Chronic Valproate Treatment Enhances Postischemic Angiogenesis and Promotes Functional Recovery in a Rat Model of Ischemic Stroke

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Background and Purpose—Enhanced angiogenesis facilitates neurovascular remodeling processes and promotes brain functional recovery after stroke. Previous studies from our laboratory demonstrated that valproate (VPA), a histone deacetylase inhibitor, protects against experimental brain ischemia. The present study investigated whether VPA could enhance angiogenesis and promote long-term functional recovery after ischemic stroke.

Methods—Male rats underwent middle cerebral artery occlusion for 60 minutes followed by reperfusion for up to 14 days. Assessed parameters were: locomotor function through the Rotarod test; infarct volume through T2-weighted MRI; microvessel density through immunohistochemistry; relative cerebral blood flow through perfusion-weighted imaging; protein levels of proangiogenic factors through Western blotting; and matrix metalloproteinase-2/9 activities through gelatin zymography.

Results—Postischemic VPA treatment robustly improved the Rotarod performance of middle cerebral artery occlusion rats on Days 7 and 14 after ischemia and significantly reduced brain infarction on Day 14. Concurrently, VPA markedly enhanced microvessel density, facilitated endothelial cell proliferation, and increased relative cerebral blood flow in the ipsilateral cortex. The transcription factor hypoxia-inducible factor-1α and its downstream proangiogenic factors, vascular endothelial growth factor and matrix metalloproteinase-2/9, were upregulated after middle cerebral artery occlusion and significantly potentiated by VPA in the ipsilateral cortex. Acetylation of histone-H3 and H4 was robustly increased by chronic VPA treatment. The beneficial effects of VPA on Rotarod performance and microvessel density were abolished by hypoxia-inducible factor-1α inhibition.

Conclusions—Chronic VPA treatment enhances angiogenesis and promotes functional recovery after brain ischemia. These effects may involve histone deacetylase inhibition and upregulation of hypoxia-inducible factor-1α and its downstream proangiogenic factors vascular endothelial growth factor and matrix metalloproteinase-2/9. (Stroke. 2012; 43:00-00.)

Key Words: angiogenesis ■ cerebral ischemia ■ hypoxia-inducible factor-1 ■ matrix metalloproteinase ■ MRI ■ valproate ■ vascular endothelial growth factor

Angiogenesis is a key component of poststroke neurovascular remodeling processes in which new capillaries are formed through directed proliferation and migration of endothelial progenitor cells from pre-existing blood vessels. Postmortem studies reveal that angiogenesis can be observed several days after cerebral ischemic stroke; notably, higher microvessel density correlates with longer patient survival. In the rodent brain, endothelial cell proliferation and capillary sprouting begin as early as 24 to 48 hours after ischemic injury. Poststroke angiogenesis increases collateral circulation and restores oxygen and nutrient supply to the injured tissue. Moreover, newly generated vessels provide neurotrophic support to concurrent neurogenesis and synaptogenesis, and these ultimately lead to functional recovery. Therefore, strategies that enhance poststroke angiogenesis hold great promise for the treatment of stroke.

Vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) are key proangiogenic factors that increase after ischemia in both rodent and human brains. VEGF induces endothelial cell proliferation and mediates the effects of other proangiogenic factors. MMPs set the stage for endothelial cell migration by degrading the...
Western Blotting
The protein levels of HIF-1α, VEGF, and acetylated histone-H3 and H4 in the ipsilateral cortex were detected by Western blotting on Days 7 and 14. Detailed procedures are available in the online-only Data Supplement Methods.

Gelatin Zymography
The activities of MMP-2 and MMP-9 were measured by gelatin zymography, and the pro- and active forms of MMP-2/9 were identified by reference to their respective standards as previously described. Detailed procedures are available in the online-only Data Supplement Methods.

Statistical Analyses
Data are expressed as mean±SEM. For Rotarod data, 2-way repeated-measures analysis of variance was performed to analyze the overall difference between treatment groups over time. Bonferroni-corrected post hoc comparisons were then used to analyze the difference between treatment groups at each time point. Comparisons between 2 groups and multiple groups were evaluated by Student t test and one-way analysis of variance followed by Tukey post hoc comparisons, respectively. Differences were considered statistically significant at P<0.05.

Results
VPA Reduced Brain Infarct Volume and Promoted Functional Recovery
On Day 14, T2-weighted images showed that infarction comprised approximately 18% of the whole brain in MCAO rats. VPA treatment significantly reduced the infarct size to 13% (Figure 1A–B). To confirm the effect of VPA on MCAO-induced brain infarction, we further measured the infarct area on Day 14 using hematoxylin and eosin staining. The infarct area in the VPA-treated group was significantly smaller compared with the untreated MCAO rats (11.2±1.3 versus 17.3±1.4 mm²; online-only Data Supplement Figure 1). The length of time that rats were able to stay on the accelerating Rotarod was markedly reduced on Days 7 and 14 after MCAO. Postischemic treatment with VPA robustly ameliorated this motor function deficit by prolonging Rotarod retention time (Figure 1C).

VPA Enhanced Postischemic Angiogenesis
To investigate whether VPA enhanced postischemic angiogenesis, microvessel density was first analyzed by immunostaining for CD31, an endothelial cell marker, in the ipsilateral cortex of MCAO rats. On Days 7 and 14 after MCAO, microvessel density in the ipsilateral cortex was increased to 136.1±4.0% and 155.3±4.0% of the corresponding contralateral side, respectively, compared with sham-operated rats (100%±1.5% for Day 7 and 100%±2.4% for Day 14; Figure 2A). VPA treatment further enhanced microvessel density to 148.4±3.9% and 182.6±6.4% on Days 7 and 14, respectively. Microvessel density in the contralateral cortex was not affected by MCAO alone or with VPA treatment (data not shown). Endothelial cell proliferation was examined by double staining Ki67 and RECA-1. VPA markedly increased Ki67 and RECA-1 double-positive cells in the ipsilateral cortex of MCAO rats from 5.3±0.8% to 8.8±0.9% of Ki76-positive cells on Day 14 (Figure 2B), indicating that VPA promoted endothelial cell proliferation.

Materials and Methods

MCAO and Drug Administration
All animal experiments were performed according to protocols approved by the National Institute of Mental Health Animal Care and Use Committee. Male Sprague-Dawley rats (200–220 g; Charles River Laboratories, Wilmington, MA) underwent right MCAO under inhalational anesthesia (1.5% isoflurane in 70% N2O and 30% O2) as previously described. Detailed procedures are available in the online-only Data Supplement Methods.

VPA (200 mg/kg, intraperitoneally; Sigma, St Louis, MO) was administrated immediately after ischemic onset, 12 hours later, and then once daily for up to 14 days. 2-methoxyestradiol (2ME2, 5 mg/kg, intraperitoneally; Sigma) was dissolved in 1% dimethyl sulfoxide and cojected with VPA once daily for 10 days beginning on Day 4 after reperfusion.

Accelerating Rotarod Test
An accelerating Rotarod apparatus (San Diego Instruments, San Diego, CA) was used to measure motor skill learning and coordination in the MCAO rats; the speed was accelerated from 0 to 40 rpm over 4 minutes. Rats received once-daily training sessions of 3 trials separated by 30-minute intervals for 3 consecutive days before MCAO. The longest amount of time each rat remained on the rod was recorded as baseline. Seven and 14 days after MCAO, rats underwent 3 trials on the Rotarod, and the best performance of each rat was recorded for that day.

Magnetic Resonance Imaging
All MRI experiments were performed on a 7-T (Bruker Avance, Billerica, MA), 210-mm horizontal scanner. Infarct volume and relative cerebral blood flow (rCBF) on Day 14 after MCAO were evaluated using T2-weighted MRI and perfusion-weighted imaging, respectively. Detailed procedures are available in the online-only Data Supplement Methods.

Immunohistochemistry
Microvessel density in the ipsilateral cortex was assessed by CD31 immunostaining on Days 7 and 14. Endothelial cell proliferation was evaluated by double immunofluorescent staining with RECA-1 (an endothelial cell marker) and Ki67 (a cell proliferation marker) on Day 14. Detailed procedures are available in the online-only Supplement Methods.

Extracellular matrix is regulated by the transcription factor hypoxia-inducible factor-1 (HIF-1), which regulates gene transcription to facilitate adaptation and survival after hypoxia–ischemia. Late inhibition of the HIF-1α subunit appears to worsen ischemic outcomes. Therefore, enhanced HIF-1 activation and HIF-1-dependent transcription could be advantageous in ischemic stroke.

Valproate (VPA), a histone deacetylase (HDAC) inhibitor, is commonly used to treat seizures and bipolar disorder. Studies have shown that VPA has protective properties in cellular and animal models of neurodegenerative diseases, including stroke. We have recently demonstrated that postischemic VPA treatment markedly attenuates blood–brain barrier disruption and brain edema in a rat model of middle cerebral artery occlusion (MCAO), and this protection persists at least 3 days after ischemia. However, the effects of VPA on later-phase recovery remain unclear. The present study investigated whether chronic VPA treatment can enhance postischemic angiogenesis and promote functional recovery in a rat MCAO model.

VPA Enhanced Postischemic Angiogenesis
Microvessel density in the ipsilateral cortex from 5.3% of Ki76-positive cells on Day 14 (Figure 2B), indicating that VPA promoted endothelial cell proliferation.
The effects of VPA on enhanced microvessel density were more pronounced on Day 14 than 7 after MCAO. Therefore, perfusion-weighted imaging maps were quantitatively analyzed for the hemodynamic changes on Day 14. Figure 3A shows representative perfusion-weighted imaging maps from an untreated and a VPA-treated MCAO rat (corresponding T2 maps are presented in Figure 1A). Perfusion-weighted imaging analysis was carried out in 3 regions of interest (ROIs) indicated by the circled numbers on the lesional side of the brain in Figure 3B. ROI-1 and 3 were located in the cortex and ROI-2 was in the striatum. The T2 value was higher in ROI-3 compared with the values in ROI-1 and 2 (online-only Data Supplement Figure II), indicating that the severity of the lesion was greatest in ROI-3. The rCBF ratio of the ipsilateral hemisphere to the contralateral hemisphere was calculated at each ROI. On Day 14 after MCAO, the rCBF ratios in all 3 ROIs declined to 60% to 70% of their respective contralateral areas, and ROI-3 had the lowest rCBF ratio. Notably, VPA treatment robustly increased the rCBF ratio in all 3 ROIs (99.9% ± 4.6% versus 75.7% ± 5.0% for ROI-1, 98.3% ± 7.5% versus 68.5% ± 5.6% for ROI-2, and 108.1% ± 7.1% versus 63.1% ± 7.6% for ROI-3), suggesting that VPA enhanced postischemic angiogenesis in both the ipsilateral cortex and striatum.

### VPA Upregulated Proangiogenic Factors and Inhibited HDACs

As an essential part of the HIF-1 complex, HIF-1α is rapidly degraded under normoxia and becomes stabilized after hypoxia–ischemia. As expected, the protein levels of HIF-1α

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**Figure 1.** Postischemic chronic treatment with VPA reduced brain infarct volume and improved functional recovery in MCAO rats. A–B, Brain infarction was detected by T2-weighted MRI. VPA significantly reduced the brain infarct volume in MCAO rats on Day 14. N=5 per group. The infarction is shown in the dotted areas. C, Behavioral deficits were evaluated by the Rotarod test. VPA markedly increased the retention time of MCAO rats on an accelerating Rotarod on Days 7 and 14 after ischemia. N=8 per group. **P<0.01 compared with sham control; *P<0.05, **P<0.01 compared with MCAO group. VPA indicates valproate; MCAO, middle cerebral artery occlusion.

**Figure 2.** VPA enhanced postischemic angiogenesis in the ipsilateral cortex. A, CD31 microvessel staining in the boxed area. MCAO increased microvessel density on Days 7 and 14, and VPA markedly enhanced this increase. N=10 per group. B, VPA significantly increased endothelial cell proliferation on Day 14. N=7 per group. **P<0.01 compared with the sham control, *P<0.05, **P<0.01 compared with MCAO group. VPA indicates valproate; MCAO, middle cerebral artery occlusion.
gradually increased in the ipsilateral cortex of MCAO rats on Days 7 and 14. This increase was further augmented by VPA treatment (201.3% ± 22.7% versus 127.5% ± 12.4% for Day 7 and 278.4% ± 13.3% versus 203.5% ± 7.5% for Day 14 compared with sham-operated rats; Figure 4A).

To study the effects of VPA on proangiogenic factors after brain ischemia, the protein levels of VEGF and activities of MMP-2/9 were examined using Western blotting and gelatin zymography, respectively. VEGF protein levels gradually increased in the ipsilateral cortex of MCAO rats on Days 7 and 14 compared with sham-operated rats (Figure 4A). VPA treatment further enhanced the MCAO-induced upregulation of VEGF protein levels (134.2% ± 9.4% versus 115.6% ± 6.2% on Day 7 and 175.1% ± 14.4% versus 159.9% ± 16.8% on Day 14 compared with sham-operated rats). In the zymography gel, MMP-2 had a much stronger signal than MMP-9. Total MMP-2 activity in MCAO rats sustained a 1.6-fold increase on Days 7 and 14 compared with sham-operated rats, whereas MCAO induced a relatively mild increase in total MMP-9 activity (Figure 4B). VPA treatment more than doubled total MMP-2 activity and also significantly upregulated total MMP-9 activity.

Acetylated histone-H3 and/or H4 (Ac-H3 and/or Ac-H4) is an index of HDAC inhibition. In MCAO rats, a gradual increase in Ac-H3 protein levels was noted from Days 7 to 14 (onefold and 1.5-fold compared with sham-operated rats, respectively), although this increase did not reach statistical significance. VPA treatment robustly amplified Ac-H3 expression to 1.4-fold and 2.8-fold on Days 7 and 14, respectively (Figure 4A). Ac-H4 was also mildly increased on Day 7 and returned to baseline on Day 14. VPA treatment markedly potentiated Ac-H4 levels on Day 14 (1.5-fold for Day 7 and 2.3-fold for Day 14; online-only Data Supplement Figure III).

HIF-1α Inhibition Abolished the Beneficial Effects of VPA on Postischemic Angiogenesis and Functional Recovery

2ME2 is a natural metabolite of estradiol and has been shown to inhibit hypoxia-dependent HIF-1α stabilization and tran-
VPA's ability to enhance postischemic angiogenesis. Our findings suggest that (1) chronic VPA treatment markedly enhanced the upregulation of VEGF and MMP-2/9, strongly potentiated both microvessel density and rCBF in the ipsilateral cortex, and (2) the proangiogenic effects of VPA likely involve HDAC inhibition and upregulation of HIF-1α and proangiogenic factors VEGF and MMP-2/9.

Accumulating evidence has established that angiogenesis naturally occurs after brain ischemia in humans and animals, potentially functioning as an endogenous mechanism to restore oxygen and nutrient supply to affected brain tissue. However, postischemic angiogenesis is often insufficient to improve clinical outcomes. Our results demonstrated that despite higher microvessel density, rCBF was significantly lower in the ipsilateral versus contralateral hemisphere on Day 14 after MCAO. This finding echoes a clinical study demonstrating that the infarct region of postmortem stroke patients contains a higher proportion of empty microvessels than normal brain tissue. Additionally, a rat MCAO study indicated that ischemia induces a transient population of leaky microvessels. Taken together, these observations suggest that the newly formed microvessels may not be perfused or functional. In the current study, VPA treatment strongly potentiated both microvessel density and rCBF in the ischemic hemisphere, suggesting that VPA may facilitate microvessel formation as well as perfusion. Furthermore, it has been proposed that ischemia-triggered angiogenesis requires additional factors such as brain-derived neurotrophic factor for long-term stabilization. VPA can induce brain-derived neurotrophic factor in rat cortical neurons by activating brain-derived neurotrophic factor promoter IV through HDAC inhibition, suggesting that VPA may also stabilize posts ischemic angiogenesis by inducing brain-derived neurotrophic factor expression.

VEGF expression rapidly increases within hours and remains elevated for weeks after ischemia in rodent and human brains. Late administration of exogenous VEGF in the ischemic penumbra of rats enhances angiogenesis and improves neurological recovery. MMP-9 activity is elevated 2 to 4 days after stroke in the postmortem human brain, whereas MMP-2 activity is elevated at later intervals. Delayed MMP inhibition in the peri-infarct cortex suppresses neurovascular remodeling and functional recovery on Day 14 in ischemic rats. Consistent with these studies, we found that VEGF protein levels and MMP-2/9 activities were significantly increased on Days 7 and 14 after MCAO. VPA treatment markedly enhanced the upregulation of VEGF and MMP-2/9, suggesting that VEGF and MMP-2/9 contribute to VPA's ability to enhance postischemic angiogenesis.

Both VEGF and MMPs increase blood–brain barrier permeability in the acute phase and subsequently facilitate neurovascular remodeling after stroke. In the present study, VPA enhanced postischemic angiogenesis by upregulating VEGF protein expression and MMP-2/9 activities on Day 14 after MCAO. We recently demonstrated that VPA attenuates blood–brain barrier disruption and brain edema by suppressing MMP-9 induction and tight junction degradation 24 hours after MCAO. In a swine hemorrhagic shock.

Discussion

In this study, VPA markedly reduced infarct volume and improved functional recovery on Day 14 after MCAO in rats. Concurrently, VPA treatment enhanced posts ischemic angiogenesis by increasing microvessel density, facilitating endothelial cell proliferation, and upregulating rCBF in the ipsilateral cortex. In addition to enhancing HDAC inhibition, VPA potentiated MCAO-induced HIF-1α accumulation and upregulated VEGF protein levels and MMP-2/9 activities in the ipsilateral cortex on Days 7 and 14 post-MCAO. Furthermore, HIF-1α inhibition reversed the enhanced postischemic angiogenesis and functional recovery observed after VPA treatment. Our findings suggest that (1) chronic VPA treatment enhances posts ischemic angiogenesis and promotes long-term functional recovery in an experimental model of ischemic stroke; and (2) the proangiogenic effects of VPA likely involve HDAC inhibition and upregulation of HIF-1α and proangiogenic factors VEGF and MMP-2/9.

Figure 5. HIF-1α inhibition abolished the beneficial effects of VPA on posts ischemic angiogenesis and functional recovery. A, 2-methoxyestradiol (2ME2), an effective HIF-1α inhibitor, robustly inhibited the VPA-induced elevation of HIF-1α and VEGF protein levels and MMP-2/9 activities in the ipsilateral cortex on Day 14. B, RECA-1 staining shows that 2ME2 completely abolished the VPA-enhanced increase in microvessel density on Day 14. Nuclei were counterstained with DAPI (blue). N = 12 per group. C, 2ME2 completely reversed the beneficial effects of VPA on the Rotarod performance of MCAO rats. N = 8 per group. *P < 0.05, **P < 0.01 compared with MCAO group; ***P < 0.01 compared with VPA-treated MCAO group. HIF indicates hypoxia-inducible factor; VPA, valproate; MMP, matrix metalloproteinase; DAPI, diaminido phenyl indole; MCAO, middle cerebral artery occlusion.
model, VPA mitigates pathological endothelial cell function by attenuating the overexpression of VEGF and its receptor 6 hours after resuscitation.24 Therefore, VPA appears to have a dual role in preserving postischemic endothelial cell function: it limits cell damage by inhibiting MMP-9 and VEGF in the early phase, whereas it enhances angiogenesis by upregulating VEGF and MMP-2/9 in the later recovery phase.

HIF-1 regulates proangiogenic genes after hypoxia–ischemia, and its activation is predominantly controlled by α subunit stabilization.12 In this study, VPA treatment significantly enhanced MCAO-induced HIF-1α protein accumulation in the ipsilateral cortex on Days 7 and 14 after MCAO. 2ME2, a HIF-1α inhibitor, completely abolished the ability of VPA to increase microvessel density and improve Rotarod performance. These findings suggest that the proangiogenic effects of VPA involve regulation of HIF-1α and are critical for the ability of VPA to improve long-term functional recovery after ischemia. It is suggested that VEGF and MMP-2/9 are under transcriptional regulation of HIF-1.12 In support of this notion, we found that HIF-1α inhibition suppressed VEGF and MMP-2/9 upregulation induced by VPA treatment in MCAO rats. To confirm that HIF-1α can directly upregulate VEGF and MMP-2/9, HIF-1α was induced in primary rat brain microvascular endothelial cells, and VEGF and MMP-2/9 mRNA levels were measured by quantitative real-time polymerase chain reaction. Cobalt chloride is widely used to mimic hypoxia by stabilizing HIF-1α,25,26 Cobalt chloride treatment increased HIF-1α protein levels with a peak observed at 6 hours (online-only Data Supplement Figure VA). At this time point, there was also a significant increase in VEGF and MMP-2/9 mRNA levels in rat brain microvascular endothelial cells (online-only Data Supplement Figure VC). Pretreatment with 2ME2 significantly suppressed HIF-1α upregulation (online-only Data Supplement Figure VB). Consequently, 2ME2 almost completely inhibited cobalt chloride-induced VEGF mRNA increase, partially reduced the MMP-9 mRNA increase, but did not affect MMP-2 mRNA levels (online-only Data Supplement Figure VC). Together with the in vivo data, these findings indicate that upregulation of HIF-1α results in the elevation of VEGF and MMP-2/9, although it is possible that HIF-1α differentially regulates the transcription of these 3 genes.

The precise mechanisms underlying VPA-enhanced HIF-1α accumulation and angiogenesis in the ischemic brain remains unclear. VPA is a pan-inhibitor of HDAC Class I (1, 2, 3, 8 isoforms) and IIa (4, 5, 7, 9 isoforms).14 VPA-induced HDAC inhibition results in histone hyperacetylation, chromatin relaxation, and gene transcription. As an index of HDAC inhibition, acetylation of histone-H3 and H4 was robustly increased by VPA treatment, especially on Day 14 after MCAO, suggesting that HDAC inhibition may participate in the proangiogenic effects of VPA after ischemia. In support of this notion, a recent in vitro study shows that HDAC inhibitors, VPA and suberoylanilide hydroxamic acid, greatly enhance VEGF-induced spheroid sprout formation in endothelial cells and that VPA displays a trend toward increasing endothelial cell migration.27 Additionally, VPA potentiates extracellular signal-regulated kinase 1/2 activation in endothelial cells, which is known to promote cell survival and angiogenesis.27 Furthermore, HDAC inhibition can induce the differentiation of multipotent adult progenitor cells into endothelial cells, with or without VEGF costimulation.28 Besides endothelial cells, microvascular pericytes also play an important role in optimizing postsischemic angiogenesis. In human microvascular pericytes, a quantitative polymerase chain reaction angiogenesis array showed that VPA leads to a general increase in genes associated with vessel stabilization and maturation such as endothelial survival, endothelial tube formation/stabilization/branching, and maintenance of direct cell–cell contacts between endothelial cells and pericytes.29 Taken together, these findings suggest that VPA-induced HDAC inhibition may modulate proangiogenic gene expression and contribute to postsischemic angiogenesis.

Interestingly, HDAC inhibitors are currently undergoing clinical evaluation as potential anticancer therapies because of their antiangiogenic effects in tumors.30 VPA has been shown to inhibit HIF-1α stabilization and tumor angiogenesis in diverse cancer cell lines.31,32 However, there is no evidence showing the effects of VPA on HIF-1α and angiogenesis in noncancerous brain cells, especially after ischemia. In line with the in vivo findings, in an in vitro oxygen–glucose deprivation model, we found that 3 hours oxygen–glucose deprivation and 24 hours reperfusion increased HIF-1α protein levels in rat brain microvascular endothelial cells, and this increase was significantly augmented by treatment with 1 mmol/L VPA (online-only Data Supplement Figure VIA). In addition, VPA also significantly increased cell viability after oxygen–glucose deprivation insult (online-only Data Supplement Figure VIB). VPA alone did not affect HIF-1α protein levels or cell viability under normoxic conditions. These findings provide complementary evidence supporting the proangiogenic effects of VPA after brain ischemia, conditions that likely differ significantly from those in cancer models.

Notably, existing studies suggest the likelihood that each HDAC isoform interacts differently with angiogenic pathways under specific conditions. There are also controversial findings regarding specific HDAC isoforms associated with HIF-1α in different cancer cell lines. For instance, one study found that HDAC7 directly interacts with HIF-1α and increases its transcriptional activity in HEK293 cells.33 Another study in C2 cells showed that VPA inhibits HIF-1α only at high concentrations that are effective against Class II HDACs and further demonstrated that HDAC4 and 6, instead of HDAC7, are involved.32 In contrast, protein kinase D-dependent phosphorylation and nuclear export of HDAC5 and 7 mediate VEGF-induced angiogenesis in endothelial cells.34,35 Additionally, HDAC5 silencing has been shown to increase endothelial cell migration, sprouting, and tube formation, whereas HDAC5 overexpression decreases sprout formation.36 An electron microscopy study has demonstrated that the pattern of new blood vessels in the ischemic brain is similar to that in normal brain but differs from that in growing tumors.37 Therefore, it is conceivable that interactions between HDAC isoforms and HIF-1α may be dissimilar in endothelial cells and cancer cells under different oxygen tension conditions and that the pan-inhibition of HDACs by VPA would affect the postsischemic angiogenesis differently than it would affect angiogenesis in cancer.
To our knowledge, this study is the first to demonstrate that VPA enhances postischemic angiogenesis in vivo. This may contribute to its observed effects in improving long-term functional outcome after ischemic stroke. Our findings lead to a better understanding of the beneficial effects of VPA against ischemic stroke and pave the way for potential clinical trials.

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Disclosures
None.

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SUPPLEMENTAL METHODS

Middle cerebral artery occlusion (MCAO)

Male Sprague-Dawley rats (200-220 g, Charles River Laboratories, Wilmington, MA) underwent right MCAO under inhalational anesthesia (1.5% isoflurane in 70% N₂O and 30% O₂) as described previously. Briefly, the right common, external and internal carotid arteries were exposed through a midline neck incision. A 4-0 nylon suture with flame-rounded tip was inserted through the right common carotid artery into the right internal carotid artery and then to the Circle of Willis to occlude the origin of the right middle cerebral artery. Reperfusion was performed by withdrawal of the suture 60 minutes after occlusion. Core body temperature was maintained at 37.0 ± 0.5°C using a heating blanket during surgery. Sham-operated rats underwent neck surgery without arterial occlusion. Neurological severity score was evaluated immediately after rats were reperfused and fully recovered from anesthesia. Rats that did not show neurological deficits were excluded from the study. MRI and behavioral studies were performed by investigators who were blinded to the group assignment.

Magnetic resonance imaging (MRI)

All studies were performed in compliance with the guide for the care and use of Laboratory Animal Resources (1996), National Research Council, and approved by the NINDS/NIMH Animal Care and Use Committee. On day 14 after MCAO, rats were anesthetized (1.5% isoflurane), intubated and stationed on a mechanical ventilator (SAR/830/P, CWE Inc., Ardmore, PA). Body core temperature was maintained at 37.0 ± 0.5°C with a heated circulating water pad. Ventilator and rectal temperature lines were interfaced to a single monitor (Biopak systems Inc., Goleta, CA) and monitored throughout MR scanning. Femoral arterial and venous lines were placed for blood gas analysis and for systemic access, if necessary (e.g., CO₂ infusion to control blood pH). The rat was then placed in a stereotaxic holder and mounted in a 72 mm volume (transmit)/25 mm surface (receive) radio frequency coil ensemble for MRI. The MRI experiments were performed on a horizontal bore 7 Tesla scanner operating on a Bruker Avance platform (Bruker Biospin Inc., Bellerica, MA).

Infarct volume was evaluated by T2-weighted imaging. Three mutually perpendicular scout images were acquired through the brain to localize the infarction site. Subsequently, 13-15 T2 weighted axial slices (1 mm thick), encompassing the entire damaged area (field-of-view [FOV] = 32 × 28 mm, matrix size = 256 x 224, in-plane resolution of 125 μm, echo time [TE] = 12 ms, repetition time [TR] = 3500 ms, echo train length = 16, number of averages [NA] = 2, and total imaging time of 26 minutes), were acquired using a spin-echo pulse sequence for delineating anatomical details and calculating qualitative T2 maps.
Relative cerebral blood flow (rCBF) was measured by perfusion-weighted imaging, using continuous arterial spin labeling-ASL-coupled with a four segmented echo planner imaging pulse sequence in a single 1.5-mm axial slice through a selected infarct site. In subsequent rats, similar anatomical location was identified when placing the labeling slice. A 2-second adiabatic labeling pulse was applied at a plane 2-cm caudal to the chosen plane for spin inversion and thus to label arterial spins (FOV = 32 mm, matrix size = 256 × 256, in-plane resolution = 125 µm, TE = 31.9 ms, and TR = 2166 ms, 2-ms delay between labeling pulse and acquisition, NA = 16, and total imaging time of 4.5 minutes). The same experiment was repeated 10 minutes after 10% CO₂ was infused as a global vasodilator.

T2 and rCBF data were processed and analyzed using software routines written in MATLAB (Mathworks Inc., Natick, MA). T2 values were evaluated by assuming single exponential decay and fitting weighted images into respective relaxation equations. The control ASL image (IC), obtained to compensate for magnetization transfer effects due to interactions with macromolecules in the selected brain slice, and the ASL (IL) CBF-weighted images were processed to calculate rCBF maps corresponding to ([IC – IL]/ IC). In the rCBF and T2 maps, three different regions of interests (ROIs) were drawn in the infarct areas with similar locations on the contralateral side, and the corresponding values were calculated.

**Hematoxylin and eosin Staining**

Hematoxylin and eosin staining was performed to detect the brain infarct area. Briefly, brain slices were washed in PBS and stained in hematoxylin and eosin (Sigma-Aldrich) followed by washing in running tap water. After differentiation in 1% acid alcohol followed by washing, dehydrating and clearing, stained slices were scanned with an Epson scanner and the infarct area was calculated using ImageJ software (Free download at http://rsbweb.nih.gov/ij/).

**Immunohistochemistry**

Microvessel density and endothelial cell proliferation in the penumbra cortex were detected by immunohistochemistry. Brains were fixed with 4% formaldehyde by transcardial perfusion, dehydrated in 30% sucrose, frozen and cryo-cut coronally at 30 µm. Free-floating sections were incubated overnight at 4°C with the following primary antibodies: mouse anti-CD31 (1:1,000, Millipore, Billerica, MA), rabbit anti-Ki67 (1:500, Abcam, Cambridge, MA), or mouse anti-RECA-1 (1:500, Abcam). After washing, for CD31, sections were incubated for 2 hours at room temperature with the biotinylated antibody, visualized with the VECTASTAIN ABC and DAB kits (Vector Laboratories, Burlingame, CA), and cleared with xylene. For Ki67 and RECA-1, sections were incubated with appropriate fluorescence dye-conjugated secondary antibodies (1:200, Invitrogen). Nuclei were stained with 4’, 6-diamidino-2-phenylindole (DAPI) included in the mounting medium (Vector). Immunolabeling signals were captured by an Olympus BX61 microscope (Center Valley, PA) or LSM510 laser-scanning confocal microscope (Carl Zeiss, Göttingen, Germany). ImageJ was used to quantify the results.

**Western blotting**

The cerebral cortex was dissected and sonicated in T-PER tissue protein extraction reagent (Thermo Scientific, Rockford, IL) containing protease (Roche Diagnostic, Mannheim, Germany) and phosphatase inhibitor cocktails (Sigma). The lysates were centrifuged at
12,000 rpm for 10 minutes at 4°C and the supernatants were used for immunoblotting. Protein concentrations were determined using the BCA method (Thermo Scientific). Samples with equal total protein (1 µg/µl) were separated on a 4-12% Nupage Bis-Tris gel (Invitrogen, Carlsbad, CA) and transferred onto a nitrocellulose membrane (Invitrogen). The blots were blocked and incubated overnight at 4°C with goat anti-HIF-1α (1:500, Santa Cruz, Santa Cruz, CA), rabbit anti-VEGF (1:1,000, Abcam), rabbit anti-acetylated histone-H3 at K9 and K14 (1:3,000, Millipore), rabbit anti-acetylated histone-H4 at K12 (1:500, Millipore), or mouse anti-β-actin (1:20,000, Sigma) in blocking buffer (LI-COR, Lincoln, NE) with 0.1% Tween 20. After washing, the membranes were incubated with appropriate IRdye 680 or 800CW conjugated secondary antibodies (1:10,000, LI-COR) for 1 hour at room temperature. Blotted proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR).

Gelatin Zymography

MMP-2/9 activities were measured by gelatin zymography as described previously. Briefly, brain tissues were homogenized in a lysis buffer (50 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 5 mmol/L CaCl$_2$, 0.05% BRIJ-35, 0.02% NaN$_3$, 1% Triton X-100), and centrifuged. Aliquots containing equal amounts of protein (4 µg/µl) from each sample were incubated for 1 hour with gelatin-Sepharose 4B (GE Healthcare, Little Chalfont, UK) with constant shaking. The pellets were washed with a working buffer (lysis buffer without Triton X-100) and resuspended in 100 µl of elution buffer (working buffer with 10% dimethylsulfoxide) for 30 minutes and then centrifuged. The samples were loaded on 10% Zymogram Gelatin Gels (Invitrogen, Carlsbad, CA, USA). After electrophoresis, the gels were incubated in Renaturing Buffer (Invitrogen) for 1 hour at room temperature and then Developing Buffer (Invitrogen) overnight at 37°C. The gels were stained for 1 hour in 1% Coomassie blue (Invitrogen) and then washed with water to obtain the clearest background for photography. Pro- and active forms of MMP-2/9 were identified by reference to their respective standards in gelatin zymography.

Rat brain microvascular endothelial cells (RBMVECs)

Cryopreserved primary RBMVECs were purchased at the 2nd passage from Cell Applications (San Diego, CA). Attachment factor solution (Cell Applications, San Diego, CA) was used to coat the cell culture flasks and multi-well plates at a concentration of 0.1 ml/cm$^2$. Cells were plated at a density of 10,000 cells/cm$^2$ and expanded in rat brain endothelial cell growth medium (Cell Applications) at 37°C in a humidified atmosphere containing 95% air and 5% CO$_2$, according to the manufacturer’s instructions. RBMVECs used in this study were from the 3rd to 5th passage.

Cobalt chloride (CoCl$_2$) and 2ME2 treatment

To determine the optimal CoCl$_2$ treatment time for inducing HIF-1α, RBMVECs were treated with 1 mmol/L CoCl$_2$ for 3, 6, 9 and 24 hours, and cell lysates were collected for Western blotting. To inhibit CoCl$_2$-induced HIF-1α activation, RBMVECs were pre-treated with 2ME2 (10 µmol/L) for 24 hours and then co-treated with 1 mmol/L CoCl$_2$ for 6 hours. Cells lysates were collected for RNA isolation or Western blotting.

RNA isolation, reverse transcription, and quantitative real-time PCR
Total RNA was extracted from RBMVECs using an RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA synthesis was performed from 2 µg of total RNA for each sample using a cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) as per the provided protocols. Real time PCR reactions were run in duplicate for each sample on a 7500 Real Time PCR System (Applied Biosystems), as previously described. Taqman primers (VEGF, MMP-2 and MMP-9) were used with β-actin internal control primers (Applied Biosystems) for each sample.

**Oxygen-glucose deprivation (OGD)**

To induce OGD, the culture medium was washed out with PBS and replaced with deoxygenated glucose-free DMEM medium. Cultures were then placed in a Modular Incubator Chamber (Billups-Rothenberg, Del Mar, CA) containing a gas mixture of 5% CO₂ and 95% N₂ at 37°C for 3 hours. Control cells were placed in glucose-containing DMEM medium for the same time interval under normoxic condition. After 3 hours, all culture medium was replaced with fresh rat brain endothelial cell growth medium and cells were returned to normoxic conditions for 24 hours of reperfusion. For VPA-treated cells, the drug was added to the cells at the beginning of OGD at a final concentration of 1 mmol/L, and was supplemented again immediately after reperfusion. Cell viability was measured and cell lysates were collected for Western blotting.

**Measurement of cell viability**

Cell viability was evaluated by the mitochondrial dehydrogenase activity to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), as previously described. RBMVECs were incubated with 125 µg/ml MTT for 3 hours at 37°C. The medium was then aspirated, and the formazan product was dissolved in dimethylsulfoxide and quantified spectrophotometrically at 540 nm. Results are expressed as a percentage of viability of the control cultures.
SUPPLEMENTAL FIGURES

Figure S1. Post-ischemic VPA treatment significantly reduced infarct area in MCAO rats. Hematoxylin and eosin staining was used to measure brain infarct area on day 14 after MCAO. Top: representative brain slices. Bottom: quantified results. N = 10 per group; **P<0.01 compared with MCAO group.

Figure S2. Regions of interest (ROIs) for T2-weighted imaging analysis. T2 values were analyzed at three ROIs indicated by the circled numbers on the ipsilateral hemisphere and the corresponding contralateral hemisphere on day 14 after MCAO. ROI-1 and 3 were located in the cortex and ROI-2 was in the striatum. The T2 value in ROI-3 was higher than the values in ROI-1 and ROI-2, indicating that the severity of the lesion was greatest in ROI-3.
**Figure S3.** VPA robustly increased acetylated histone-H4 (Ac-H4) levels on day 14 after MCAO. N = 6 per group. **P<0.01 compared with MCAO group.

**Figure S4.** The HIF-1α inhibitor 2ME2 had no effect on the rotarod performance of MCAO rats on days 7 and 14.
Figure S5. VEGF, MMP-2 and MMP-9 mRNA levels were upregulated by CoCl₂, a HIF-1α stabilizer. (A) HIF-1α protein levels were upregulated following CoCl₂ treatment, with peak at 6 hours. Quantified data are from two independent experiments. (B) 2ME2, a HIF-1α inhibitor, markedly inhibited CoCl₂-induced HIF-1α protein upregulation. Quantified data are from three independent experiments. (C) VEGF, MMP-2 and MMP-9 mRNA levels were robustly upregulated after 6-hour CoCl₂ treatment. 2ME2 almost completely reversed the VEGF mRNA increase, partially inhibited the MMP-9 mRNA increase, but did not affect MMP-2 mRNA levels. Data are from three independent experiments. **P<0.01 compared with control; ***P<0.01 compared with OGD group.
Figure S6. VPA enhanced OGD-induced HIF-1α upregulation and protected RBMVECs from OGD insult. (A) OGD for 3 hours followed by reperfusion for 24 hours significantly upregulated HIF-1α protein levels, and VPA treatment potentiating this upregulation. VPA did not affect HIF-1α protein levels under control conditions. Quantified data are from four independent experiments. (B) OGD significantly reduced RBMVECs viability. VPA treatment at 1 mmol/L significantly increased cell viability under OGD insult, but had no effect under control conditions. Data are from three independent experiments performed in triplicate. *P<0.05, **P<0.01 compared with control; *P<0.05 compared with OGD group.
SUPPLEMENTAL REFERENCES


4. Leng Y, Chuang DM. Endogenous alpha-synuclein is induced by valproic acid through histone deacetylase inhibition and participates in neuroprotection against glutamate-induced excitotoxicity. *J Neurosci.* 2006;26:7502-7512