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 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, and normoxic cultures were maintained in 95% air/5% CO2 in humidified 95°C. Hypoxic cultures were maintained in 95% air/5% CO2. P02 in culture medium was 156 mm Hg in normoxic cultures and 63±3 mm Hg in hypoxic cultures 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). Endothelial cell dysfunction or death was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).13

Real-Time Polymerase Chain Reaction
mRNA was extracted, reverse transcribed, and quantified by real-time polymerase chain reaction (PCR) as described. Endoglin-specific primers were 5'-GCTGGAGTCGTAGGCCAAGT-3' (forward) and 5'-GCTGGAGTCGTAGGCCAAGT-3' (reverse); mouse vWF-specific primers were 5'-ACCGAGCGTTGCTACAGCTT-3' (forward) and 5'-ACCGAGCGTTGCTACAGCTT-3' (reverse); and mouse vWF-specific primers were 5'-ACCGAGCGTTGCTACAGCTT-3' (forward) and 5'-ACCGAGCGTTGCTACAGCTT-3' (reverse). Primary antibodies were the aforementioned endoglin antibody and rabbit polyclonal anti-vWF (Sigma; 1:500); secondary antibodies were rhodamine-conjugated goat anti-rat IgG.

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 (M KK3) [pRC/RSV-Flag-MKK3(Glu)] and dominant negative MKK3 [pRC/RSV-Flag-MKK3(Ala)] were provided by Dr Roger Davis, University of Massachusetts Medical School. Constitutively active JNK1 [pCMV-Flag-JNK1], was provided by Dr Roya Khoosravi-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 diaminobenidine 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.

Figure 1. Expression of endoglin mRNA and protein in bEND.3 and TS11 cells. A, Total RNA from bEND.3 cells transfected with control vector (PMEP) or TSP-1 (TS11) was reverse transcribed. cDNA was used in real-time PCR to measure expression of endoglin relative to β-actin, and fluorescence intensity was plotted against PCR cycle number. NTC indicates no-template control. B, Values in A (mean±SE; n=3) were expressed relative to PMEP samples and normalized to β-actin (**P<0.01 vs PMEP). C, Western blotting was used to detect endoglin protein expression in the 2 cell lines.
Jackson ImmunoResearch; 1:200) and fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (Vector Laboratories; 1:200). 4',6-Diamidino-2-phenylindole (DAPI; Vector) was used to counterstain nuclei, and fluorescence signals were detected with a Nikon E800 microscope at excitation/emission wavelengths of 535/565 nm (rhodamine), 470/505 (fluorescein isothiocyanate), and 360/400 (DAPI). Results were recorded with a Magnifire digital camera (ChipCoolers). Controls included omission or preabsorption of the primary antibody or omission of the secondary antibody.

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.

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. Results were recorded with a Magnifire digital camera (ChipCoolers). Controls included omission or preabsorption of the primary antibody or omission of the secondary antibody.

Figure 2. Ischemia increases endoglin protein expression in mouse brain. Endoglin immunoreactivity (brown) is shown in ischemic (A left and B) and nonischemic (A right and C) cerebral hemispheres after 90 minutes of MCAO and reperfusion for 28 days. Outlined area in A is ischemic core; fields shown in B and C are from this area and the same area on the contralateral side, respectively. D through G show colocalization of endoglin (ENG) and vWF, with DAPI used to stain nuclei. Bar = 250 μm in A; bar = 150 μm in B and C; bar = 50 μm in D through G.

To determine the effect of hypoxia on endoglin expression, bEND.3 cells were exposed to hypoxia for 0 to 24 hours, and total RNA was extracted and reverse transcribed. cDNA was used in real-time PCR to examine expression of endoglin 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 0 hours). B, Cells were exposed to normoxia (C) or hypoxia (H) for 8 or 24 hours, and 50-μg protein samples from whole cell extracts were transferred to PVDF membranes for Western analysis of endoglin expression. Blots were stripped and reprobed with anti-β-actin. C, Endoglin protein expression was quantified by computer densitometry, normalized to β-actin, and expressed as percent control expression at 0 hours (mean ± SE; n = 3; *P < 0.01 vs normoxic control).

Figure 3. Hypoxia increases endoglin mRNA and protein in bEND.3 cells. A, Cells were exposed to hypoxia for 0 to 24 hours, and total RNA was extracted and reverse transcribed. cDNA was used in real-time PCR to examine expression of endoglin 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 0 hours). B, Cells were exposed to normoxia (C) or hypoxia (H) for 8 or 24 hours, and 50-μg protein samples from whole cell extracts were transferred to PVDF membranes for Western analysis of endoglin expression. Blots were stripped and reprobed with anti-β-actin. C, Endoglin protein expression was quantified by computer densitometry, normalized to β-actin, and expressed as percent control expression at 0 hours (mean ± SE; n = 3; *P < 0.01 vs normoxic control).
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 \(\mu\)mol/L), p38 inhibitor SB203580 or SB202190 or the inactive analogue SB202474 (all 5 \(\mu\)mol/L), or the JNK inhibitor dicumarol (100 \(\mu\)mol/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\(^{25}\) or constitutively active JNK1, and measured levels of endoglin mRNA 48 hours later. In normoxic cultures, endoglin mRNA expression was increased by approximately 45% by constitutively active MKK3(Glu) and by approximately 40% by constitutively active JNK1 and was reduced by approximately 40% by dominant negative MKK3(Ala) (Figure 6). In hypoxic cultures, dominant negative MKK3(Ala) markedly reduced hypoxic upregulation of endoglin mRNA, while constitutively active MKK3(Glu) or JNK1 enhanced the effect of hypoxia (Figure 6). These results suggest that activation of p38, and perhaps JNK1, is involved in hypoxic induction of endoglin expression in bEND.3 cells.
of endoglin, activates p38-α and -γ.34 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 synthase35 and hypoxia-inducible factor-1α.36

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