Mild Cerebral Ischemia Induces Loss of Cyclin-Dependent Kinase Inhibitors and Activation of Cell Cycle Machinery before Delayed Neuronal Cell Death

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After mild ischemic insults, many neurons undergo delayed neuronal death. Aberrant activation of the cell cycle machinery is thought to contribute to apoptosis in various conditions including ischemia. We demonstrate that loss of endogenous cyclin-dependent kinase (Cdk) inhibitor $p16^{INK4a}$ is an early and reliable indicator of delayed neuronal death in striatal neurons after mild cerebral ischemia *in vivo*. Loss of $p27^{Kip1}$, another Cdk inhibitor, precedes cell death in neocortical neurons subjected to oxygen–glucose deprivation *in vitro*. The loss of Cdk inhibitors is followed by upregulation of cyclin D1, activation of Cdk2, and subsequent cytoskeletal disintegration. Most neurons undergo cell death before entering S-phase, albeit a small number (~1%) do progress to the S-phase before their death. Treatment with Cdk inhibitors significantly reduces cell death *in vitro*. These results show that alteration of cell cycle regulatory mechanisms is a prelude to delayed neuronal death in focal cerebral ischemia and that pharmacological interventions aimed at neuroprotection may be usefully directed at cell cycle regulatory mechanisms.

Key words: cell cycle; cerebral ischemia; cyclin-dependent kinases; delayed neuronal cell death; $p16^{INK4a}$; $p27^{Kip1}$

Neurons enter and remain in a terminally differentiated or "resting" state after final cell division. If the control of the resting state breaks down, neurons may reenter the cell cycle, a process that is presumably lethal (Lee et al., 1992; Herrup and Busser, 1995; Gill and Windebank, 1998). Thus, aberrant activation of the cell cycle machinery is thought to cause apoptosis in postmitotic neurons after various insults, including cerebral ischemia and Alzheimer's disease (Busser et al., 1998; Osuga et al., 2000; Sakurai et al., 2000). Increases in cyclin D1 and cyclin-dependent kinase (Cdk) 4 expression occur after focal ischemia in mice and rats (Li et al., 1997), in global ischemia in rats (Timsit et al., 1999), and in a rabbit spinal cord ischemia model (Sakurai et al., 2000). In these models the induction of cyclin D1 occurs in neurons either before nuclear condensation and the appearance of chromosomal DNA fragmentation (Timsit et al., 1999; Osuga et al., 2000) or after the appearance of an apoptotic phenotype (Guégan et al., 1997). Neuronally differentiated PC12 cells, sympathetic neurons, and primary neuronal cells in culture upregulate cell cycle proteins before their apoptotic death when DNA damage occurs or when trophic support is withdrawn (Farinelli and Greene, 1996; Park et al., 1996, 1997a,b, 1998; Padmanabhan et al., 1999).

On the other hand, expression of cell cycle markers after ischemia might be a sign of potentially beneficial mechanisms. Recovery from ischemic damage may recapitulate ontogeny, and the expression of developmental proteins in the penumbra zone to some degree may indicate active reconditioning that could promote cell survival (Li et al., 1997, 1998; Cramer and Chopp, 2000).

The aim of the present study was to define the temporal and spatial relationship between expression of cell cycle proteins including cyclin D1, Cdk2, Cdk4, the endogenous Cdk inhibitors $p16^{INK4a}$ and $p27^{Kip1}$, and delayed neuronal death after mild focal cerebral ischemia in mice (Endres et al., 1998b). We also sought to determine the cell cycle event during which neuronal death is triggered to gain an understanding of the link between cell cycle regulation and delayed neuronal death. We found that loss of endogenous Cdk inhibitors is a likely trigger for reentry of postmitotic neurons into the cell cycle. Neurons that survive the ischemia do not show any loss of Cdk inhibitors.

MATERIALS AND METHODS

Animal experiments. All experimental procedures that were performed on laboratory animals conformed to institutional guidelines for the care and use of laboratory animals. $129/Sv_{EvTacBr}$ wild-type mice (18–22 gm; Taconic Farms, Germantown, NY) were administered 1 $mg \cdot hr^{-1} \cdot kg^{-1}$ bromodeoxyuridine (BrdU; Sigma, St. Louis, MO) via subcutaneously

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implanted osmotic mini-pumps (flow rate 1 μl/hr; Alzet, Cupertino, CA). This dose is nontoxic (Gould et al., 1997; Liu et al., 1998; Kempermann et al., 1998). The administration of BrdU via osmotic mini-pumps was at least as effective as a more established method of daily intraperitoneal injections (50 mg/kg body weight) as determined by comparing the number of BrdU-positive cells using the two protocols (data not shown).

Ischemia model. Mice were anesthetized for induction with 1.5% halothane and maintained in 1.0% halothane in 70% N_2O and 30% O_2 using a vaporizer. Cerebral ischemia was induced with a 8.0 nylon monofilament coated with a silicone resin/hardener mixture (Xantopren M Mucosa and Activator NF Optosil Xantopren; Haereus Kulzer) as described previously (Endres et al., 1998a,b, 1999, 2000). The filament was introduced into the left internal carotid artery up to the anterior cerebral artery. Thereby the middle cerebral artery and anterior choroidal arteries were occluded. Filaments were withdrawn after 30 min of ischemia to allow reperfusion. Regional cerebral blood flow measured using laser-Doppler-flowmetry (Perimed, Jarfälla, Sweden) fell to <20% during ischemia and returned to ~100% within 5 min after reperfusion in either group ($p > 0.05$). Core temperature during the experiment was maintained at 36.5°C ± 0.5°C with a feedback temperature control unit.

Primary neuronal cell culture. Primary neuronal cultures of cerebral cortex were obtained from E17 Wistar rats (Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, Germany). Cultures were prepared according to Brewer (1995) with some modifications as described previously (Bruer et al., 1997; Lautenschlager et al., 2000). Whole cerebral cortices were dissected, incubated for 15 min in trypsin/EDTA (0.05/0.02% w/v in PBS) at 36.5°C, rinsed twice with PBS and once with dissociation medium (modified Eagle's medium with 10% fetal calf serum, 10 mM HEPES, 44 mM glucose, 100 U penicillin + streptomycin/ml, 2 mM L-glutamine, 100 IE insulin/l), dissociated by Pasteur pipette in dissociation medium, pelleted by centrifugation (at 210 × *g* for 2 min at 21°C), redissociated in starter medium (neurobasal medium with supplement B27, 100 U penicillin + streptomycin/ml, 0.5 mM L-glutamine, 25 μM glutamate), and plated in 24-well plates at a density of 200,000 cells/cm^2. Wells were pretreated by incubation with poly-L-lysine (0.5% w/v in PBS) at room temperature for 1 hr, then rinsed with PBS, followed by incubation with coating medium (dissociation medium with 0.03‰ w/v collagen G) for 1 hr at 37°C, then rinsed twice with PBS, before cells were seeded in starter medium. Cultures were kept at 36.5°C and 5% CO_2 and were fed with cultivating medium (starter medium without glutamate) by replacing half of the medium twice a week beginning from the fourth day *in vitro* (DIV). By choosing a serum-free culture condition, we were able to maintain cultures with a very low percentage of glia (Lautenschlager et al., 2000). Neurobasal medium and supplement B27 were obtained from Life Technologies (Eggenstein, Germany); modified Eagle's medium, PBS, HEPES buffer trypsin/EDTA, penicillin/streptomycin, L-glutamine, collagen G, and poly-L-lysine were from Biochrom (Berlin, Germany), and multi-well plates were from Falcon (Franklin Lakes, NJ).

Oxygen–glucose deprivation. Serum-free primary neuronal cultures were treated after 10–14 DIV. The condition of cells at various time points after treatment was determined morphologically by phase-contrast microscopy. Before oxygen–glucose deprivation (OGD), the medium was removed from the cultures and preserved. Cultures were rinsed twice with PBS, then subjected to OGD for 90 min in a balanced salt solution at $P_{O_2} < 2$ mmHg, followed by replacement of the preserved medium as described previously (Bruer et al., 1997; Harms et al., 2000). For control conditions, cells were placed in a balanced salt solution with 20 mM D-glucose for 120 min in normoxic atmosphere with 5% CO_2. Neuronal injury was quantitatively assessed by the measurement of lactate dehydrogenase (LDH) at various time points in the medium (Koh and Choi, 1987). The enzyme standard for kinetic LDH test was obtained from Sigma Chemie GmbH (Deisenhofen, Germany). Representative photographs were taken with phase-contrast microscopy 24 hr after OGD.

Olomoucine treatment protocol. The Cdk inhibitor olomoucine (Alexis, Grünberg, Germany), dissolved in DMSO (50 mM), was used at final concentrations of 1, 10, and 100 μM in the medium. Olomoucine was applied to cortical cell cultures 1 hr before and during OGD. The vehicle-treated cultures received 0.2% DMSO.

Tissue preparation and immunocytochemistry. In the *in vivo* experiments, the brains were perfusion fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.4, and post-fixed in the same fixative overnight at 4°C. Coronal 40 μm sections were cut on a Vibratome (Technical Products, St. Louis, MO). The sections were incubated in a blocking solution containing 10% normal goat serum and 0.3% Triton X-100 for 30 min followed by the primary antibodies [rabbit polyclonal anti-p16^{INK4a}, anti-p27^{Kip1}, anti-cyclin D1 (Santa Cruz, Heidelberg, Germany); 1:250] overnight at 4°C. After three washes with PBS, the sections were incubated in biotinylated secondary antibody (goat anti-rabbit, 1:250; Vector Laboratories, Burlingame, CA) for 90 min at room temperature and developed with Texas Red-labeled streptavidin (1:200; Molecular Probes, Leiden, Holland). Sections were washed in PBS and processed for double labeling with neuronal markers as follows. The sections were incubated with antibodies against neuronal markers, mouse monoclonal anti-MAP-2 (1:2000; Roche, Grenzach-Wyhlen, Germany) or mouse monoclonal anti-NeuN (1:100; Chemicon, Hofheim, Germany) overnight at room temperature. This was followed by incubation in Alexa 488-conjugated goat anti-mouse IgG (1:250; Molecular Probes) for 90 min at room temperature.

For terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) histochemistry, unfixed brains were snap-frozen in isopentane at −40°C. Coronal 10 μm sections were cut on a cryostat (Microm, Heidelberg, Germany), thaw-mounted on glass slides, and stored at −20°C. TUNEL was performed using a fluorescence ApopTag Kit (Biogen, Heidelberg, Germany) according to the manufacturer's instructions. For double labeling with TUNEL and cell-type specific markers, sections were first incubated in blocking solution containing 10% normal serum and 0.1% Triton in PBS and then incubated overnight at 4°C with anti-NeuN (mouse monoclonal, 1:100; Chemicon), anti-GFAP (astroglial marker, rabbit polyclonal, 1:500; Dako, Hamburg, Germany), or anti-MAC-1 (microglial marker, rat monoclonal, 1:1000; Serotec, Oxford, UK) antibodies, followed by incubation with corresponding biotinylated secondary antibodies, and finally with Texas Red-labeled streptavidin (Molecular Probes; 1:250). The sections were then thoroughly rinsed in PBS and processed for TUNEL staining.

For BrdU and TUNEL double labeling, the sections were first processed for TUNEL staining. Then, the DNA was hydrolyzed by 2N HCl into single strands, and the sections were incubated with rat monoclonal anti-BrdU antibody (1:500; Harlan, Borchen, Germany) overnight at 4°C and reacted with biotinylated rabbit anti-rat IgG followed by Texas Red-labeled streptavidin. For immunocytochemical analysis of cell cultures, cells were seeded onto glass coverslips, fixed with freshly prepared 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.3% Triton X-100 in PBS, and exposed to blocking solution (PBS containing 10% goat serum and 1% bovine serum albumin) for 30 min at room temperature. Cultures then were incubated with the rabbit polyclonal antibodies to p16^{INK4a}, p27^{Kip1}, or cyclin D1 (1:100) for 1 hr at room temperature and developed with Texas Red-labeled goat anti-rabbit IgG (1:500) for 30 min at room temperature. After rinsing with PBS, the glass coverslips were incubated with anti-NeuN (1:100) for 1 hr at room temperature, followed by incubation in Alexa 488-conjugated goat anti-mouse (1:500). For control studies, sections were treated the same way except that TdT (for TUNEL studies) or primary antibodies (for immunoreactivity studies) were omitted, resulting in no visible staining.

Confocal laser scan microscopy. A Nikon Optiphot/Bio-Rad MRC 600 (Hempstead, UK) confocal laser scanning microscope equipped with an argon/krypton laser was used. The images were acquired using CoMOS software program (Version 7.0a, Bio-Rad) and imported into Adobe Photoshop 5.0 (Adobe Systems, Mountain View, CA).

Cell counting protocols. To estimate the number of p16^{INK4a}-negative MAP-2-positive neurons, four sections 80 μm apart at the level of anterior commissure were chosen from each animal. Seven randomly chosen nonoverlapping high-power fields (400×) from the striatum were examined from each section ($n = 3$ for each reperfusion time points at 9, 18, 48, and 72 hr as well as for sham-operated controls). All MAP-2-positive neurons in each high-power field were counted, and the number of MAP-2 positive that were p16^{INK4} negative was recorded. TUNEL-positive cells were counted in a single section at the same level in seven randomly chosen, nonoverlapping high-power fields ($n = 3$ for each time point). The cells were classified as TUNEL positive only when they showed strong nuclear signal with condensed nuclei with clumped chromatin without cytoplasmic staining (see Fig. 1*B*, *arrowheads*). Colocalization of TUNEL with BrdU was examined 72 hr after 30 min middle cerebral artery occlusion (MCAo)/reperfusion ($n = 4$) in three randomly chosen sections at the level of anterior commissure, whereby 12 high-power fields within each ischemic striatum were examined. The proportion of TUNEL and BrdU double-labeled cells was calculated.

Electron microscopy. The mice were transcardially perfused with 0.1% glutaraldehyde/2% paraformaldehyde. After overnight post-fixation, 60-μm-thick Vibratome sections were processed for BrdU immunohistochemistry using ABC kit (Vector) and DAB (Sigma) as a chromagen.

The sections were post-fixed in 2.5% glutaraldehyde in PBS and 1% osmium tetroxide, rinsed in 0.1 M sodium acetate, and stained with 2% uranyl acetate for 1 hr. The sections were dehydrated in ethanol and embedded in resin. Ultrathin sections were examined in a Zeiss EM10 electron microscope.

Immunoblots. Proteins were denatured by boiling in 30 μl sample buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% w/v DTT, 2% w/v SDS, and 0.01% w/v bromophenol blue) for 3 min. Samples (30 μl) were subjected to 10% SDS-PAGE, and the dried gels were subjected to autoradiography. Cells were lysed in 130 μl RIPA buffer (10 mM Na_2HPO_4, pH 7.0, 300 mM NaCl, 0.1% w/v SDS, 1% v/v NP40, 1% w/v Na-deoxycholate, 2 mM EDTA, 1 mM DTT, and protease/phosphatase inhibitors as described) for 30 min on ice. Proteins were denatured by boiling in 30 μl sample buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% w/v DTT, 2% w/v SDS, and 0.01% w/v bromophenol blue) for 3 min. Samples (15 μl) were electrophoretically separated, transferred to polyvinylidene difluoride membranes and blocked, and primary antibodies (0.2–1.0 μg/ml) were incubated overnight at 4°C on a rotary platform with gentle agitation. They were subsequently probed with secondary HRP-conjugated anti-mouse or anti-rabbit IgG antibodies (diluted 1:5000; Amersham Pharmacia Biotech, Braunschweig, Germany). Equal loading was confirmed by resolving 20 μg total protein by SDS-PAGE and probing with anti-actin antibody (1:2000). Detection was performed using the enhanced chemiluminescence assay (Amersham). To provide semiquantitative analysis of band intensity, band densitometry was determined from scanned images of nonsaturated immunoblot films, using Scion Image, version Beta 4.0.2 software (Scion Corporation, Frederick, MD). To compare at least three different experiments, for each protein and brain region, pixel intensities of the bands obtained in each experiment were added and set as 100%. The individual band was calculated as percentage of total signals.

Histone kinase assays. Anti-Cdk2 immunocomplexes were washed twice in lysis buffer and once with ice-cold kinase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1.0 mM DTT) and resuspended in 50 μl kinase buffer supplemented with 10 μg lysine-rich histone HIIIS (Sigma), 10 μCi [γ-^{32}P]ATP (111 MBq/mmol; NEN, Boston, MA). Control reactions were run in parallel with the omission of antibody. After incubation for 60 min at 37°C with continuous agitation, the reaction was terminated by addition of 25 μl SDS sample buffer. Boiled samples (30 μl) were separated by 15% SDS-PAGE and Coomassie stained for evaluation of equal protein loading, and the amount of incorporated radioactive label was quantified using a phosphorimager and TINA software (Raytest).

Statistical evaluation. Data are shown as mean ± SEM. To avoid possible variations of the cell cultures depending on the quality of dissection and seeding procedures, data were pooled from at least three representative experiments. For statistical analyses, one-way ANOVA was followed by Tukey's *post hoc* test. $p < 0.05$ was considered statistically significant.

RESULTS

Mild ischemia leads to delayed neuronal death

After 30 min MCA occlusion and reperfusion in 129/SV mice, cell death was prominent in the striatum and spared the cortex (Fig. 1*A*). TUNEL staining first appeared in the striatum at 18 hr, increased by 36 hr, and peaked by 72 hr (Fig. 1*B*). When double labeling for TUNEL and cell-type specific markers was performed at the 72 hr time point, almost 100% of the TUNEL-positive cells were also NeuN positive (Fig. 1*C–E*). However, not any of the TUNEL-positive cells were GFAP positive (Fig. 1*F–H*). Also, there was no MAC-1/TUNEL double labeling detected, with the exception of some cells that most likely represent engulfed nuclei of dead neurons (Fig. 1*I–K*). Interestingly, TUNEL and MAP-2 double labeling was not detected at 72 hr. MAP-2, a neuron-specific cytoskeletal marker, was profoundly downregulated in the ischemic region at the 72 hr time point (Fig. 1*A*); it is known to be extremely sensitive to ischemia (Li et al., 1998; Endres et al., 1999). MAP-2-positive cells were TUNEL negative and morphologically intact. These cells most likely represent neurons that survive the ischemic insult. We have reported previously that only ~15% of the neurons, identified as such by morphological criteria, survived at 72 hr, and this percentage did not change significantly at 21 d (Endres et al., 1998b, 2000; Fink et al., 1998).

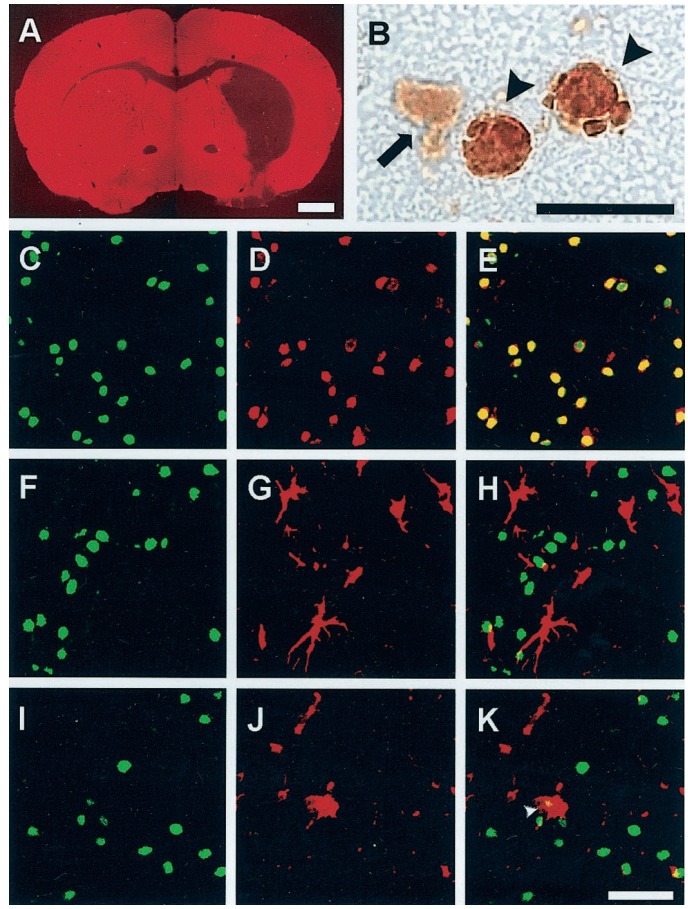

Figure 1. Selective neuronal death in the mouse striatum 72 hr after an episode of 30 min MCAo and reperfusion. The low-power view of MAP-2 immunostaining indicates striatal lesion and cortical sparing (*A*). At higher magnification (*B*), TUNEL-positive cells (*arrowheads*) show in DAB staining condensed nuclei with clumped chromatin. Cells showing weak diffuse DAB-positive cytoplasmic staining are not considered TUNEL positive (*arrow*). Sections were double stained for TUNEL (*C*, *F*, *I*) and cell type-specific markers NeuN (*D*), GFAP (*G*), or MAC-1 (*J*) and examined in a confocal microscope. Cell-specific labeling was visualized using antibodies conjugated with fluorescein (*C*, *F*, *I*: *green*) or Texas Red (*D*, *G*, *J: red*). No double labeling was detected for TUNEL and the astrocytic marker, GFAP (*H*). Moreover, there was no double labeling for TUNEL and the microglial marker, MAC-1 (*K*), with the exception of some cells that most likely represent engulfed nuclei of dead neurons (*K*, *arrowhead*). By contrast, most of the TUNEL-positive cells were also immunoreactive for the neuronal marker NeuN (*E*), indicating neuronal origin of the TUNEL-positive cells. Scale bars: *A*, 1 mm; *B*, 10 μm; *C–K*, 30 μm.

Loss of p16^{INK4a} and p27^{Kip1} after ischemia/reperfusion

We analyzed cellular localization of p16^{INK4a} and p27^{Kip1}, endogenous Cdk inhibitors, in the striata of normal mice *in vivo*. We detected strong immunoreactivity for the Cdk4 inhibitor p16^{INK4a}, a member of the INK4 family, in striatal neurons. In fact, p16^{INK4a}/NeuN double-labeling experiments confirmed that all striatal neuronal nuclei expressed p16^{INK4a} (Fig. 2*A–C*). No staining was obtained with antibodies against p27^{Kip1}, a member of the CIP/KIP family that inhibits a wide range of Cdks (data not shown). By contrast, cortical neurons in culture demonstrated

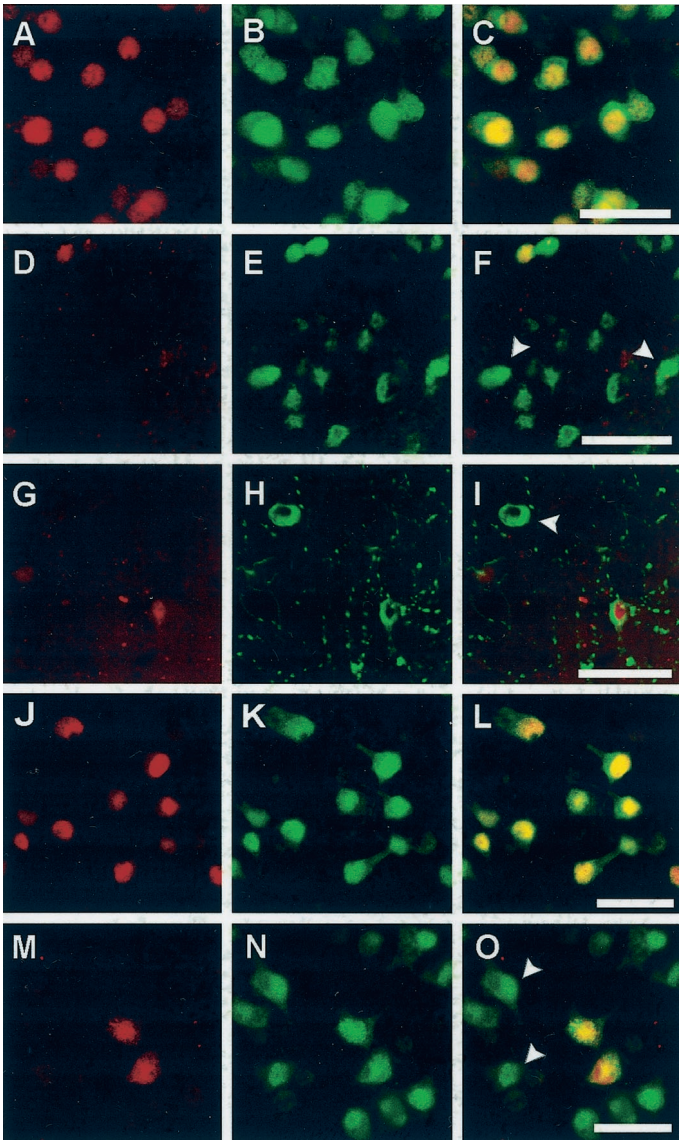

Figure 2. Expression of $p16^{INK4a}$ and $p27^{Kip1}$, endogenous inhibitors of cyclin-dependent kinases, after 30 min MCAo and reperfusion in the mouse striatum (*A–I*, $p16^{INK4a}$) and after OGD in rat primary cortical neurons (*J–O*, $p27^{Kip1}$). Immunoreactivity for $p16^{INK4a}$/$p27^{Kip1}$ was visualized with Texas Red (*A*, *D*, *G*, *J*, *M*: *red*), and neuronal marker NeuN was visualized with Alexa 488 (*B*, *E*, *H*, *K*, *N*: *green*), with colocalization resulting in a *yellow* color (*C*, *F*, *I*, *L*, *O*). Strong nuclear expression of $p16^{INK4a}$ was seen in all neurons in the normal (non-ischemic) striatum as shown by double labeling with $p16^{INK4a}$ and NeuN (*A–C*). $p16^{INK4a}$ immunoreactivity was lost in ischemic striatal neurons at 9 hr after MCAo/reperfusion as shown by the appearance of NeuN-positive p16-negative cells (*D–F*, *arrowheads*). Double labeling of $p16^{INK4a}$ with the ischemia-sensitive neuronal marker MAP-2 demonstrated that the loss of $p16^{INK4a}$ expression occurred in cytoarchitectonically intact neurons at 9 hr (*G–I*; *arrowhead* in *I*). Strong nuclear $p27^{Kip1}$ immunoreactivity was detected in all neurons in primary neuronal culture (*J–L*). Two hours after OGD the majority of neurons downregulated $p27^{Kip1}$, as indicated by *arrowheads* (*M–O*). Scale bars, 30 μm.

nuclear staining for both $p16^{INK4a}$ and $p27^{Kip1}$. $p27^{Kip1}$/NeuN double labeling confirmed strong nuclear expression of $p27^{Kip1}$ in cultured neurons (Fig. 2*J–L*), whereas $p16^{INK4a}$ immunoreactivity was weaker (data not shown). Expression of $p16^{INK4a}$ and $p27^{Kip1}$ protein was confirmed by immunoblot analysis.

After cerebral ischemia there was a profound and early down-

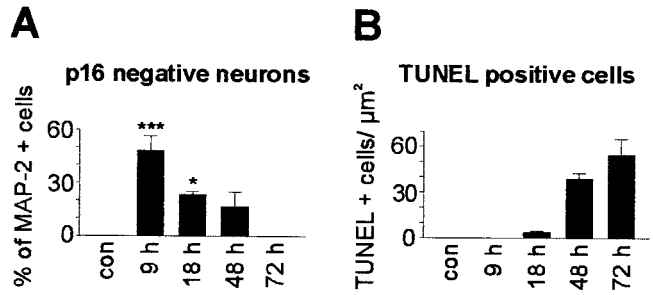

Figure 3. Time course of $p16^{INK4a}$ downregulation and delayed neuronal death after 30 min MCAo. *A*, The number of $p16^{INK4a}$-negative and MAP-2 positive cells is presented as a percentage of all MAP-2-positive cells at 9, 18, 48, and 72 hr after MCAo as well as in sham-operated, control mice. In controls and at 72 hr after MCAo, all MAP-2-positive cells were also $p16^{INK4a}$ positive. *B*, The number of TUNEL-positive cells per square millimeters was determined at the same time points as in *A*. No TUNEL positivity was observed in sham-operated animals and at 9 hr after MCAo, whereas the maximum number of TUNEL-positive cells was observed at 72 hr after MCAo; $n = 3–6$. Data are mean values ± SEM. $^*p < 0.05$; $^{***}p < 0.001$.

regulation of endogenous Cdk inhibitors. As early as 9 hr after 30 min MCAo/reperfusion, $p16^{INK4a}$ was downregulated in the ischemic striatum (Fig. 2*D–F*). This early downregulation did not simply reflect cell death or cytoskeletal disintegration because $p16^{INK4a}$-negative neurons were strongly MAP-2 positive and intact by morphological criteria at 9 and 18 hr (Fig. 2*G–I*). We analyzed the temporal and spatial relationship between $p16^{INK4a}$ downregulation, MAP-2 staining, and markers of cell death. At 9 hr, when no TUNEL-positive cells were detected in the ischemic striatum, 48.0 ± 8.3% of all MAP-2-positive cells were $p16^{INK4a}$ negative. Thus, some MAP-2-expressing, apparently intact neurons downregulated $p16^{INK4a}$ well before TUNEL labeling became apparent. Concomitant with the increase of TUNEL-positive cells, the amount of p16-negative and morphologically intact neurons decreased (23.3 ± 1.8% at 18 hr vs 16.7 ± 8.3% at 48 hr) (Fig. 3). At 72 hr all remaining morphologically intact MAP-2-positive neurons were $p16^{INK4a}$ positive. Moreover, TUNEL and $p16^{INK4a}$ double-labeled cells were not detected at any time point. Thus, $p16^{INK4a}$ downregulation preceded MAP-2 downregulation and neuronal death.

Similar to the early downregulation of $p16^{INK4a}$ *in vivo*, $p16^{INK4a}$ and $p27^{Kip1}$ were downregulated in cultured cortical neurons as early as 2 hr after OGD, as shown by immunohistochemistry (Fig. 2*M–O*) and immunoblots ($p16^{INK4a}$: 44.9 ± 9.9% decrease at 4 hr, $p < 0.001$; $p27^{Kip1}$: 34.6 ± 9.0 and 57.2 ± 6.5% decrease at 2 and 4 hr, $p < 0.05$ and $p < 0.001$, respectively) (Fig. 4).

When our *in vivo* and *in vitro* data are taken together, endogenous Cdk inhibitors such as $p16^{INK4a}$ or $p27^{Kip1}$ appear to be reliable markers of neuronal survival and their loss a reliable indicator of neuronal death after cerebral ischemia.

Cell cycle protein expression after ischemia/hypoxia

Next, we investigated the expression of the G_1 phase cyclin, cyclin D1, in striatal neurons *in vivo* after focal ischemia and in neocortical neurons *in vitro* after OGD. We detected no cyclin D1 immunoreactivity in striatal neurons from sham-operated controls (data not shown), whereas cortical neurons in control cultures expressed cyclin D1 in the cytosol (Fig. 5*A–C*). Cyclin D1 is thought to be inactive in the cytosol and requires nuclear translocation to activate Cdks (Yang and Kornbluth, 1999). Indeed,

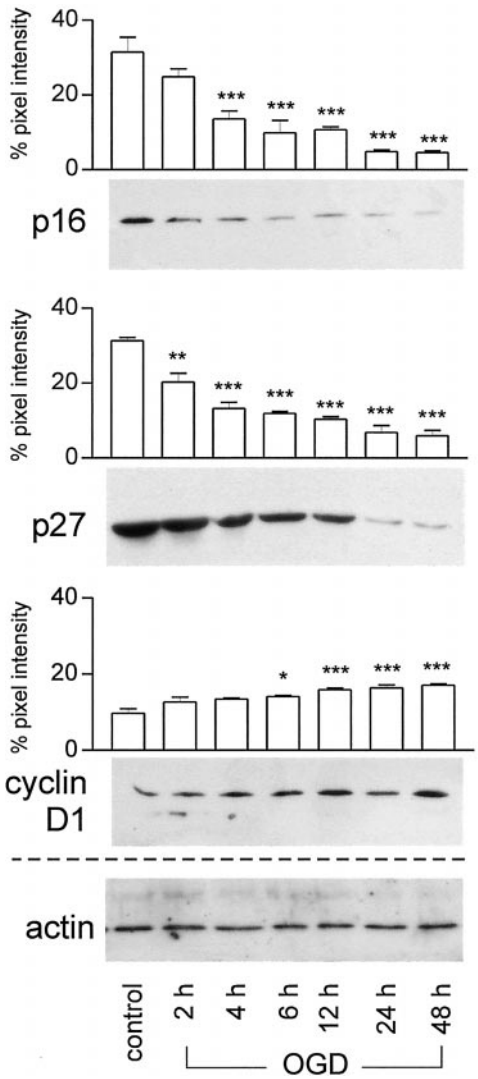

Figure 4. Immunoblots showing time-dependent changes of cell cycle-related proteins in primary cortical neurons after 90 min oxygen–glucose deprivation. Cell lysates (20 μg) were subjected to SDS-PAGE, and membranes were probed with antibodies against $p16^{INK4a}$, $p27^{Kip1}$, and cyclin D1 (0.2–1.0 μg/ml). Actin served as internal control. The experiment was repeated three times; a representative experiment is shown. For semiquantitative analysis, the intensity of each band was quantitated from scanned images of nonsaturated immunoblot films using Scion Image (Scion Corporation, Frederick, MD). The pixel intensity of the bands obtained in each experiment was summed and set as 100%, and the individual band was calculated as percentage of total signals. The graphs show a significant downregulation of $p27^{Kip1}$ starting at 2 hr and of $p16^{INK4a}$ at 4 hr after OGD compared with controls. Cyclin D1 levels were significantly upregulated at 6 hr and further increased at 48 hr. Mean value ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

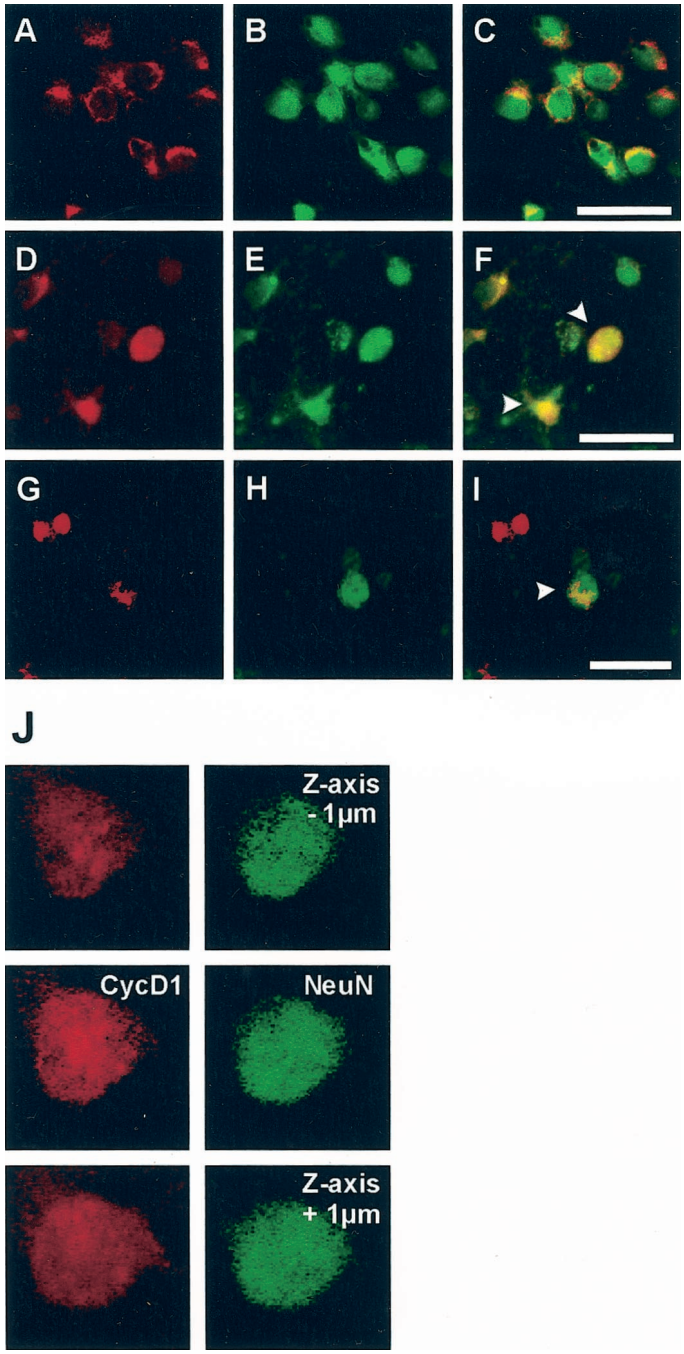

Figure 5. Cyclin D1 expression in primary cortical neurons in culture after OGD (*A–F*) and in striatal neurons of mice after MCAo/reperfusion (*G–I*). Immunoreactivity for cyclin D1 was visualized with Texas Red (*A*, *D*, *G*; *red*), and neuronal marker NeuN was visualized with Alexa 488 (*B*, *E*, *H*; *green*). Double labeling for cyclin D1 and NeuN demonstrates that cyclin D1 is expressed exclusively in the cytoplasm of cultured neurons under control conditions (*A–C*). Two hours after OGD cyclin D1 is strongly upregulated, and its immunoreactivity translocates to the nucleus (*D–F*, *arrowhead*). Confocal Z-series images (step = 1 μm) confirm the nuclear expression of cyclin D1 in OGD-treated neurons (*J*). Cyclin D1 is not expressed in normal striatal neurons (data not shown). Forty-eight hours after MCAo/reperfusion, nuclear cyclin D1 immunoreactivity is detected in neurons in the ischemic striatum (*G–I*, *arrowhead*). Scale bars: *A–I*, 30 μm.

after cerebral ischemia *in vivo* and OGD *in vitro*, we detected nuclear expression and an increase in immunoreactivity of cyclin D1 (Fig. 5*D–I*). Z-series confocal images confirmed nuclear expression of cyclin D1 (Fig. 5*J*). Immunoblot analyses confirmed the increase in cyclin D1 *in vitro* detected by immunohistochemistry (150.8 ± 21.0% at 6 hr, $p < 0.05$) (Fig. 4). As expected, some glial cells exhibited strong cyclin D1 immunoreactivity *in vivo*.

Cyclin D1 activates Cdk4, which in turn phosphorylates the retinoblastoma protein in mid G_1 phase, followed by the activation of Cdk2 around the G_1–S transition. We performed histone kinase assays to determine Cdk2 activity in cultured cortical

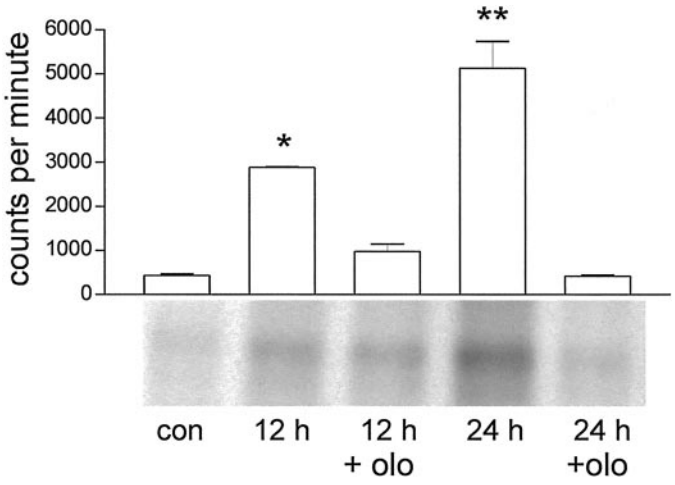

Figure 6. Cdk2 activity increases in cortical neurons in culture after 90 min OGD. Cell lysates were immunoprecipitated with anti-Cdk2 antibody, and the resultant complexes were allowed to incubate with [γ-^{32}P]ATP and histone HIIIS as substrate. In each lane, 30 μg protein was separated. There was a clear increase in kinase activity at 12 and 24 hr after OGD as compared with control. This kinase activation at 12 and 24 hr was completely abolished when the cells were pretreated with 10 μM olomoucine (*olo*) 1 hr before and during OGD. To estimate Cdk2 activity, the amount of incorporated radioactive label was quantified using a phosphorimager and TINA software. The values of three independent experiments are graphically represented as mean value ± SEM. *$p < 0.01$; **$p < 0.001$ versus control.

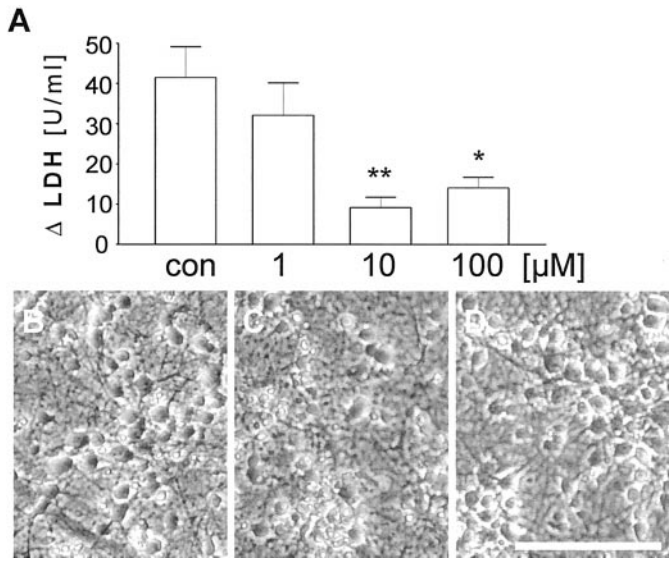

Figure 7. The Cdk inhibitor olomoucine protects primary neuronal cultures subjected to OGD. *A*, Quantitative assessment of neuronal injury by measurement of LDH release 24 hr after OGD presented as the difference between control cultures and cultures subjected to OGD. Olomoucine treatment produced significant protection at 10 and 100 μM, whereas higher concentrations were toxic to the cultures (data not shown). The results are presented as the mean value ± SEM from three independent experiments performed in triplicate. *$p < 0.05$ and **$p < 0.01$ versus vehicle-pretreated sister cultures exposed to OGD; one-way ANOVA followed by Tukey's *post hoc* test. *B*, Phase-contrast micrograph of primary cortical neurons in culture. *C*, Twenty-four hours after 90 min OGD and pretreatment with vehicle (0.02% DMSO). *D*, Cultures exposed to the same insult as in *C* but pretreated with olomoucine (10 μM). Scale bar, 70 μm.

neurons after OGD to determine whether the molecular machinery necessary for G_1–S transition was being mobilized (Fig. 6). Cdk2 activity increased at 12 hr (658.3 ± 4.3% increase, $p < 0.05$) and peaked at 24 hr (1171.0 ± 137.5% increase, $p < 0.01$). When cells were pretreated with olomoucine (1–100 μM), a synthetic Cdk inhibitor, Cdk activation at 12 and 24 hr was completely abolished (Fig. 6). Hence, we demonstrate that Cdks are activated in neurons after ischemia/hypoxia, and olomoucine administration effectively inhibits Cdk activation in this model. Immunoblot experiments showed that protein levels of Cdk4 and Cdk2 did not change significantly over time (data not shown).

Inhibition of Cdk activity protects from oxygen–glucose deprivation

Olomoucine is a purine derivative that inhibits Cdks 1, 2, and 5 and ERK1/MAP-kinase and blocks G_1–S-phase transition (Vesely et al., 1994; Abraham et al., 1995). Cultured cortical neurons were pretreated with olomoucine (1, 10, or 100 μM) before OGD. Cell damage after OGD was determined by quantifying LDH release into the culture medium. Olomoucine pretreatment significantly protected neurons from OGD and reduced LDH release at 24 hr (Fig. 7*A*). The strongest protection was achieved with 10 μM concentration (~75% reduction in LDH release; 41.5 ± 7.6 vs 9.2 ± 2.6 U/ml medium; $n = 24$ in three independent experiments; $p = 0.004$). The decrease in LDH release (Fig. 7*A*) correlated with higher numbers of viable neurons at 72 hr as assessed by phase-contrast microscopy (Fig. 7*B–D*). Thus, inhibition of Cdk activity protects cultured cortical neurons against OGD.

Progression to S-phase is a rare event

We used BrdU as a marker for DNA synthesis (Nowakowski et al., 1989) to determine whether downregulation of Cdk inhibitors and upregulation of cyclin D1 caused striatal neurons to enter S-phase after ischemic damage *in vivo*. After 30 min MCAo and reperfusion, BrdU-positive cells first appeared in the ischemic striatum at 36 hr. Their numbers increased at 48 and 72 hr. Most of the BrdU-labeled cells had the morphological appearance of microglia/macrophages and could be labeled with antibodies against MAC-1. A small number of NeuN-labeled cells were BrdU positive at 72 hr (<1%; data not shown). In fact, electron microscopy confirmed that some BrdU-positive cells were indeed neurons (Fig. 8*A*). A small fraction of TUNEL-positive cells was BrdU positive (0.957 ± 0.172% of all TUNEL-positive cells; 72 hr; $n = 4$ animals), indicating that some of the TUNEL-positive cells had entered S-phase (Fig. 8*B–G*). As demonstrated in Figure 1, virtually all TUNEL-positive cells are NeuN positive at 72 hr. Together, these data suggest that some neurons indeed entered S-phase before their death.

We were not able to detect any mitotic figure, however, either *in vivo* or *in vitro*, although the presence of mitotic figures has been reported after a hypoxic insult *in vitro* (Bossenmeyer-Puorié et al., 1999). It appears that although some neurons enter S-phase, they do not proceed beyond the G_2–M checkpoint within 72 hr after the insult.

DISCUSSION

We provide evidence that cell cycle protein expression is altered in postmitotic neurons in response to focal cerebral ischemia. The earliest event is the downregulation of the Cdk inhibitors $p16^{INK4a}$ and $p27^{Kip1}$ followed by upregulation and nuclear expression of cyclin D1 and activation of Cdks. We consider these events as "attempts" at cell cycle reentry. Because none of the neurons enters M-phase within the 72 hr period, we conclude that

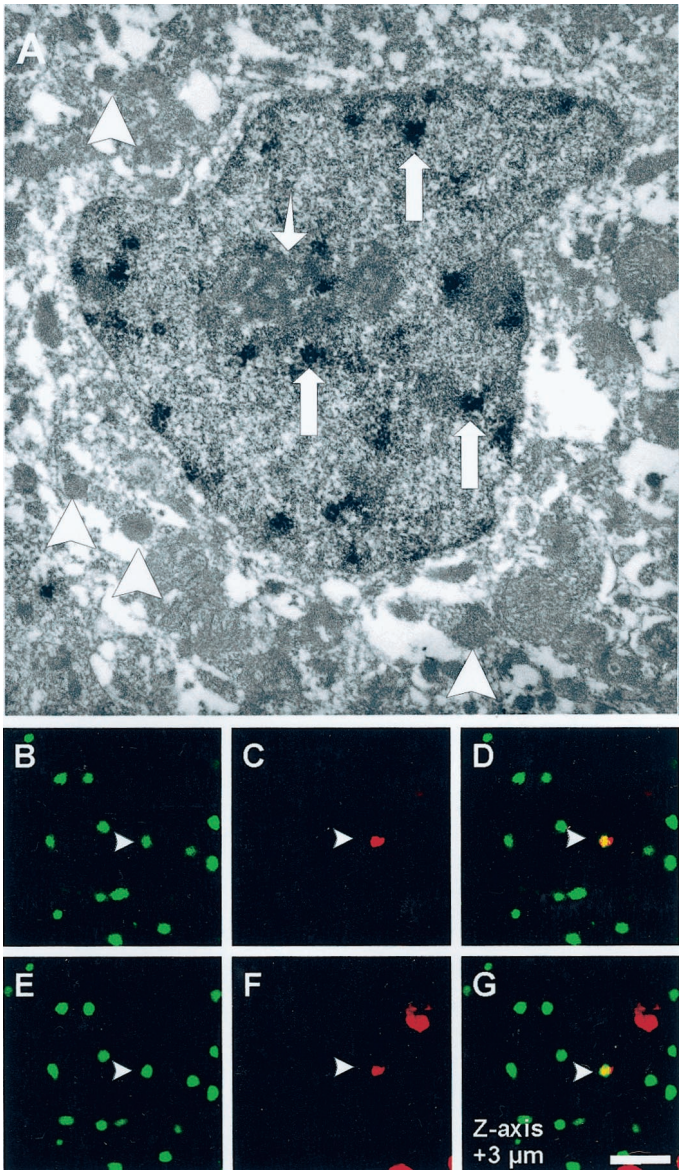

Figure 8. TUNEL/BrdU double labeling. BrdU was administered via subcutaneous osmotic mini-pumps in 129/SV mice (1 $mg \cdot hr^{-1} \cdot kg^{-1}$ body weight). Mice were subjected to 30 min of MCAo and 72 hr reperfusion. *A*, For electron microscopy studies, Vibratome sections of ischemic striatum were immunostained for BrdU using DAB as a chromagen. Electron micrograph shows a cell with numerous electron-dense osmiophilic granules (*large arrows*) within the nucleus corresponding to the BrdU labeling. The cell has a central nucleus with a prominent nucleolus (*small arrow*) and multiple vesicles (*arrowheads*) in its cytoplasm and lacks glial filaments, indicating that it is of neuronal origin. Original magnification: 11,000×. TUNEL/BrdU double labeling was performed on fresh-frozen cryosections (10 μm) using ApopTag Kit and rat monoclonal anti-BrdU antibody. TUNEL was visualized with fluorescein (*B*, *E*: *green*), and Texas Red was used for BrdU immunoreactivity (*C*, *F*: *red*). Of all TUNEL-positive cells, 0.957 ± 0.172% stain positive for the S-phase marker BrdU ($n = 4$ animals). Z-series of confocal images through the nucleus (1 μm steps) confirms the costaining for both markers (*D*, *G*: *yellow*). Scale bar, 30 μm.

these attempts fail. These findings show that neurons attempt cell cycle reentry after cerebral ischemia, most likely because of loss of endogenous Cdk inhibitors, and that the loss of Cdk inhibitors is a preamble for neuronal death rather than for cell division or survival, or both.

Loss of endogenous cell cycle inhibitors may be an early trigger for cell cycle activation

$P16^{INK4a}$ and $p27^{Kip1}$ induce cell cycle arrest by inhibiting Cdk activity (Johnson and Walker, 1999; Vidal and Koff, 2000), and they promote cell cycle exit during development (Zindy et al., 1997; Watanabe et al., 1998). We show that virtually all neurons in the striatum express $p16^{INK4a}$ and that all postmitotic neurons in cortical cultures express $p27^{Kip1}$ in the nucleus. Nuclear expression of $p16^{INK4a}$ *in vivo* and $p27^{Kip1}$ *in vitro*, but not vice versa, may relate to differences in cell maturation. The *in vivo* studies were performed on 6-week-old mice, whereas the neurons *in vitro* were obtained from embryonic day 17 rats and cultured for up to 14 d. $p27^{Kip1}$ is the predominant Cdk inhibitor and the only Cdk inhibitor to decrease 5 hr after KCl withdrawal in primary cultures of cerebellar granule neurons at 5–6 d *in vitro* (Padmanabhan et al., 1999). By contrast, increased levels of $p16^{INK4a}$ are associated with cell senescence (Huschtscha and Reddel, 1999), and its expression may relate to a more mature state.

After an ischemic insult, $p16^{INK4a}$ and $p27^{Kip1}$ were profoundly downregulated. We propose that the downregulation acts as an early trigger of attempted cell cycle reentry and subsequent neuronal death. The loss of $p16^{INK4a}$ is a specific event and not related to general protein degradation, because at early time points, $p16^{INK4a}$-negative neurons are morphologically intact and express MAP-2. Most neurons downregulate $p16^{INK4a}$ between 9 and 18 hr after ischemia (Figs. 3, 4). Later on, $p16^{INK4a}$-negative neurons undergo cytoskeletal disintegration and become TUNEL positive, whereas virtually every neuron that maintains high levels of $p16^{INK4a}$ remains viable at least for 72 hr. Hence, $p16^{INK4a}$ may be a survival factor, and its early downregulation may predict neuronal death. Similarly, Sindbis virus-induced expression of $p16^{INK4a}$/$p27^{Kip1}$ protected sympathetic and cortical neurons from death induced by DNA-damaging compounds (Park et al., 1998).

Our *in vitro* evidence showed unequivocally that the loss of $p27^{Kip1}$ was paralleled by nuclear expression of cyclin D1, closely followed by Cdk2 activation, which is thought to be crucial for G_1–S transition (Sherr, 1994). Some ischemic neurons progressed to S-phase in our *in vivo* model (see below). These data are in agreement with several studies on brain tumors showing that the loss of endogenous Cdk inhibitors is sufficient to precipitate uncontrolled cell proliferation (Nishikawa et al., 1995; Ueki et al., 1996). Similarly, expression of Cdk inhibitors was investigated in Alzheimer's disease and in support of the hypothesis that an aborted attempt to activate the cell cycle in terminally differentiated neurons might be a critical event in the pathomechanism of Alzheimer's disease (Arendt et al., 1996, 1998; McShea et al., 1997, 1999; Nagy et al., 1997).

Cell cycle protein expression and delayed neuronal death

We demonstrate upregulation and, more importantly, nuclear expression of cyclin D1 in neurons after ischemia/hypoxia, extending previous reports (Freeman et al., 1994; Kranenburg et al., 1996; Li et al., 1997, 1998; McShea et al., 1997; Nagy et al., 1998; Timsit et al., 1999). Nuclear expression of cyclin D1 was followed by Cdk activation. Inhibition of Cdk activation protected neurons from death, a finding that is in accordance with studies that exposed cultured neurons to various insults, including DNA damage, trophic factor deprivation, and β-amyloid toxicity (Park et al., 1997a; Bossenmeyer-Puorié et al., 1999; Stefanis et al., 1999). In preliminary experiments, we also detected Cdk4 expression in

striatal neurons *in vivo*. Although we did not study the effects of olomoucine *in vivo*, a recent study convincingly demonstrated that Cdk inhibitors protect neurons from death after focal cerebral ischemia (Osuga et al., 2000).

The mechanisms that cause cell death after Cdk activation have remained elusive. They may relate to release of cytochrome *c* and activation of caspase 9 and eventually caspase-3 (Stefanis et al., 1999). We have demonstrated previously that activation of caspase 3 contributes to cell death after mild ischemia (Endres et al., 1998b; Fink et al., 1998) and OGD (Harms et al., 2000), and the time course of caspase activation is compatible with cell cycle events reported in this study. Another possibility is that Cdk activation might mediate cell death by converting p35 to neurotoxic p25 (Patrick et al., 1999)

S-phase progression after cerebral ischemia

The expression of cell cycle-related proteins after ischemia may signify their function in the apoptotic machinery rather than in the cell cycle (Heintz, 1993; Padmanabhan et al., 1999; Park et al., 1998; Stefanis et al., 1999). To further analyze cell cycle progression, we used BrdU as S-phase marker (Nowakowski et al., 1989; Takahashi et al., 1993; Gage et al., 1995). After ischemia some cells were double labeled with TUNEL and BrdU in our *in vivo* model. Because virtually all TUNEL cells were NeuN positive by 72 hr (Fig. 1), we postulate that the TUNEL and BrdU double-labeled cells are neurons. Indeed, we identified BrdU-positive cells as neurons by electron microscopy (Fig. 8*A*). Similarly, TUNEL/BrdU double labeling was also used in previous studies in the developing nervous system to demonstrate S-phase progression before apoptotic death (Thomaidou et al., 1997; ElShamy et al., 1998). Reentry of neurons into S-phase before cell death was a rare event: ~1% of the TUNEL-positive cells were BrdU positive. Thus, the majority of neurons died before S-phase after Cdk activation. Accordingly, in preliminary experiments we did not detect any expression of cyclin A and cyclin B in ischemic neurons, which are indicative of S–G_2 and G_2–M transition, respectively. Another caveat may be that BrdU incorporation indicated DNA repair rather than DNA synthesis. However, a significant number of TUNEL-positive cells (i.e., >99% at 72 hr) are not labeled with BrdU (which would be the case if BrdU were the marker of DNA damage) (Thomaidou et al., 1997). Moreover, other markers of proliferation such as cyclin D1 are expressed. There is good evidence in the literature from both *in vitro* as well as *in vivo* experiments that the concentration of BrdU used in this study is not sensitive enough to detect DNA repair (Gobbel et al., 1998; Parent et al., 1999; Palmer et al., 2000). Thus, we show that a small number of neurons enter S-phase before undergoing delayed cell death.

The fact that we were not able to identify mitotic figures argues against the possibility that BrdU labeled-neurons were newly born during the 72 hr period (Gu et al., 2000). Furthermore, it is unlikely that 72 hr would be a sufficient interval for a progenitor cell to give rise to a mature neuron (Kuhn et al., 1996; Palmer et al., 2000). Moreover, we were not able to detect any obvious migration of BrdU-positive cells from the subventricular zone of neuronal progenitor cells to the ischemic striatum by 72 hr.

In conclusion, we show that endogenous Cdk inhibitors are constitutively expressed in quiescent neurons but that they are downregulated early after cerebral ischemia/hypoxia. We show that the loss of Cdk inhibitors may be the trigger for cell destruction. The downstream events leading to delayed neuronal death after Cdk activation remain to be determined.

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