Interactions Between p38 Mitogen-Activated Protein Kinase and Caspase-3 in Cerebral Endothelial Cell Death After Hypoxia-Reoxygenation

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Background and Purpose—The emerging concept of the neurovascular unit in stroke reemphasizes the need to focus on endothelial responses in brain. In this study we examined the role of mitogen-activated protein (MAP) kinase signaling in the regulation of hypoxic cell death in cerebral endothelial cells.

Methods—Human cerebral microvascular endothelial cells were exposed to 4 to 12 hours of hypoxia followed by 12 to 24 hours of reoxygenation. Cytotoxicity was measured by quantifying lactate dehydrogenase release. DNA laddering and caspase-3 activity were assessed to document a role for caspase-dependent cell death. zVAD-fmk and zDEVD-fmk were used to inhibit caspases. Activation of extracellular signal–regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) was assessed with Western blotting and kinase activity assays. U0126, SB203580, and SP600125 were used to interrupt the ERK, p38, and JNK pathways, respectively.

Results—Endothelial cell death occurred primarily during reoxygenation. DNA laddering and caspase activation were observed, and cytotoxicity was ameliorated by caspase inhibitors (20 \( \mu \)M of zVAD-fmk or zDEVD-fmk). Among the 3 major MAP kinases, only p38 was transiently activated during reoxygenation, and inhibition with 10 \( \mu \)M of SB203580 significantly reduced cytotoxicity. No effects were observed with other MAP kinase inhibitors. Cytoprotection with SB203580 was not accompanied by caspase downregulation. In contrast, cytoprotection with zVAD-fmk was associated with a decrease in p38 activation. Furthermore, cleavage of MEKK1 (an upstream kinase of p38) was significantly reduced by zVAD-fmk.

Conclusions—Cerebral endothelial cell death after hypoxia-reoxygenation is mediated by interactions between caspases and p38 MAP kinase. Surprisingly, p38 pathways lie downstream of caspase mechanisms in this model system. (Stroke. 2003;34:2704-2709.)

Key Words: apoptosis \textbullet; blood-brain barrier \textbullet; cerebral ischemia \textbullet; cerebrovascular disorders \textbullet; signal transduction

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A t the recent Stroke Progress Review convened by the National Institute of Neurological Disorders and Stroke, the neurovascular unit was proposed as a conceptual framework for stroke research.\textsuperscript{1} This concept emphasizes the fact that although neuronal and glial cell death ultimately mediate brain tissue injury, proximal interactions with endothelial dysfunction play a critical role. A recent study in a primate model of focal cerebral ischemia demonstrated that regional neuronal injury was statistically correlated with local loss of microvascular integrity,\textsuperscript{2} suggesting an important link between vascular and parenchymal compartments in maintaining overall tissue homeostasis. Presumably, stroke disrupts these homeostatic interactions, thus triggering cascades of cell death in brain.\textsuperscript{3} Hence, a focus on cerebral endothelial cells as primary targets seems justified.

Previous investigations have suggested that apoptotic-like pathways exist in nonbrain endothelium.\textsuperscript{4–6} However, in contrast to the neuronal literature, studies of similar cell death pathways in cerebral endothelial cells are relatively few. In cerebral endothelial cultures, oxidative stress can trigger caspase-mediated pathways of cellular demise.\textsuperscript{7–9} Because oxidative stress plays a critical role in ischemia and reperfusion, it is likely that similar caspase-mediated pathways operate in the vascular compartment after stroke. Nevertheless, the signaling pathways that regulate caspase activity during cerebral endothelial cell death remain to be fully elucidated.

In mammalian cells, response to external stress and signaling is regulated by 3 major mitogen-activated protein (MAP) kinase systems: the extracellular signal–regulated kinase ERK and the 2 stress-activated protein kinases p38 and c-Jun N-terminal kinase (JNK).\textsuperscript{10–12} In this study we examined the role of ERK, p38, and JNK in primary human cerebral endothelial cells after hypoxia-reoxygenation. Cell death in
this model was found to be caspase dependent, and biochemical and pharmacological inhibition experiments demonstrated that p38 activation was deleterious during reoxygenation. Surprisingly, the data also suggested that downstream caspases were not regulated by p38 but instead that caspases act upstream of the p38 pathway via cleavage and activation of the MAP kinase kinase kinase MEKK1.

Materials and Methods

Cell Culture
Human cerebral microvascular endothelial cells were purchased from Cell Systems Corporation. Cells (passage 4 to 9) were grown on dishes coated with attachment factor in complete medium.

Hypoxia-Reoxygenation
Cells were replaced with serum-free medium for 18 hours followed by hypoxia induced with a modular chamber (Billups-Rothenberg) perfused with 90% N2/5% CO2/5% H2. The chamber was sealed and placed at 37°C for indicated time periods for hypoxia. After hypoxia, cells were removed from the chamber and maintained in the regular incubator for reoxygenation periods. Control cultures were incubated under normoxic conditions for equivalent durations.

Western Blot Analysis
Immunoblotting was performed according to previous protocols. Cells were lysed with lysis buffer (Cell Signaling), and protein concentration was determined with the Bradford assay (Bio-Rad). Equal amounts of proteins were separated on sodium dodecyl sulfate–polyacrylamide gels and transferred onto polyvinylidene fluoride. The membranes were probed with antibodies against phospho-ERK1/2, phospho-p38, phospho-SAPK/JNK, ERK1/2, SAPK/JNK, cleaved caspase-3 antibody (all from Cell Signaling Technology), and p38 (Promega). Immune complexes were visualized by enhanced chemiluminescence (Amersham).

Kinase Assay
p38 activation was assayed with a p38 kinase assay kit (Cell Signaling Technology). Briefly, cell lysates containing 250 μg protein were immunoprecipitated with phospho-p38 antibody and protein A/G agarose beads, and the activity of p38 was evaluated with the use of exogenous ATF-2 as a substrate. Phosphorylation of ATF-2 was detected by immunoblotting.

Lactate Dehydrogenase Release Assay
Cytotoxicity was quantified by a standard measurement of lactate dehydrogenase (LDH) release with the use of the LDH assay kit (Roche). In our model, 40% LDH release after 4 hours of hypoxia and 24 hours of reoxygenation is approximately equivalent to 40% cytotoxicity.

DNA Fragmentation Assay
Genomic DNA isolation was performed with the apoptotic DNA ladder detection kit (Chemicon). The cells were incubated with Tris/EDTA buffer solution containing RNase A at 37°C for 10 minutes and Proteinase K at 55°C for 30 minutes. DNA was precipitated at −20°C for 2 hours with 3 mol/L ammonium acetate, and the DNA ladder was visualized under UV light with ethidium bromide staining.

Measurement of Caspase-3 Activity
Caspase-3 activity was measured with the use of ApoAlert kit (Clontech). Lysed cells were incubated in reaction buffer with

Figure 1. Cytotoxic effects of hypoxia-reoxygenation in cerebral endothelial cells. Similar levels of LDH release (A) were obtained after varying periods of hypoxia (mean±SD; n=3 experiments per time point). Cells were exposed to normoxia (N) or 4 to 12 hours of hypoxia (H) followed by 12 to 24 hours of reoxygenation (R). B, After 4 hours of hypoxia, LDH release progressively increased during reoxygenation (mean±SD; n=4 experiments per time point). C, DNA electrophoresis showing DNA laddering after 4 hours of hypoxia and 24 hours of reoxygenation.

Statistical Analysis
Quantitative data were analyzed with ANOVA followed by Tukey honestly significant difference tests between individual groups. Data were expressed as mean±SD. P<0.05 was considered significant.

Results

Induction of Cerebral Endothelial Cell Death During Reoxygenation After Hypoxia
Varying periods of hypoxia-reoxygenation were examined to determine the temporal profile of endothelial cell death in our model system. Cells that were exposed to 4 to 12 hours of hypoxia showed similar levels of LDH release (Figure 1A). In contrast, when endothelial cells were exposed to 4 hours of hypoxia followed by varying times of reoxygenation, cytotoxicity increased steadily over time (Figure 1B). Taken together, these data indicate that in this model system, endothelial cell death occurred primarily during reoxygenation. Cell death was accompanied by a change in morphology (rounding) with eventual detachment from the plate (data not shown). A DNA fragmentation assay demonstrated evidence of laddering (Figure 1C).
Involvement of Caspase-3 in Endothelial Cell Death After Hypoxia-Reoxygenation

To analyze the contribution of caspases in the death signal triggered by hypoxia-reoxygenation, the proteolytic activity of caspase-3 was assessed. Western blots showed that cleaved activated caspase-3 was increased after 4 hours of hypoxia. Although there may have been a slight decrease immediately on reoxygenation, cleaved caspase-3 levels remained elevated above baseline up to 24 hours after reoxygenation (Figure 2A). Correspondingly, cell death was significantly reduced by pretreatment with 20 μmol/L of the specific caspase-3 inhibitor zDEVD-fmk or 20 μmol/L of the wide-spectrum inhibitor zVAD-fmk (Figure 2B).

Involvement of p38 MAP Kinase in Cerebral Endothelial Cell Death After Hypoxia-Reoxygenation

To study cell death–related signaling pathways activated in cerebral endothelial cells after hypoxia-reoxygenation, 3 major MAP kinases (ERK1/2, p38 kinase, JNK) were examined by measuring levels of phosphorylated kinases on Western blots. Phosphorylation of ERK1/2 and JNK was not changed during hypoxia-reoxygenation (Figure 3A and 3B). However, p38 kinase was markedly phosphorylated during reoxygenation after 4 hours of hypoxia (Figure 4A). A p38 enzyme activity assay demonstrated elevated kinase activity (Figure 4B), consistent with the Western blot results. To further confirm the role of these MAP kinases, we compared the effects of various MAP kinase inhibitors on hypoxia-reoxygenation injury. Pretreatment with the p38 kinase inhibitor SB203580 significantly protected against cell death (Figure 4C). This endothelial protection was sustained even when SB203580 was administered in a delayed fashion, after 4 hours of hypoxia at the onset of reoxygenation (Figure 4C).

Cross Talk Between p38 MAP Kinase and Caspase-3

p38 kinase is known to act as an upstream regulator of caspase in many cell death models. To investigate the interactions between p38 and caspase-3, we examined the effect of SB203580 on active caspase-3 measured with the use of Western blot and caspase activity assays. Treatment with 20 μmol/L of zVAD-fmk decreased cleaved caspase-3 levels and caspase-3–like activity, confirming that our detection systems were working well (Figure 5A and 5B). However, cytoprotective concentrations of SB203580 (10 μmol/L) did not have a detectable effect on cleaved caspase-3 as measured by Western blot (Figure 5A) or caspase activity (Figure 5B). In contrast, cytoprotective concentrations of zVAD-fmk significantly reduced the activity of p38 kinase after hypoxia-reoxygenation (Figure 5C). MEKK1 was subsequently examined as a candidate mechanism for cross talk between p38 kinase and caspase pathways. MEKK1 is a 196-kDa upstream kinase that can activate p38. MEKK1 may be cleaved and activated by caspase-3. Western blots showed that hypoxia-reoxygenation induced cleavage of MEKK1 protein, as indicated by partial loss of full-length MEKK1 and the emergence of the cleaved active form (Figure 5D). Levels of cleaved MEKK1 were significantly reduced by 20 μmol/L of zVAD-fmk (Figure 5D). Finally, cotreatment with SB203580 plus zVAD-fmk did not yield any additional or synergistic protection compared with treatment with each inhibitor alone (Figure 5E), suggesting that independent pathways were not involved.

Discussion

Caspase-mediated cell death is regulated in part by MAP kinase cascades. The standard model suggests that ERK sustains cell viability, whereas the 2 major stress-activated protein kinases, p38 and JNK, may promote cell death. However, many exceptions to this general rule exist. For
example, although JNK is known to mediate Fas-induced apoptosis in neuronal cells, it can also be beneficial by interrupting p38 kinase and preventing cell death after tumor necrosis factor-α exposure in cardiomyocytes. Similarly, both deleterious and beneficial actions of p38 signaling have been documented. Contrasting effects have also been described for ERK. Whereas ERK activation prevented apoptosis after growth factor withdrawal in cerebellar neurons and PC12 cells, our laboratory and others have shown that ERK inhibition protected against cortical neuronal injury. Overall, the literature suggests that contributions of individual MAP kinases depend on the cell types and the nature and severity of the insult involved.

In this study we found that p38 was the dominant pathway mediating cytotoxicity in cerebral endothelial cells after hypoxia-reoxygenation, and the relatively selective p38 inhibitor SB203580 reduced cell death. In contrast, no effects were detectable when U0126 and SP600125 were used to target the ERK and JNK pathways, respectively. These data are consistent with the endothelial literature describing connections between stress-activated protein kinases (p38, JNK) and caspases. In liver endothelium, activation of Fas and tumor necrosis factor death receptors induces p38- and caspase-dependent toxicity. In human venous endothelial cells, oxidative stress and high glucose levels activate JNK and trigger downstream caspase-mediated cytotoxicity. It was somewhat surprising that we did not detect activation of JNK in our model. Cerebral endothelial cells may behave differently from nonbrain endothelial cells, or, alternatively, our detection methods may not have been sensitive enough to measure subtle changes in JNK.

In many cell death models, p38 acts upstream of caspase execution. A major finding here was that cytoprotective p38
inhibition surprisingly did not decrease caspase-3 activity. Instead, protection with caspase inhibitors decreased p38 activity and reduced cleavage of MEKK1, an upstream MAP kinase kinase kinase that activates both p38 and JNK pathways. MEKK1 is a kinase that is activated when cleaved by caspase-3. Hence, it is conceivable that in our model, caspase-3 operates upstream of p38 pathways by cleaving and activating the upstream MAP kinase kinase kinase. Nevertheless, our data cannot exclude the possibility that multiple interactions exist between p38 and caspases. Although MEKK1 is a caspase-3 substrate, constitutively active mutant MEKK1 upregulates caspase. Ultimately, it is likely that MEKK1 serves as a feedback loop that amplifies caspase-mediated cell death via p38. Because our combination inhibitor experiment showed no additional protection, independent pathways are not likely to be involved in our model.

Regardless of precisely how p38 interacts with caspase, our data suggest that caspase-independent pathways may also contribute to p38-mediated cell death because effective protection with SB203580 was not associated with caspase downregulation. In endothelial cells, the nuclear factor-κB (NF-κB) pathway is active after oxidative stress. Indeed, blocking NF-κB with molecular decoys reduces cerebral endothelial dysfunction after hypoxic or oxidative stress. Whether NF-κB may contribute to p38 pathways in our cerebral endothelial system remains to be determined.

Taken together, the findings in this study suggest that p38 may be a potential target for reducing cerebrovascular damage in stroke. However, several caveats remain. First, selective inhibition of endothelial p38 may be difficult in vivo. Further studies in animal models will be needed to extend the present in vitro data because other cell types in brain (neurons, astrocytes, oligodendrocytes) may have different MAP kinase responses. Second, although we have chosen MAP kinase inhibitor concentrations on the basis of our previous experience and the existing literature, one cannot exclude the possibility that the inhibitors selected here may have affected other kinases, especially given the extensive cross talk that exists between signaling pathways. A third caveat is the acute 24-hour time frame of this study. We may have missed delayed apoptotic responses that may be particularly critical for stroke in vivo. Further experiments investigating longer-term outcomes will be useful. Finally, it is important to note that maximal cytoprotection achieved by either caspase or p38 inhibition was only approximately 40% in this study. Clearly, other pathways of endothelial cell death operate after hypoxia-reoxygenation. One possibility may involve group I metabotropic glutamate receptors; activation of these receptors protected endothelial cells against nitric oxide–induced toxicity independently of p38. A clearer delineation of how various p38-dependent and -independent mechanisms interact may yield combination therapies that maximize efficacy.

In summary, the major findings in this study were that (1) caspases mediated endothelial cytotoxicity during reoxygenation after hypoxia; (2) only the p38 MAP kinase was activated during reoxygenation, and inhibition of p38 decreased cell death; and (3) p38 appeared to function downstream of caspase activation in this model system. These results suggest that p38 is a potential therapeutic target for cerebrovascular injury. The precise molecular pathways that possibly mediate caspase-independent actions of p38 warrant further investigation.

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