VEGF Stimulates the ERK 1/2 Signaling Pathway and Apoptosis in Cerebral Endothelial Cells After Ischemic Conditions

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Background and Purpose—Cerebral endothelial cells that line microvessels play an important role in maintaining blood flow homeostasis within the brain-forming part of the blood–brain barrier. These cells are injured by hypoxia-induced reperfusion, leading to blood–brain barrier breakdown and exacerbation of ischemic injury. We investigated the roles of vascular endothelial growth factor (VEGF) and the downstream extracellular signal-regulated kinase (ERK) protein after oxygen-glucose deprivation (OGD) in primary endothelial cells.

Methods—Primary mouse endothelial cells were isolated and subjected to OGD. Western analysis of VEGF and ERK 1/2 protein levels was performed. Cells were transfected with VEGF small interference RNA. A terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate-biotin nick end labeling (TUNEL) assay and DNA fragmentation assay were used on mouse endothelial cells that overexpress copper/zinc-superoxide dismutase (SOD1).

Results—VEGF protein expression was induced and its receptor, Flk-1, was stimulated by OGD. Phosphorylation of ERK 1/2 protein levels was upregulated. Inhibition of phosphorylated ERK (pERK) expression by U0126 reduced endothelial cell death by OGD. Transfection of small interfering RNA for VEGF also inhibited an increase in pERK, suggesting that VEGF acts via ERK. The TUNEL and DNA fragmentation assays showed a significant decrease in TUNEL-positivity in the SOD1-overexpressing endothelial cells compared with wild-type cells after OGD.

Conclusions—Our data suggest that OGD induces VEGF signaling via its receptor, Flk-1, and activates ERK via oxidative-stress-dependent mechanisms. Our study shows that in cerebral endothelial cells the ERK 1/2 signaling pathway plays a significant role in cell injury after OGD. (Stroke. 2009;40:00-00.)

Key Words: endothelial cells | oxidative stress | superoxide dismutase | VEGF

The blood–brain barrier (BBB) comprises endothelial cells, pericytes, microglia, astrocytes, and basal lamina. Interactions among these cells are necessary for the induction or maintenance of the specialized function of the BBB and maintenance of the microenvironment of the central nervous system. Cerebral endothelial cells (CEC) that line microvessels play a crucial role in maintaining normal blood flow within the brain. They can be damaged after a hypoxic episode induced by stroke or oxygen deprivation and this damage can lead to BBB breakdown and exacerbation of ischemic injury.1 Cerebral endothelial injury or death after ischemia contributes to postischemic secondary injury with increased vascular permeability and vasogenic brain edema. This secondary injury enhances leukocyte adhesion and serves as a source of oxygen-derived free radicals. The early phase of BBB breakdown is associated with generation of free radicals and activation of matrix metalloproteinases, and in the later phase there is production of proinflammatory cytokines and regulators of leukocyte trafficking.2

Reactive oxygen species (ROS) are formed as a result of oxidative stress. Overproduction of ROS leads to vascular injury and contributes significantly to the development of neurodegenerative diseases. ROS trigger intracellular signaling that leads to enhanced angiogenesis in vivo and activation of endothelial cells in vitro. Moreover, the superoxide anion, nitric oxide (NO), and hydrogen peroxide play an important role in mediating angiogenic signals initiated by growth factors such as vascular endothelial growth factor (VEGF). NO has been reported to mediate VEGF response during BBB disruption. Although VEGF is potent in angiogenesis, it can have detrimental effects such as an increase in endothelial permeability3 leading to vasogenic edema. Early administration of VEGF exacerbates BBB leakage, whereas late administration leads to angiogenesis.4 It induces various cell signaling pathways that result in cell permeability and migration and exerts its biological effects by binding to its respective transmembrane receptors, VEGF receptor 1 and
VEGFR receptor 2 (Flk-1). VEGF induces mitogen-activated protein kinase (MAPK) signaling pathways via its Flk-1 receptor. MAPK comprises a ubiquitous group of signaling proteins that play a role in regulating cell proliferation, differentiation, and adaptation. Many of these kinases have been reported to play a role in various diseases and have been implicated in cell injury. Activation of extracellular signal-regulated kinase (ERK) 1/2 protects neurons from apoptosis after growth factor withdrawal, as well as being detrimental in ischemic brain and cortical neuron injury. Phosphorylated ERK (pERK) 1/2 is increased in substantia nigra neurons of patients with Parkinson disease. Increased ERK 1/2 phosphorylation has been noted in the vulnerable penumbra after acute ischemic stroke in humans as well as in mouse models.

In the present study, we examined the activation of VEGFR and ERK 1/2 phosphorylation, which exacerbates endothelial cell death after oxygen-glucose deprivation (OGD).

Materials and Methods
Experiments were performed in accordance with the National Institutes of Health guidelines and were approved by Stanford University’s Administrative Panel on Laboratory Animal Care.

Mouse CEC Culture
Cerebral cortices of both wild-type (WT) and copper/zinc-superoxide dismutase (SOD1) transgenic (Tg) mice were minced and digested with collagenase/displace. The samples were centrifuged to remove the fatty layer. The cell pellet was resuspended in M199 and further digested with collagenase/displace for 30 minutes. Cells were then isolated using a 50% Percoll gradient and plated onto collagen-coated plates in endothelial cell medium with supplements (BD Biosciences).

OGD Treatment of the Cultures
We used OGD reoxygenation, an in vitro model that best mimics in vivo cerebral ischemia reperfusion. Endothelial cells were subjected to OGD by replacing the medium with buffered salt solution without glucose. The plates were placed in an anaerobic chamber (Plas Labs, Lansing, Mich) at 37°C. After 8 hours the medium was replaced with endothelial cell medium and the plates were returned to a 5% CO2/95% air incubator for different reoxygenation periods. Endothelial cells were treated with 10 μg/mL of neutralizing VEGF antibody or 1 μmol/L or 10 μmol/L of the MAPK/ERK inhibitor, U0126, when necessary.

Transient Focal Cerebral Ischemia
Tg mice carrying the SOD1 gene with a CD-1 background were derived from the founder stock with a 3-fold overexpression of SOD1 activity in brain cells. Three-month-old male mice (35–40 grams) were subjected to transient focal cerebral ischemia (FCI) and reperfusion. After 60 minutes of proximal middle cerebral artery occlusion, blood flow was restored by removing the suture. Control normal mice did not undergo surgery. Physiological parameters were monitored throughout the studies and values were normal.

Assessment of Cell Death
Cell viability after OGD was estimated by quantification of lactate dehydrogenase release after 24 hours of reoxygenation using a cytotoxicity detection kit (LDH kit; Roche Diagnostics). Viability was assessed with a LIVE/DEAD Viability/Cytotoxicity kit (Invitrogen) using calcein AM and ethidium homodimer-1 staining (Invitrogen). DNA fragments were quantified using mouse monoclonal antibodies directed at DNA and histone of cell lysates using a cell-death detection kit (Roche Diagnostics).

Western Blot Analysis
Ten micromgrams of the samples in 1X lysis buffer were electrophoresed on 4% to 20% Tris-glycine gels and immunoblotted with the specific antibodies (VEGF and Flk-1, Santa Cruz Biotechnology; ERK 1/2 and pERK 1/2, Cell Signaling Technology). Equal protein loading was confirmed by immunostaining of blots with β-actin.

Transfection of Small Interfering RNA
Small interfering RNA (siRNA) oligonucleotides of VEGF or nonfunctional negative control siRNA were obtained from Ambion. Bend.3 cells (mouse CEC line) that were 60% confluent were used for transfection according to instructions provided by Ambion. The transfection mixture of SiPORT lipid reagent and siRNA was added to the antibiotic-free cell culture medium to a final concentration of 50 nmol/L siRNA and 2 μL/mL SiPORT lipid. Eagles minimum essential medium supplemented with 10% fetal bovine serum was added after 4 hours of incubation for a final volume of 1 mL. After 48 hours the cells were subjected to OGD.

TUNEL Assay
CEC subjected to OGD or untreated cells were fixed with 4% paraformaldehyde. TUNEL staining was performed using an apoptotic peroxidase in situ apoptosis detection kit (Chemicon International).

Quantification and Statistical Analysis
All data were obtained from 3 to 5 independent experiments and expressed as mean±SEM. Comparisons between 2 groups were achieved with 1-way ANOVA with Fisher protected least significant difference test using StatView version 5.01 (SAS Institute). P≤0.05 was considered statistically significant.

Results
Assessment of CEC Death After OGD
We examined the effects of OGD in mouse CEC. Cell death increased significantly in a time-dependent manner. The lactate dehydrogenase assay showed a 38% death in cells subjected to 4 hours of OGD. This increased with longer periods of OGD, 60% to 60% after 6 hours, 65% after 8 hours, and 70% after 12 hours (Figure 1A). We determined the extent of DNA fragmentation by quantitation of cytoplasmic histone-associated DNA fragments using a cell death enzyme-linked immunosorbent assay kit. DNA fragmentation also increased significantly with increasing periods of OGD (Figure 1B). Cleaved caspase-3, an apoptotic marker, was also elevated after 8 hours of OGD and 2 hours of reoxygenation (data not shown).

Fluorescent staining with calcein and ethidium homodimer-1 (LIVE/DEAD Viability kit) confirmed cell death after OGD in which the ethidium dye entered the dying cells producing a bright red fluorescence (Figure 1C). In the live cells, the intact plasma membrane excluded the dye and stained with calcein.

Expression of Hypoxia-Inducible Factor-1α, VEGF, and Flk-1
OGD in endothelial cells induced hypoxia-inducible factor-1α. It was transiently induced within 5 minutes of reoxygenation after 8 hours of OGD and decreased to control levels within 30 minutes (Figure 2A). ROS, which are generated as a result of OGD, exert their cellular effects through modulation of vascular genes such as VEGF, which plays a crucial role in vascular response. Induction of VEGF was observed after OGD (Figure 2A). Western analysis also
showed an induction of its receptor, Flk-1. Quantitative analysis showed a 2-fold increase in VEGF and Flk-1 levels after OGD (Figure 2B).

Upregulation of ERK 1/2 Phosphorylation After OGD in WT but Not in SOD1-Overexpressing Endothelial Cells

We examined the VEGF-induced intracellular signaling pathway, which is regulated by its receptor Flk-1. Flk-1 is associated downstream with the MAPK signaling cascade in which the kinase ERK 1/2 plays a role. Western blot analysis showed that there was a 1.5-fold increase in pERK 1/2 protein expression in cells subjected to OGD compared with controls (Figure 3A).

To investigate whether activation of ERK 1/2 was caused by ROS, CEC from SOD1-overexpressing mice were subjected to OGD compared with WT CEC (Figure 3A), suggesting activation of ERK 1/2 occurs if superoxide radicals, produced after OGD, are present. Moreover, in the WT mice subjected to transient FCI after 4 hours of reperfusion, pERK 1/2 was expressed in the endothelial cells (red fluorescence in Figure 3B). This expression was not observed in the SOD1 Tg mice subjected to transient FCI. Furthermore, Western analysis showed that VEGF expression in the WT mice subjected to transient FCI was significantly upregulated 1.5-fold, whereas this was not observed in the SOD1 mice (Supplemental Figure IA, IB, available online at http://stroke.ahajournals.org).

SOD1 Overexpression Decreased Apoptosis After OGD in CEC

Apoptosis was measured by TUNEL staining, which demonstrated fewer positive SOD1-overexpressing cells than WT cells after OGD (Figure 4). This suggests that ROS via ERK 1/2 play a significant role in cell death after OGD.

ERK 1/2 Signaling Is Activated Via VEGF and Plays a Role in Cell Death by OGD

Blocking VEGF with a neutralizing VEGF antibody resulted in inhibition of ERK 1/2 activation (Figure 5A). Quantitative analysis of the Western blotting is shown in Supplemental Figure II. The effect on cell death of blocking VEGF was observed using the lactate dehydrogenase assay. As shown quantitatively, blocking VEGF reduced cell death by approximately 40% (Figure 5B). To determine whether ERK phosphorylation was downstream of VEGF activation, we used siRNA-mediated knockdown of VEGF. Blocking VEGF protein expression in Bend.3 endothelial cells resulted in a significant decrease in the pERK protein, but not in the levels of phosphorylated p38 or phosphorylated c-Jun N-terminal kinase. This substantiated the finding that VEGF exerts its effect via ERK 1/2 activation (Figure 5C and Supplemental Figure III).

Treatment of the cells with 10 μmol/L of U0126, a specific MAPK/ERK inhibitor affecting ERK 1/2 (Figure 6A and Supplemental Figure IV), reduced injury in CEC after OGD. U0126 reduced cell death by 40% after OGD. This suggests that activation of ERK 1/2 is detrimental to endothelial cells subjected to OGD (Figure 6B).

Discussion

Recent studies have led to the concept that endothelial dysfunction plays a role in ischemic injury via its interactions with the more well-studied neuronal and glial cells. In the current study we looked at endothelial cell death using a well-established in vitro ischemic model and subjected cells to OGD. We demonstrated that significant cell death occurs with 8 hours of OGD. We also observed an increase in VEGF and its receptor, Flk-1, within 2 hours of reoxygenation. Finally, by silencing VEGF and blocking it with a neutralizing VEGF antibody, we demonstrated that VEGF activates signaling pathways via the phosphorylation of ERK. Inhibition of VEGF also resulted in reduced cell death.
VEGF is induced in the ischemic core and penumbra after middle cerebral artery occlusion. It is critical for angiogenesis and promotes endothelial integrity by stimulating NO production. Whereas the effects of VEGF that promote late recovery are thought to be mediated by stimulation of VEGF, its acute effects on cerebral ischemia are more complicated. Zhang and Chopp found that early postischemic administration of VEGF significantly increased BBB permeability. VEGF-induced NO production may react strongly with superoxide ions to generate peroxynitrite, which causes tissue damage. A recent study demonstrated that endothelial NO generation, in addition to neuronal NO generation, may be detrimental by causing peroxynitrite formation. Thus, it is possible that the negative effect of VEGF is linked to the production of free radicals and oxidative stress. We looked at one of the signaling pathways affected by VEGF activation. In endothelial cells VEGF has been shown to exert its biological mitogenic activities and vasopermeability responses mainly through one of its receptors, Flk-1. This receptor then activates signaling cascades, including the MAPK pathway, by phosphorylating ERK 1/2. Members of the MAPK family play an important role in the regulation of cell growth, differentiation, and control of cellular responses to cytokines and stress. They are activated by phosphorylation, which then phosphorylates other intracellular enzymes and transcription factors. ROS are also known to contribute to activation of the ERK 1/2 pathway. Although it is generally thought that activation of ERK 1/2 can confer a survival advantage to cells, there is growing evidence suggesting that activation of ERK 1/2 also contributes to cell death. Persistent activation of ERK leads to cell death, whereas short-lived activation of ERK is associated with cell proliferation.

Activated ERK phosphorylates Elk-1 and triggers other transcription factors, like nuclear factor-κB and activator
protein-1. ERK has been shown to be neuroprotective in vitro, but in vivo studies suggest a deleterious effect of ERK activation after FCI. In the present study, we showed an increase in pERK 1/2 expression as early as 2 hours after OGD, and this increase was maintained until 24 hours of reoxygenation. By silencing VEGF using siRNA knockdown and a neutralizing VEGF antibody, we demonstrated that pERK 1/2 expression decreased after OGD in endothelial cells that had decreased VEGF levels. By blocking VEGF, we also observed a reduction in cell death. Furthermore, U0126, an ERK 1/2 inhibitor, decreased phosphorylation of ERK 1/2 and significantly reduced cell death. Thus, ERK 1/2 activation is important in OGD injury in CEC.

Because ROS are known to be involved in activation of ERK, we examined the effect of overexpression of SOD1 on ERK phosphorylation. Antioxidant enzymes have been reported to be one of the major mechanisms by which cells counteract the deleterious effects of ROS. We have shown that SOD plays a protective role against FCI. We reported the attenuation of cytochrome c release from mitochondria and subsequent DNA fragmentation after FCI in Tg mice that overexpress SOD1. Endothelial cells have been shown to release cytochrome c after OGD. In the present study, TUNEL positivity was significantly decreased in SOD1-overexpressing endothelial cells compared with the WT cells after OGD, suggesting that ROS are critical during OGD. There was no significant change in VEGF and ERK phosphorylation in the SOD1 endothelial cells compared with the WT cells. MAPK/ERK was shown to be activated by ROS after FCI and in vascular smooth muscle cells in vitro. Moreover, antioxidants prevented both ERK activation and cell death induced by Zn\(^{2+}\), which generated ROS. In this study, we showed that VEGF exacerbates injury and this may involve ERK 1/2. Because ERK 1/2 phosphorylation was decreased using VEGF siRNA and a neutralizing antibody against VEGF, this study suggests that activation of ERK is independent of the Ras/Raf pathway and that the effect is mainly caused by activation of the VEGF receptor, Flk-1. There is evidence that binding of VEGF to Flk-1 results in its tyrosine phosphorylation, which is thought to then activate protein kinase C, mediating ERK 1/2 phosphorylation. In the present study, no increase in ERK 1/2 phosphorylation was observed in endothelial cells from the SOD1 Tg mice, suggesting that ROS and oxidative stress are critical in ERK activation. SOD1 is known to contribute to inhibition of apoptosis after various injuries, including focal ischemia, and ERK 1/2 phosphorylation may have a crucial role in cell injury. Inhibition of phosphorylation of ERK 1/2 by U0126 also caused a decrease in endothelial cell death after OGD.
thus suggesting that ERK 1/2 activation promotes cell death. In summary, our study shows that the ERK 1/2 signaling pathway plays a significant role in CEC injury after OGD and that ERK 1/2 can be considered a molecular target for stroke therapy.

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Disclosures
None.

References


Figure I. Representative Western blot (A) and quantitative analysis (B) of VEGF after OGD in WT and SOD1 Tg endothelial cells. An increase in VEGF was observed in WT cells with 2 hours of reoxygenation after OGD. There was no upregulation of VEGF levels in SOD1 Tg endothelial cells. Beta-actin was used as an internal control. C indicates control; AU, arbitrary units. *P<0.05.

Figure II. Quantitative analysis of Western blots from Figure 5A showing that levels of pERK are significantly reduced after treatment of endothelial cells with 10 μg/mL of the neutralizing VEGF antibody. O indicates OGD; V, VEGF antibody. *P<0.05.
Figure III. Quantitative analysis of Western blots from Figure 5C. Blocking VEGF with siRNA showed a simultaneous decrease in pERK but not in phosphorylated p38 or phosphorylated c-Jun N-terminal kinase. Con indicates control; Scr, scrambled RNA; Si, siRNA against VEGF. *P<0.05.

Figure IV. Quantitative analysis of Western blot from Figure 6 showing decreased levels of pERK after treatment of cells with the ERK inhibitor, U0126. U indicates U0126. *P<0.05.
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