Treatment with OA and kinase inhibitors was as described above except that PI was omitted from the medium and incubation was stopped at 0, 4, 8, and 24 hr, after which cultures were removed from the coverslips into Eppendorf tubes and frozen in liquid nitrogen. Each tissue sample was pooled from 5–10 cultures. The tissue was thawed on ice and mixed with 100 μl of homogenization buffer containing 50 mM 3-[N-morpholino]propane-sulfonic acid/HCl and 2.0 mM DTT, pH 7.6, 3.0 mM EGTA, 0.5 mM magnesium acetate, 0.1 mM sodium orthovanadate, 0.1 mM PMSF, 20 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ pepstatin A, 5 $\mu\text{g/ml}$ aprotinin, and 0.32 M sucrose. The tissue was sonicated while the samples were kept cold by repeatedly cooling the sonicator tip in liquid nitrogen.

Twenty to one-hundred microliters (10–50 μg protein) of each sample were mixed with Laemmli sample buffer (Bio-Rad, Hercules, CA) with the addition of 5% mercaptoethanol. The samples were centrifuged at 3000 rpm for 3 min, boiled for 3 min, and separated by SDS-PAGE (12 hr) before blotting onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad).

Immunostaining and detection. The membranes were washed in Tris-buffered saline with 0.2% Tween 20 (TBS-T) before incubation for 2 hr in 3% bovine serum albumin or nonfat milk powder (Nestlé) in TBS-T at room temperature. The membranes were then incubated with primary antibody diluted 1:1000 in 3–5% BSA or milk powder at 4°C overnight, washed, and incubated with a secondary antibody coupled to horseradish peroxidase (Amersham, Arlington Heights, IL) diluted 1:2000 in 3% BSA at 4°C for 1 hr. Labeling was detected using the enhanced chemiluminescence technique (ECL). After detection the membranes were stripped in buffered mercaptoethanol and reprobbed.

RESULTS

Okadaic acid induces selective neuronal death

None of the cultures exhibited significant cell death in any of the hippocampal regions at the start of the experiment (0 hr) (Fig. 1*A*). In controls some cell death was observed in the dentate gyrus (DG) at 24 and 48 hr, but not in CA1 or CA3 (Fig. 1*A,B*). Treatment with OA caused a dose- and time-dependent increase in cell death. In the DG a significant increase in cell death was observed at 24 hr in cultures that had been treated with >10 nM OA (Fig. 1*A*). At 300 nM some structural disintegration of the cultures caused an apparent reduction in cell death in this region (because dead cells became more dispersed). At these doses cell death was pronounced also in the CA3 region, but increased only minimally in the CA1 region (Fig. 1*A*). After incubation for 48 hr (Fig. 1*B*), the cell death was massive in the DG and in the CA3 region in those cultures treated with >10 nM OA and also extended, albeit less pronounced, into the CA1 region.

A quantitative assessment of the dose-response relationship for the OA-induced cell death is given in Figure 2. Okadaic acid significantly increased cell death in all regions at doses >10 nM. The spontaneous cell death in DG of control cultures was seen as a higher “baseline” fluorescence in this region at 24 and 48 hr than that seen in CA3 and CA1. Nevertheless, treatment with

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slice cultures [CA1, CA3, and dentate gyrus (DG)]. The other images are from cultures incubated for 24 hr after treatment with okadaic acid (concentrations indicated). Cultures (13 DIV) were incubated in serum-free medium with PI and OA (0–300 nM). PI fluorescence indicates cell death. *B*, Similar images as in *A* obtained at 48 hr.

Figure 2. Dose–response curves of OA-induced cell death. The experimental conditions were as in Figure 1. PI fluorescence intensities were measured from the outlined regions illustrated in Figure 1*A* (DG, CA3, and CA1) after incubation with OA (0–300 nM). Student's *t* test showed a significant increase in cell death in all regions at OA concentrations >10 nM ($p < 0.05$, Student's *t* test). Error bars indicate SEM; $n = 5$.

>10 nM OA caused a dramatic increase in cell death in the DG. Cell death was dose-dependent, with no further increase observed at doses >100 nM at 24 hr and >30 nM at 48 hr.

Although there was very little spontaneous cell death in the CA3 region, cell death after treatment with OA closely followed that observed in the DG. Thus, doses >10 nM caused significant cell death, with no further increase at doses >100 nM at 24 hr and >30 nM at 48 hr (Fig. 2).

In the CA1 region only a very slight increase in fluorescence was seen in cultures treated with >10 nM OA at 24 hr. At 48 hr >10 nM okadaic acid caused significant cell death. This was never as pronounced as that observed in the CA3 or DG and appeared to reach maximum at 100 nM OA (Fig. 2).

Electron microscopy of control cultures revealed ultrastructural features closely resembling those observed in the hippocampus *in situ*. The major difference was the less ordered layout of the stratum pyramidale in both regions, and particularly in the CA1 where it was much broader than *in situ*. The interrelationship of glial cells and neurons was preserved in this type of culture, and synapses appeared to be normal (data not shown).

After treatment with 100 nM OA for 24 hr, few changes were seen in the CA1 region. The CA3 pyramidal cells exhibited a reduced electron density of the cytoplasm and an aggregation of endoplasmic reticulum and mitochondria around the nuclei (Fig. 3*A*). Some cells appeared to have a disrupted plasma membrane. The nuclei of pyramidal cells sometimes displayed a slight indentation and chromatin condensation, but this was nowhere pronounced. Nerve terminals displayed a loss of vesicle clustering, and there was swelling of dendritic spines (Fig. 3*C*).

At 48 hr, CA1 pyramidal cells exhibited ultrastructural changes similar to those observed in the CA3 region at 24 hr (Fig. 3*B*). However, the changes were less extensive, and completely disrupted cells were seldom seen. The CA3 pyramidal cells were severely damaged at 48 hr (Fig. 4*A*). The cells had either disappeared or showed full disruption of the cytoplasm. Mitochondria were still present in many cases. Sometimes spherical bodies, the size of mitochondria and consisting of concentric osmophilic lamellae, were seen surrounding the nuclei (Fig. 4*C*). These could possibly be degenerating mitochondria. The nuclei were morphologically intact (i.e., did not exhibit fragmentation and/or condensation of chromatin) in those cells still present.

Figure 3. Okadaic acid-induced cell damage. Ultrastructural changes in hippocampal pyramidal cells. Cultures (13 DIV) were treated with 100 nM OA in serum-free medium for 24 hr, after which they were fixed by immersion in 2.5% glutaraldehyde and 1% paraformaldehyde and prepared for flat-embedding in epoxy resin. Ultrathin sections were studied with a Philips CM10 transmission electron microscope. *A*, The ultrastructural changes seen in the pyramidal cells (*p*) of the CA3 region at 24 hr involved slight nuclear indentations (arrow), aggregations of mitochondria around the nuclei (asterisk), and an increased amount of endoplasmic reticulum. Many cells also exhibited gross damage. Scale bar, 5 μ m. *B*, At 48 hr, similar but less extensive changes were seen in pyramidal cells (*p*) in the CA1 region. Scale bar, 5 μ m. *C*, Synaptic contacts in the CA1 region at 48 hr exhibit swollen dendritic spines (*s*) and nerve terminals (asterisk) with dispersed vesicles. Scale bar, 1 μ m.

Glial cells in the CA3 region, identified by their content of fibrils, appeared to undergo apoptotic changes with condensation of the chromatin and nuclear fragmentation (Fig. 4*B*).

In semithin sections, granule cell death in the control cultures appeared to be apoptotic with nuclear condensation and fragmentation. The OA-treated cultures displayed a more heterogeneous picture (data not shown). Because of this cell death in controls, the granule cell death after OA treatment was not further investigated in the electron microscope.

Protein kinase inhibition can ameliorate okadaic acid-induced cell death

Several protein kinase inhibitors were tested in an attempt to block the OA toxicity and elucidate the pathways involved. The drugs that we used are listed in Materials and Methods. Some of

Figure 4. Okadaic acid-induced cell damage. Electron microscopic images from the CA3 region after incubation with 100 nM OA for 48 hr. *A*, Pyramidal cells (*p*) exhibited extensive damage with disrupted cytoplasm. The nuclei were usually morphologically intact. Scale bar, 5 μ m. *B*, Glial cell (asterisk) in the CA3 region with morphological features reminiscent of apoptosis, i.e., multiple nuclear indentations and condensation of the chromatin. Scale bar, 5 μ m. *C*, Enlarged image of framed area in *A* showing multiple spherical cytoplasmic bodies (arrows) composed of concentric osmophilic lamellae, possibly degenerating mitochondria, surrounding the nucleus. Scale bar, 0.5 μ m.

the drugs were selected because they are excellent OA antagonists in hepatocytes (Holen et al., 1992, 1993; Gordon et al., 1995). Many of these drugs are potent Ca^{2+} /calmodulin-dependent kinase II inhibitors.

The first drug found to exert a protective effect was the bacterial alkaloid K-252a (Figs. 5, 6), a general protein kinase inhibitor. A dose of 100–1000 nM was necessary to significantly reduce the OA-induced cell death. When used alone, the drug was toxic in a dose- (>100 nM) and time-dependent manner but nevertheless protective when used in combination with OA (Fig. 6). At 24 hr the protective effect was seen mainly in the CA3 region, whereas at 48 hr reduction in cell death was observed in all hippocampal subfields.

Figure 5. K-252a protects against okadaic acid-induced cell death. False color-coded images of propidium iodide-labeled slice cultures incubated for 24–48 hr. Cultures (13 DIV) were incubated in serum-free medium containing PI and the nonspecific kinase inhibitor K-252a (0–1000 nM). Images were obtained with or without addition of 100 nM OA and at 24 and 48 hr.

Another inhibitor, KT5926, has a somewhat similar pharmacological profile but did not exert any protective effect on the OA-treated cultures (data not shown).

The specific Ca^{2+} /calmodulin-dependent kinase II inhibitors KN-62 and KN-93 were without any protective effect against OA-induced cell death in hippocampal slice cultures (data not shown). Also, two inhibitors of protein kinase C, H7 and staurosporine, as well as the tyrosine kinase inhibitor genistein, proved ineffective. Naringin, a flavanoid with OA-antagonistic effects in hepatocytes, was likewise without any detectable effect in the slice cultures.

Evidence for involvement of the MAP kinase pathway in okadaic acid-induced neuronal death

The specific MAP kinase kinase (MEK1/2) inhibitor PD98059 caused a significant reduction of the OA-induced cell death at 10 and 50 μ M (Figs. 7, 8). The drug did not cause any cell death on its own and, importantly, was also without effect on the spontaneous apoptotic cell death in the DG. At 24 hr, PD98059 prevented cell death in the CA3 region and reduced cell death in DG in OA-treated cultures. At 48 hr, PD98059 reduced cell death in all regions. In the CA1 region, cell death was almost abolished,

Figure 6. K-252a protects against okadaic acid-induced cell death. Dose–response curves illustrate both the toxic effects and amelioration of OA-induced cell death by K-252a. Experimental conditions were as in Figure 5. Significant reduction in OA-induced cell death was seen in the CA3 region at 24 hr in cultures treated with 100–1000 nM K-252a and in all regions at 48 hr ($p < 0.05$, SEM; $n = 5$; Student's t test).

whereas that in the CA3 region and DG was reduced by ~50% (Fig. 8).

Western blots of cultures treated with OA are shown in Figure 9. Cultures to which OA had been added and that had quickly been placed (i.e., within 10–15 min) in liquid nitrogen are labeled 0 hr on the blots. OA caused a very rapid (within 15 min) and sustained increase in the tyrosine phosphorylation of two bands at 44 and 42 kDa corresponding to the p44/42^{mapk} (ERK1/2), as demonstrated with an antibody specific for these kinases (Fig. 9A,C). No other bands exhibited increased tyrosine phosphorylation. A band at 49 kDa was observed, but the labeling did not exhibit any dose- or time-dependent changes in intensity. An antibody specific to the phosphorylated form of ERK1/2 confirmed that the tyrosine phosphorylation was at the MAP kinase (Fig. 9B). The phosphorylation occurred immediately after OA addition and increased up to 8 hr, after which no further increase was observed. This was long before any destructive changes were observed in the slice cultures. The protein level was unaffected by the OA treatment (Fig. 9C).

Western blots from cultures treated with OA and the protein kinase inhibitors K-252a (1.0 μ M) and PD98059 (10 and 50 μ M) (Fig. 10) showed that the two drugs potentially reduced the hyperphosphorylation of MAP kinase. PD98059 appeared to be the

Figure 7. PD98059 protects against okadaic acid-induced cell death. False color-coded images of propidium iodide-labeled slice cultures incubated for 24–48. Cultures (13 DIV) were preincubated for 1 hr in serum-free medium containing PI and the MEK1/2 inhibitor PD98059 (10 μ M). Images were obtained after addition of OA (0 or 30 nM) and at 24 and 48 hr.

more potent drug. The tyrosine kinase inhibitor genistein (10 μ M) had no effect on the OA-induced hyperphosphorylation of MAP kinase (Fig. 10D).

DISCUSSION

In the present study we have used organotypic slice cultures to investigate the effects of sustained hyperphosphorylation in nerve tissue. This has allowed us to demonstrate, for the first time, a differential susceptibility of specific neuron types to such an insult. Furthermore, we found evidence indicating that the toxic effect of OA was mediated by a sustained activation of the MAP kinase cascade.

The cell death was selective in that the CA3 pyramidal cells and the granule cells in the dentate gyrus were much more sensitive to OA than the pyramidal cells in the CA1 region. The type of cell death in the CA3 region as well as that seen later in the CA1 region were nonapoptotic from a morphological point of view, whereas that in the dentate gyrus was more difficult to classify because of significant cell death in the controls.

We found that the cell death was reduced or prevented by drugs that also reduced the hyperphosphorylation of MAP kinase (p44/42^{mapk}/ERK1/2). OA has previously been shown to activate the

Figure 8. PD98059 protects against okadaic acid-induced cell death. Dose-response curves illustrate the amelioration of OA-induced cell death by PD98059. Experimental conditions were as in Figure 7. Significant reduction in OA-induced cell death was seen in all regions at 24 and 48 hr in cultures treated with 10–50 μ M PD98059 ($p < 0.05$, SEM; $n = 5$; Student's t test).

proposed tau-directed MAP kinase p42 (p42^{mapk}/ERK2) in neocortical slices (Garver et al., 1995). The amelioration of cell death by the MEK1/2 inhibitor PD98059 directly implicates the MAP kinase cascade in the cell death induced by OA. K-252a is a nonspecific protein kinase inhibitor, and a high dose (100–1000 nM) was necessary to antagonize the effect of OA. Another drug, KT5926, which acts on many of the same kinases as K-252a, was without any protective effect. K-252a is an inhibitor of receptor tyrosine kinases (Tapley et al., 1992). However, the tyrosine kinase inhibitor genistein did not ameliorate the OA-induced cell death, nor did it affect the hyperphosphorylation of ERK1/2. It is therefore possible that K-252a exerted its protective effect at the level of the MAP kinase cascade itself (Bird et al., 1992). This would indicate that OA exerts its toxic effects by inhibiting phosphatases that normally dephosphorylate the MAP kinase cascade, and not its upstream regulators. A likely target of dephosphorylation in this case is MEK1/2, which is dephosphorylated by protein phosphatase 2A (Denhardt, 1996).

Importantly, PD98059 and K-252a did not affect the spontaneously occurring apoptotic cell death in the DG. Along with a nonapoptotic morphology in the CA3 region of OA-treated cultures, this highlights the fact that different mechanisms are involved in the OA-induced cell death and apoptosis.

Figure 9. Okadaic acid-induced hyperphosphorylation of MAP kinase. Cultures (13 DIV) were incubated in serum-free medium. After addition of OA (0–100 nM) or vehicle (DMSO) to all the cultures, some were removed into Eppendorf tubes and frozen in liquid nitrogen. As much as 15 min may have passed from the beginning of the addition of OA until the first group of cultures was frozen. Lanes with labeled tissue from these cultures are labeled 0h on the blots. Other cultures were removed at 4 hr, 8 hr, and 24 hr. Each tissue sample was pooled from five cultures and subjected to SDS-PAGE. *A*, Western blot of OA-treated cultures labeled with an antibody against phosphotyrosine. *B*, Same blot as in *A* but with an antibody recognizing the phosphorylated form of MAP kinase (*phospho-MAP kinase*). *C*, Same blot as in *A* but with an antibody against unphosphorylated MAP kinase (ERK1/2–p44/42^{mapk}). Note mobility shift at high doses of OA.

K-252a itself caused cell death at high concentrations, but was still protective in combination with high concentrations of OA. This highlights the yin–yang relationship of kinases and phosphatases (Hunter, 1995). The pathways involved in the K-252a-mediated toxicity remain to be explored.

It has been reported that the protein kinase C inhibitors H7, H8, and H9 (Candeo et al., 1992; Cagnoli et al., 1996a,b) ameliorated the toxic effects of OA and that the hyperphosphorylation of tau was prevented by KN-62, an inhibitor of Ca²⁺/calmodulin-dependent kinase II (Harris et al., 1993). In our laboratory, H7 and KN-62 were ineffective in preventing OA-induced damage, as were other inhibitors of Ca²⁺/calmodulin-dependent kinase II, protein kinase C, and the tyrosine kinase inhibitor genistein, as well as the OA antagonist naringin (Gordon et al., 1995). These discrepancies as well as the relative resistance in our study of CA1 pyramidal cells to OA-induced injury may result from a differential complement of protein kinases and phosphatases between different cell types (Pei et al., 1994; Hunter, 1995). The ultimate targets of the signaling cascades may also differ.

Figure 10. Reduction in MAP kinase phosphorylation with the protein kinase inhibitor K-252a and with the MEK1/2 inhibitor PD98059. Cultures (13 DIV) were incubated in serum-free medium containing the bacterial alkaloid K-252a (1 μ M) (*A*) or the MEK1/2 inhibitor PD98059 (10 and 50 μ M) (*B*, *C*). After addition of OA (0–100 nM), some cultures were removed and frozen. This procedure took ~15 min (labeled 0 hours on the blot). Others were removed after 8 hr. Each tissue sample was pooled from five cultures and subjected to SDS-PAGE. *D*, Cultures were treated essentially as in Figure 9 but with the addition of the tyrosine kinase inhibitor genistein (10 μ M).

What is the pathophysiological relevance of our findings? The neuroprotective effect of PD98059 and K-252a, with proven efficacy against enzymes in the protein kinase cascades, strongly implies that OA acts by perturbing these pathways, and in particular the MAP kinases. A perturbed MAP kinase signaling may in turn affect several cellular processes such as gene regulation, cytoskeletal turnover, and receptor function (Seeger and Krebs, 1995). The fact that MAP kinase activation is seen early whereas cell death occurs at a much later stage indicates that downstream targets of the MAP kinase cascade, rather than the MAP kinase itself, are responsible for the cell death.

Sustained MAP kinase activation is seen after brief ischemic episodes in the CA3 region of the hippocampus (Hu and Wieloch, 1994), and the MAP kinase family is one group of kinases that has been implicated in the phosphorylation of tau in AD (Drewes et al., 1992; Arendt et al., 1995). Recent observations suggest a

strong association between the severity of dementia, AD, and previous ischemia (Kokmen et al., 1996; Snowden et al., 1997). Cerebral ischemia leads to activation of signal transduction cascades via glutamate release and activation of NMDA receptors, which in turn leads to calcium entry and increased production of nitric oxide (Lander et al., 1996; Wieloch et al., 1996; Xia et al., 1996). Furthermore, both oxidative stress and β -amyloid are known to induce MAP kinase activation and subsequent phosphorylation of tau (Hu and Wieloch, 1994; Greenberg and Kosik, 1995; Ferreira et al., 1997; Mizukami and Yoshida, 1997). Thus, repeated ischemic episodes (e.g., transitory ischemic attacks) might cause sustained activation of protein kinases that could trigger the development of AD-specific pathology, i.e., tau phosphorylation (Geddes et al., 1994). Our study shows that sustained activation of one of these pathways, the MAP kinase cascade, can cause considerable damage to neurons.

The hippocampal PHFs of AD are located primarily in the CA1 region (McKee et al., 1990; Price et al., 1991), the target for the Schaffer-collateral axons originating from the CA3 pyramidal cells. Sustained MAP kinase activation in CA3 neurons as observed after ischemia has previously been suggested to mediate selective resistance to ischemia in hippocampal CA3 pyramidal cells (Hu and Wieloch, 1994). Although this is not excluded by the present investigation, it is also possible that the MAP kinase may cause inappropriate protein phosphorylation in CA3 pyramidal cells and disruption of the cytoskeleton in axons terminating in the CA1 region.

In conclusion, inhibition of protein phosphatases with okadaic acid leads to a sustained activation of the MAP kinase cascade followed by selective neuronal degeneration. The pyramidal cells of the CA3 region are much more susceptible to protein phosphatase inhibition than are those in CA1. The mechanisms may involve changes in gene expression and destabilization of the cytoskeleton. The sustained MAP kinase activity leads to a nonapoptotic cell death. These findings indicate that the MAP kinase cascade, which after ischemia is selectively activated in the CA3 region, could prove to be a link between stroke/ischemia and AD and consequently an important target for adjunctive therapy in stroke management.

REFERENCES

- Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR (1995) PD 98059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *J Biol Chem* 270:27489–27494.
- Ankarcrona M, Dypbukt JM, Orrenius S, Nicotera P (1996) Calcineurin and mitochondrial function in glutamate-induced neuronal cell death. *FEBS Lett* 394:321–324.
- Arendt T, Holzer M, Grossmann A, Zedlick D, Bruckner MK (1995) Increased expression and subcellular translocation of the mitogen-activated protein kinase kinase and mitogen-activated protein kinase in Alzheimer's disease. *Neuroscience* 68:5–18.
- Benito A, Lerga A, Silva M, Leon J, Fernandez-Luna JL (1997) Apoptosis of human myeloid leukemia cells induced by an inhibitor of protein phosphatases (okadaic acid) is prevented by Bcl-2 and Bcl-X_L. *Leukemia* 11:940–944.
- Billingsley ML, Kincaid RL (1997) Regulated phosphorylation and dephosphorylation of tau protein: effects on microtubule interaction, intracellular trafficking and neurodegeneration. *Biochem J* 323:577–591.
- Bird TA, Schule HD, Delaney PB, Sims JE, Thoma B, Dower SK (1992) Evidence that MAP (mitogen-activated protein) kinase activation may be a necessary but not sufficient signal for a restricted subset of responses in IL-1-treated epidermoid cells. *Cytokine* 4:429–440.
- Blankson H, Holen I, Seglen PO (1995) Disruption of the cytoskeleton and inhibition of hepatocytic autophagy by okadaic acid. *Exp Cell Res* 218:522–530.

- Burack MA, Halpain S (1996) Site-specific regulation of Alzheimer-like tau phosphorylation in living neurons. *Neuroscience* 72:167–184.
- Cagnoli CM, Atabay C, Manev H (1996a) Protective action of isoquinolinesulfonamides in in vitro models of neuronal apoptosis. *Life Sci* 58:L295–L301.
- Cagnoli CM, Kharlamov E, Atabay C, Uz T, Manev H (1996b) Apoptosis induced in neuronal cultures by either the phosphatase inhibitor okadaic acid or the kinase inhibitor staurosporine is attenuated by isoquinolinesulfonamides H-7, H-8, and H-9. *J Mol Neurosci* 7:65–76.
- Campos-Gonzalez R, Kindy MS (1992) Tyrosine phosphorylation of microtubule-associated protein kinase after transient ischemia in the gerbil brain. *J Neurochem* 59:1955–1958.
- Candeo P, Favaron M, Lengyel I, Manev RM, Rimland JM, Manev H (1992) Pathological phosphorylation causes neuronal death: effect of okadaic acid in primary culture of cerebellar granule cells. *J Neurochem* 59:1558–1561.
- Cardell M, Wieloch T (1993) Time course of the translocation and inhibition of protein kinase C during complete cerebral ischemia in the rat. *J Neurochem* 61:1308–1314.
- Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 91:231–241.
- Davis MA, Chang SH, Trump BF (1996) Differential sensitivity of normal and H-ras oncogene-transformed rat kidney epithelial cells to okadaic acid-induced apoptosis. *Toxicol Appl Pharmacol* 141:93–101.
- Denhardt DT (1996) Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: the potential for multiplex signalling. *Biochem J* 318:729–747.
- Drake M, Friberg H, Boris-Moller F, Sakata K, Wieloch T (1996) The immunosuppressant FK506 ameliorates ischaemic damage in the rat brain. *Acta Physiol Scand* 158:155–159.
- Drewes G, Lichtenberg-Kraag B, Doring F, Mandelkow EM, Biernat J, Goris J, Doree M, Mandelkow E (1992) Mitogen activated protein (MAP) kinase transforms tau protein into an Alzheimer-like state. *EMBO J* 11:2131–2138.
- Fernández-Sánchez MT, García-Rodríguez A, Díaz-Trelles R, Novelli A (1996) Inhibition of protein phosphatases induces IGF-1-blocked neurotrophin-insensitive neuronal apoptosis. *FEBS Lett* 398:106–112.
- Ferreira A, Lu Q, Orecchio L, Kosik KS (1997) Selective phosphorylation of adult tau isoforms in mature hippocampal neurons exposed to fibrillar A β . *Mol Cell Neurosci* 9:220–234.
- Gähwiler BH (1988) Organotypic cultures of neural tissue. *Trends Neurosci* 11:484–489.
- Garthwaite J, Boulton CL (1995) Nitric oxide signaling in the central nervous system. *Annu Rev Physiol* 57:683–706.
- Garver TD, Oyler GA, Harris KA, Polavarapu R, Damuni Z, Lehman RA, Billingsley ML (1995) Tau phosphorylation in brain slices: pharmacological evidence for convergent effects of protein phosphatases on tau and mitogen-activated protein kinase. *Mol Pharmacol* 47:745–756.
- Garver TD, Lehman RA, Billingsley ML (1996) Microtubule assembly competence analysis of freshly-biopsied human tau, dephosphorylated tau, and Alzheimer tau. *J Neurosci Res* 44:12–20.
- Geddes JW, Schwab C, Craddock S, Wilson JL, Pettigrew LC (1994) Alterations in τ immunostaining in the rat hippocampus following transient cerebral ischemia. *J Cereb Blood Flow Metab* 14:554–564.
- Gordon PB, Holen I, Seglen PO (1995) Protection by naringin and some other flavonoids of hepatocytic autophagy and endocytosis against inhibition by okadaic acid. *J Biol Chem* 270:5830–5838.
- Greenberg SM, Kosik KS (1995) Secreted beta-APP stimulates MAP kinase and phosphorylation of tau in neurons. *Neurobiol Aging* 16:403–407.
- Harris KA, Oyler GA, Doolittle GM, Vincent I, Lehman RA, Kincaid RL, Billingsley ML (1993) Okadaic acid induces hyperphosphorylated forms of tau protein in human brain slices. *Ann Neurol* 33:77–87.
- Hidaka H, Inagaki M, Kawamoto S, Sasaki Y (1984) Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* 23:5036–5041.
- Holen I, Gordon PB, Seglen PO (1992) Protein kinase-dependent effects of okadaic acid on hepatocytic autophagy and cytoskeletal integrity. *Biochem J* 284:633–636.
- Holen I, Gordon PB, Seglen PO (1993) Inhibition of hepatocytic autophagy by okadaic acid and other protein phosphatase inhibitors. *Eur J Biochem* 215:113–122.
- Hu BR, Wieloch T (1994) Tyrosine phosphorylation and activation of mitogen-activated protein kinase in the rat brain following transient cerebral ischemia. *J Neurochem* 62:1357–1367.
- Hu B-R, Wieloch T (1995) Persistent translocation of Ca²⁺/calmodulin-dependent protein kinase II to synaptic junctions in the vulnerable hippocampal CA1 region following transient ischemia. *J Neurochem* 64:277–284.
- Hu BR, Kamme F, Wieloch T (1995) Alterations of Ca²⁺/calmodulin-dependent protein kinase II and its messenger RNA in the rat hippocampus following normo- and hypothermic ischemia. *Neuroscience* 68:1003–1016.
- Hunter T (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* 80:225–236.
- Ito T, Deng X, Carr B, May WS (1997) Bcl-2 phosphorylation required for anti-apoptosis function. *J Biol Chem* 272:11671–11673.
- Jacobson MD (1997) Apoptosis: Bcl-2-related proteins get connected. *Curr Biol* 7:R277–R281.
- Kase H, Iwahashi K, Nakanishi S, Matsuda Y, Yamada K, Takahashi M, Murakata C, Sato A, Kaneko M (1987) K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem Biophys Res Commun* 142:436–440.
- Kindy MS (1993) Inhibition of tyrosine phosphorylation prevents delayed neuronal death following cerebral ischemia. *J Cereb Blood Flow Metab* 13:372–377.
- Kokmen E, Whisnant JP, O'Fallon WM, Chu CP, Beard CM (1996) Dementia after ischemic stroke: a population-based study in Rochester, Minnesota (1960–1984). *Neurology* 46:154–159.
- Laake JH, Slyngstad TA, Haug F-MS, Ottersen OP (1995) Glutamine from glial cells is essential for the maintenance of the nerve terminal pool of glutamate: immunogold evidence from hippocampal slice cultures. *J Neurochem* 65:871–881.
- Lander HM, Jacovina AT, Davis RJ, Tauras JM (1996) Differential activation of mitogen-activated protein kinases by nitric oxide-related species. *J Biol Chem* 271:19705–19709.
- MacKintosh C, MacKintosh RW (1994) Inhibitors of protein kinases and phosphatases. *Trends Biochem Sci* 19:444–448.
- Malchiodi-Albedi F, Petrucci TC, Picconi B, Iosi F, Falchi M (1997) Protein phosphatase inhibitors induce modification of synapse structure and tau hyperphosphorylation in cultured rat hippocampal neurons. *J Neurosci Res* 48:425–438.
- Mawal-Dewan M, Henley J, Van de Voorde A, Trojanowski JQ, Lee VM (1994) The phosphorylation state of tau in the developing rat brain is regulated by phosphoprotein phosphatases. *J Biol Chem* 269:30981–30987.
- McKee AC, Kosik KS, Kennedy MB, Kowall NW (1990) Hippocampal neurons predisposed to neurofibrillary tangle formation are enriched in type II calcium/calmodulin-dependent protein kinase. *J Neuropathol Exp Neurol* 49:49–63.
- Merrick SE, Trojanowski JQ, Lee VY (1997) Selective destruction of stable microtubules and axons by inhibitors of protein serine/threonine phosphatases in cultured human neurons. *J Neurosci* 17:5726–5737.
- Mizukami Y, Yoshida K (1997) Mitogen-activated protein kinase translocates to the nucleus during ischaemia and is activated during reperfusion. *Biochem J* 323:785–790.
- Nakanishi S, Yamada K, Iwahashi K, Kuroda K, Kase H (1990) KT5926, a potent and selective inhibitor of myosin light chain kinase. *Mol Pharmacol* 37:482–488.
- Pei JJ, Sersen E, Iqbal K, Grundke-Iqbal I (1994) Expression of protein phosphatases (PP-1, PP-2A, PP-2B and PTP-1B) and protein kinases (MAP kinase and P34cdc2) in the hippocampus of patients with Alzheimer disease and normal aged individuals. *Brain Res* 655:70–76.
- Price JL, Davis PB, Morris JC, White DL (1991) The distribution of tangles, plaques and related immunohistochemical markers in healthy aging and Alzheimer's disease. *Neurobiol Aging* 12:295–312.
- Quick J, Ware JA, Driedger PE (1992) The structure and biological activities of the widely used protein kinase inhibitor, H7, differ depending on the commercial source. *Biochem Biophys Res Commun* 187:657–663.
- Rossini GP, Pinna C, Viviani R (1997) Inhibitors of phosphoprotein phosphatases 1 and 2A cause activation of a 53 kDa protein kinase accompanying the apoptotic response of breast cancer cells. *FEBS Lett* 410:347–350.

- Saito T, Ishiguro K, Uchida T, Miyamoto E, Kishimoto T, Hisanaga S (1995) In situ dephosphorylation of tau by protein phosphatase 2A and 2B in fetal rat primary cultured neurons. *FEBS Lett* 376:238–242.
- Seger R, Krebs EG (1995) The MAPK signaling cascade. *FASEB J* 9:726–735.
- Snowdon DA, Greiner LH, Mortimer JA, Riley KP, Greiner PA, Markesbery WR (1997) Brain infarction and the clinical expression of Alzheimer disease. The Nun Study. *JAMA* 277:813–817.
- Strijbos PJ, Leach MJ, Garthwaite J (1996) Vicious cycle involving Na^+ channels, glutamate release, and NMDA receptors mediates delayed neurodegeneration through nitric oxide formation. *J Neurosci* 16:5004–5013.
- Takagi N, Shinno K, Teves L, Bissoon N, Wallace MC, Gurd JW (1997) Transient ischemia differentially increases tyrosine phosphorylation of NMDA receptor subunits 2A and 2B. *J Neurochem* 69:1060–1065.
- Tapley P, Lamballe F, Barbacid M (1992) K252a is a selective inhibitor of the tyrosine protein kinase activity of the trk family of oncogenes and neurotrophin receptors. *Oncogene* 7:371–381.
- Tergau F, Weichert J, Quentin I, Opitz R, Von Zezschwitz C, Marwitz J, Ritz V, Steinfeldt HJ (1997) Inhibitors of ser/thr phosphatases 1 and 2A induce apoptosis in pituitary GH₃ cells. *Naunyn Schmiedeberg Arch Pharmacol* 356:8–16.
- Tremblay L, Gingras D, Boivin D, Beliveau R (1992) Tyrosine protein kinase activity in renal brush-border membranes. *Biochim Biophys Acta* 1108:183–189.
- Wang BH, Ternai B, Polya G (1997) Specific inhibition of cyclic AMP-dependent protein kinase by warangalone and robustic acid. *Phytochemistry* 44:787–796.
- Wang JZ, Grundke-Iqbal I, Iqbal K (1996) Restoration of biological activity of Alzheimer abnormally phosphorylated tau by dephosphorylation with protein phosphatase-2A, -2B and -1. *Brain Res Mol Brain Res* 38:200–208.
- Wieloch T, Hu BR, Boris-Moller A, Cardell M, Kamme F, Kurihara J, Sakata K (1996) Intracellular signal transduction in the postischemic brain. *Adv Neurol* 71:371–387.
- Xia Z, Dudek H, Miranti CK, Greenberg ME (1996) Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. *J Neurosci* 16:5425–5436.
- Yan Y, Shay JW, Wright WE, Mumby MC (1997) Inhibition of protein phosphatase activity induces p53-dependent apoptosis in the absence of p53 transactivation. *J Biol Chem* 272:15220–15226.
- Yang DD, Kuan CY, Whitmarsh AJ, Rincón M, Zheng TS, Davis RJ, Rakic P, Flavell RA (1997) Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. *Nature* 389:865–870.