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

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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
tial 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 SB20474 were from Calbiochem. JNK inhibitor dicumarol was from Sigma, and media and sera were from Cellgro.

Cell Culture

bEND.3, a polyaoma 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 cultures and 63 mm Hg in hypoxic cultures at 24 hours (n=3). Nontreated bEND.3 cells were 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. PO2 in culture medium was 156±5 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).15

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’-CTGCTAGGGCTAGCGGAGATG-3’ (forward) and 5’-ACCGAGCTGGCTACAGCTT-3’ (reverse); mouse β-actin-specific primers 5’-ACGGAGCTGGCTACAGCTT-3’ (forward) and 5’-TCAGCGAGCTCAGCTT-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)] were provided by Dr. Roger Davis, University of Massachusetts Medical School. Constitutively active JNK1 (pCMV-Flag-JNK1) was provided by Dr. Roya Khorasvi-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 diaminobenzidine 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.
(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 and 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).

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).

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), and JNK inhibitor dicumarol (100 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 and 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.
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Discussion
Angiogenesis is a well-documented consequence of focal cerebral ischemia. We found that endoglin expression correlates with endothelial cell proliferation and that hypoxia and ischemia upregulate endoglin expression, and we identified some of the signaling pathways involved. Hypoxic upregulation of endoglin expression may be mediated through p38 MAPK and JNK since hypoxic upregulation of endoglin was reduced by p38 inhibitors and by a dominant negative form of the p38-activating kinase MKK3 and was increased by constitutively active forms of MKK3 and JNK1.

bEND.3 cells are mouse brain endothelial cells formed by polyoma middle T oncogenes. As noted above, they exhibit numerous properties of untransformed endothelial cells. Like other cell lines, they have advantages and disadvantages compared with primary cell cultures; advantages include the ability to propagate indefinitely, which yields large amounts of protein for study, and cellular homogeneity, which facilitates molecular analysis. The finding that endoglin expression also increased in ischemic mouse brain in vivo helps to validate this approach.

The effects of hypoxia on MAPK activation depend on cell type and conditions. In cultured cortical neurons, hypoxia activated ERK and p38 but decreased activation of JNK. Hypoxia also activates ERK and p38, but not JNK, in PC12 cells and ERK, p38, and JNK in rat pulmonary artery. In this study ERK, p38, and JNK were all phosphoactivated by hypoxia in bEND.3 cells, but studies with kinase inhibitors and constitutively active or dominant negative enzyme constructs implicated only p38 and perhaps JNK1 in induction of endoglin expression. SB202190 and SB203580, which reduced hypoxic induction of endoglin, are potent inhibitors of the p38-α and -β but not -γ or -δ isoforms. MKK3, constitutive expression of which enhanced hypoxic induction of endoglin, activates p38-α 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.

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Stroke. 2003;34:2483-2488; originally published online August 28, 2003;
doi: 10.1161/01.STR.0000088644.60368.ED
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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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:
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