Hypoxic Induction of Endoglin via Mitogen-Activated Protein Kinases in Mouse Brain Microvascular Endothelial Cells

Yonghua Zhu, MD; Yunjuan Sun, MD; Lin Xie, BS; Kunlin Jin, MD, PhD; Nader Sheibani, PhD; David A. Greenberg, MD, PhD

Background and Purpose—Endoglin (CD105) is a membrane glycoprotein that is mutated in hereditary hemorrhagic telangiectasia (Osler-Rendu-Weber disease) and shows increased expression in proliferating endothelial cells during angiogenesis.

Methods—We investigated the effect of hypoxia on endoglin expression in murine cerebral microvascular endothelial (bEND.3) cells in vitro and the possible involvement of mitogen-activated protein kinase (MAPK) pathways.

Results—Hypoxia increased endoglin mRNA and protein expression in bEND.3 cells, which was associated with phosphoactivation of extracellular signal–related kinase (ERK), p38 MAPK, and Jun amino-terminal kinase (JNK). Inhibitors of p38 decreased hypoxic induction of endoglin expression, as did dominant negative MAPK kinase 3 (MKK3), which activates p38. In contrast, constitutively active MKK3 or JNK1 potentiated the hypoxic induction of endoglin.

Conclusions—These results indicate that hypoxia induces the expression of endoglin at both the mRNA and protein levels and that induction is regulated by the p38 and perhaps also JNK pathways. (Stroke. 2003;34:2483-2488.)

Key Words: angiogenesis ■ brain ■ endoglin ■ endothelium ■ hypoxia ■ mitogen-activated protein kinases ■ protein kinases

Hypoxia-induced angiogenesis increases blood flow and oxygen delivery to ischemic tissues and may contribute to recovery after stroke. Angiogenesis occurs in human brain after stroke, with endothelial cell proliferation after 3 to 4 days, a dense capillary network by 1 week, and neovascular infiltration by 2 to 4 weeks. Within 3 months, microvessel density in the infarct area increases relative to the contralateral side, and extracts of infarcted brain tissue induce angiogenesis in chick chorioallantoic membranes. These alterations may help to restore cerebral blood flow in stroke survivors and protect against recurrent ischemia. Therefore, investigating mechanisms of angiogenesis after cerebral hypoxia or ischemia may provide new insights into stroke pathophysiology and treatment.

Endoglin (CD105) is a homodimeric membrane protein that binds transforming growth factor (TGF)-β1 and -β3. Loss-of-function mutations in the human gene ENG cause hereditary hemorrhagic telangiectasia (Osler-Rendu-Weber disease), which produces capillary and venous telangiectases affecting the skin, mucous membranes, and respiratory, gastrointestinal, and urinary tracts. Endoglin is upregulated in tumor vasculature, and the density of endoglin-immunopositive microvessels correlates inversely with cancer survival. Endoglin is also overexpressed in endothelial cells of healing wounds, embryos, psoriatic skin, and rheumatoid synovia, suggesting that it is an endothelial proliferation marker. Vascular smooth muscle development and endothelial remodeling are defective in endoglin-knockout mice.

Hypoxic regulation of gene expression involves extracellular signal–related kinase (ERK), which is widely associated with cell survival, and p38 mitogen-activated protein kinase (MAPK) and Jun amino-terminal kinase (JNK), which are implicated in cell death. However, little is known regarding expression of endoglin in cerebral endothelial cells under hypoxic or ischemic conditions. To investigate how hypoxia and ischemia regulate endoglin expression in cerebral microvascular endothelial cells and to determine which signaling pathways are involved, we examined expression of endoglin mRNA and protein in hypoxic bEND.3 mouse brain capillary endothelial cells and in focal cerebral ischemia in mice. bEND.3 cells exhibit endothelial properties, including expression of von Willebrand factor (vWF), vascular endothelial growth factor receptors, E-selectin, platelet–endo-
thelial cell adhesion molecule-1, vascular cell adhesion molecule-1, mucosal vascular addressin cell adhesion molecule-1, endothelial NO synthase, and endomucin. bEND.3 cells can internalize acetylated low-density lipoprotein and produce capillary-like tubes in 3-dimensional cultures. They form gap junctions and respond to mechanical injury with increased expression of connexin 43. Our results show that hypoxia and ischemia induce expression of endoglin at both the mRNA and protein levels and that induction is regulated by the p38 pathway.

### Materials and Methods

#### Reagents

MAPK kinase (MEK) inhibitor PD98059, p38 inhibitors SB202190 and SB203580, and SB202474 were from Calbiochem. JNK inhibitor dicumarol was from Sigma, and media and sera were from Cellgro.

#### Cell Culture

bEND.3, a polyoma middle T-transformed mouse brain capillary endothelial cell line, was maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. TS11, a cell line derived from bEND.3 cells transfected with human thrombospondin-1, was produced as previously described and was cultured in Dulbecco’s modified Eagle’s medium containing hygromycin B (50 μg/mL, Gibco-BRL).

#### Hypoxia

Hypoxic cultures were maintained in modular incubator chambers (Billups-Rothenberg) for 0 to 24 hours at 37°C in humidified 95% N2/5% CO2. Hypoxic cultures were maintained in modular incubator chambers at 24 hours (n=3). Inhibitors were added 1 hour before hypoxia. Endothelial cell dysfunction or death was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Secondary antibodies were rhodamine-conjugated goat anti-rat IgG.

#### Real-Time Polymerase Chain Reaction

mRNA was extracted, reverse transcribed, and quantified by real-time polymerase chain reaction (PCR). Endoglin-specific primers were 5’-CTGCCAATGCTGTGCGTGAA-3’ (forward) and 5’-GCTGGAGTCGTAGGCCAAGT-3’ (reverse); mouse β-actin-specific primers 5’-ACCGAGCGTGGCTACAGCTT-3’ (forward) and 5’-TCAGGCGATCGTATACGCTT-3’ (reverse) were used as controls. The thermal profile was 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 1 minute. In each plate, a dilution series of cDNA samples from untreated bEND.3 cells was run along with unknown samples. Data were analyzed with the use of ABI PRISM 7000 Sequence Detection System 1.0. All reactions were repeated at least 3 times to ensure reproducibility. Levels relative to normoxic reference samples were reported after normalization to β-actin, which was not altered by treatment.

#### Western Blotting

Fifty-microgram protein samples were loaded on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and blotted onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad), which were probed with primary antibody at 4°C overnight and secondary antibody at room temperature for 1 hour; signals were detected by chemiluminescence. Primary antibodies were rabbit anti-p38 (Santa Cruz); rat monoclonal anti-mouse endoglin (Cymbus Biotechnology Ltd); rabbit polyclonal anti-ERK1/2 (Promega); mouse monoclonal anti-β-actin (Sigma); and rabbit polyclonal anti-phospho(Ser 15)-p53, polyclonal anti-phospho(Thr202/Tyr204)-MAPK (ERK1/2), anti-phospho(Thr180/Tyr182)-p38 MAPK, and anti-phospho-JNK (Cell Signaling Technology, Inc).

#### Plasmids and Transfection

Expression constructs encoding constitutively active MAPK kinase 3 (MKK3) [pRC/RSV-Flag-MKK3(Glu)] and dominant negative MKK3 [pRC/RSV-Flag-MKK3(Ala)]23,25 were provided by Dr. Roger Davis, University of Massachusetts Medical School. Constitutively active JNK1 (pCMV-Flag-JNK1)26 was provided by Dr. Roya Khosravi-Far, Harvard Medical School. A construct encoding green fluorescent protein (pEGFP, Clontech) was used to verify transfection efficiency and as a control. Transient transfections were performed with the use of Lipofectamin 2000 (Invitrogen).

#### Focal Cerebral Ischemia

Ischemia was induced in adult male Charles River CD1 mice by intraluminal middle cerebral artery occlusion (MCAO) with a suture, in accordance with institutional and National Institutes of Health guidelines. Physiological measurements, including arterial blood gas measurements and temperature, were performed, and the ischemic penumbra was identified by laser-Doppler flowmetry, as described. The suture was left in place for 90 minutes and then withdrawn to allow reperfusion, and mice were killed 28 days later.

#### Immunohistochemistry

Frozen sections were fixed with methanol at −20°C for 10 minutes, incubated overnight at 4°C with rat monoclonal anti-mouse endoglin (Cymbus Biotechnology Ltd: 1:10), visualized with the use of Vectastain ABC kits (Vector Laboratories Inc) with dianibenzidine as substrate, and counterstained with hematoxylin. For double-label immunocytochemistry, sections were fixed with 4% paraformaldehyde in PBS for 1 hour at room temperature, washed twice with PBS, and incubated in blocking solution with primary antibodies at 4°C overnight and with secondary antibodies at room temperature for 2 hours. Primary antibodies were the aforementioned endoglin antibody and rabbit polyclonal anti-vWF (Sigma; 1:500); secondary antibodies were rhodamine-conjugated goat anti-rat IgG.
Results

bEND.3 cells proliferate rapidly in culture. Expression of thrombospondin-1 in bEND.3, producing cells designated TS11, reduces growth rate, saturation density, fibrinolytic activity, and ability to form hemangiomas and increases cord formation.17,22 TS11 cells expressed lower levels of endoglin mRNA and protein than bEND.3 cells transfected with control vector (Figure 1), consistent with proliferation-associated induction of endoglin.

Endoglin expression after focal ischemia was examined in brain sections up to 28 days after MCAO with reperfusion. Endoglin immunoreactivity increased in the ischemic core compared with the same brain area on the contralateral side and was associated with vascular endothelial cells, as evidenced by colocalization with vWF expression (Figure 2).

Statistical Analysis

Experiments were repeated at least 3 times. Quantitative results were expressed as mean±SEM. ANOVA and t tests were used for statistical analysis, with \( P<0.05 \) considered significant.

To determine the effect of hypoxia on endoglin expression, bEND.3 cells were exposed to hypoxia for 0 to 24 hours. Upregulation of endoglin mRNA (30% increase) was observed 4 hours after hypoxia and reached highest levels (90% increase) at 24 hours (Figure 3A); endoglin protein expression also increased (Figure 3B and 3C).

To investigate mechanisms of endoglin upregulation, we used antibodies against phosphoactivated ERK, p38, and
JNK. Western blots showed phosphoactivation of ERK1/2 and p38, and to some extent JNK, after 16 to 24 hours of hypoxia (Figure 4A and 4B). Total ERK1/2 (not shown) and p38 were unchanged. Thus, hypoxia appears to activate 3 major MAPK systems in bEND.3 cells.

Next we assessed the effects of MEK inhibitor PD98059 (20 μmol/L), p38 inhibitor SB203580 or SB202190 or the inactive analogue SB202474 (all 5 μmol/L), added 1 hour before hypoxia, on hypoxic induction of endoglin in bEND.3. Both p38 inhibitors and dicumarol reduced endoglin mRNA levels in hypoxic cultures, whereas PD98059 and SB202474 did not (Figure 5A). Levels of endoglin protein in hypoxic cultures were also decreased by p38 or JNK, but not MEK, inhibitors (Figure 5B). Dicumarol also reduced MTT absorbance (Figure 5C), consistent with impaired metabolic function or reduced cell viability, and therefore no conclusion regarding the role of JNK in endoglin induction could be made. Thus, p38 but not MEK/ERK helps to mediate the effect of hypoxia on endoglin expression in bEND.3 cells.

Finally, we transfected bEND.3 cells with expression constructs encoding constitutively active or dominant negative MKK3, which specifically phosphorylates and activates p38 or constitutively active JNK1, and measured levels of endoglin mRNA 48 hours later. In normoxic cultures, endoglin mRNA expression was also decreased by p38 or JNK, but not MEK, inhibitors (Figure 6C). Dicumarol also reduced MTT absorbance (Figure 6C), consistent with impaired metabolic function or reduced cell viability, and therefore no conclusion regarding the role of JNK in endoglin induction could be made. Thus, p38 but not MEK/ERK helps to mediate the effect of hypoxia on endoglin expression in bEND.3 cells.

Figure 4. Hypoxia increases ERK, p38, and JNK phosphoactivation in bEND.3 cells. A, Cells were exposed to hypoxia for 0 to 24 hours, and 50-μg protein samples from whole cell extracts were transferred to PVDF membranes for Western analysis of phosphoprotein expression, with total p38 as a control. B, Phosphoprotein expression was quantified by computer densitometry, normalized to β-actin, and expressed as percent control expression at 0 hours (mean±SE; n=3).

Figure 5. Effects of protein kinase inhibitors on hypoxia-induced endoglin mRNA and protein expression in bEND.3 cells. Cells were treated for 1 hour with the MEK inhibitor PD98059 (PD), the p38 inhibitor SB203580 (SB1), the inactive analogue SB202474 (SB2), or the JNK inhibitor dicumarol (Di) and then exposed to hypoxia (H) for 16 hours. A, Total RNA was extracted and reverse transcribed, and cDNA was used in real-time PCR to examine endoglin mRNA expression relative to β-actin. Values (mean±SEM; n=3) are expressed relative to reference (0 hours) samples and normalized to β-actin (*P<0.05, **P<0.01 vs normoxic control at left). B, Protein samples from whole cell extracts were loaded on 10% SDS-PAGE gels and transferred to PVDF membranes for Western analysis of endoglin expression. Blots were stripped and reprobed with anti-β-actin. C indicates control.
endoglin, activates p38- and -γ. Therefore, stimulation of endoglin expression by hypoxia is likely conveyed at least partly through MKK3 and p38-α. How p38 or JNK might regulate hypoxic induction of endoglin is unclear, but p38 is implicated in hypoxic induction of other proteins, including inducible NO synthase and hypoxia-inducible factor-1α.

Acknowledgments
This study was supported by National Institutes of Health grants NS-35965 and NS-37695.

References
18. Sikorski EE, Hallmann R, Berg EL, Butcher EC. The Peyer’s patch high endothelial receptor for lymphocytes, the mucosal vascular addressin, is


Hypoxic Induction of Endoglin via Mitogen-Activated Protein Kinases in Mouse Brain Microvascular Endothelial Cells
Yonghua Zhu, Yunjuan Sun, Lin Xie, Kunlin Jin, Nader Sheibani and David A. Greenberg

Stroke. 2003;34:2483-2488; originally published online August 28, 2003;
doi: 10.1161/01.STR.000088644.60368.ED
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/34/10/2483

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/