Cerebral Capillary Endothelial Cell Mitogenesis and Morphogenesis Induced by Astrocytic Epoxideicosatrienoic Acid

Chenyang Zhang, MD, PhD; David R. Harder, PhD

Background and Purpose—Epoxyeicosatrienoic acids (EETs) are products of cytochrome P450 epoxygenation of arachidonic acid. We have previously demonstrated that astrocyte-conditioned medium induced mitogenesis in brain capillary endothelial cells. The goals of the present studies are to further define the mechanism through which this can occur and to confirm that EETs are derived from astrocytes, through which astrocytic activity can regulate cerebral angiogenesis in response to neuronal activation.

Methods—Astrocytes and cerebral capillary endothelial cells in primary cultures were cocultured to examine the interaction of the 2 cell types. We used multiple immunohistochemical techniques to characterize the multicellular nature of the capillaries, which is not simply an artifact related to the culture conditions. The mitogenic effect of EETs was determined by \(^{3}\)H-thymidine incorporation and cell proliferation assay. Endothelial tube formation was examined in vitro and in vivo with the use of a reconstituted basement membrane (Matrigel) assay.

Results—In cocultures of astrocytes and capillary endothelium, we observed morphological changes in both cell types such that each assumed certain physiological characteristics, ie, endothelial networks and astrocytes with “footlike” projections as well as intermittent gap junctions forming within the endothelial cells. EETs from astrocytes as well as synthetic EETs promoted mitogenesis of endothelial cells, a process sensitive to inhibition of tyrosine kinase with genistein. Treatments with exogenous EETs were sufficient for endothelial cells to differentiate into capillary-like structures in culture as well as in vivo in a Matrigel matrix.

Conclusions—The 2 major conclusions from these data are that astrocytes may play an important role in regulating angiogenesis in the brain and that cytochrome P450–derived EETs from astrocytes are mitogenic and angiogenic. (Stroke. 2002;33:2957-2964.)

Key Words: angiogenesis ■ capillaries ■ cells, cultured ■ cytochrome P-450 ■ gap junctions ■ rats

Astrocytes in the central nervous system are anatomically situated between neurons and the microcirculation, forming “foot processes” that impinge on arteriolar and capillary networks. Traditionally, it has been assumed that astrocytes play an organizational role, keeping neurons and capillary networks in register and supporting the blood-brain barrier. Recently, the roles of astrocytes with respect to cross talk with neurons and cerebral vasculature have been reexamined, and it has been suggested that astrocytes could be key regulatory elements in the brain.\(^{1-3}\) In the retina, astrocytes have been reported to be required for capillary angiogenesis since the migration of astrocytes into developing retina precedes formation of the retinal vasculature.\(^{4-7}\) The mechanism underlying astrocyte-involved angiogenesis in the brain is largely unknown.

In a previous communication\(^{8}\) we showed that astrocyte-conditioned culture media stimulated proliferation of cerebral microvascular endothelial cells in culture. Formation of capillary tubes in the coculture of astrocytes and endothelial cells was blocked on inhibition of cytochrome P450 (P450) enzymes.\(^{8}\) One such enzyme is encoded by the cytochrome P450 2C11 (CYP2C11) gene. We have cloned and sequenced CYP2C11 cDNA from astrocytes of rats.\(^{9}\) Epoxyeicosatrienoic acids (EETs) are biologically active metabolites of arachidonic acid (AA), the formation of which is catalyzed by P450 epoxygenase. There are 4 regioisomers of EETs, namely, 5,6-EETs, 8,9-EETs, 11,12-EETs, and 14,15-EETs.\(^{10,11}\) Previous studies have shown synthesis of EETs by astrocytes.\(^{9,12}\) Under normal conditions, EETs were found in the cerebrospinal fluid measured in micromolar concentrations.\(^{3}\) Pharmacological inhibition of EET formation blocked functional hyperemic response to glutamate infusion in the brain, suggesting a role of EETs in regulating cerebral blood flow to match neuronal activity.\(^{14-16}\) Therefore, we hypothesized that P450-derived EETs were involved in astrocyte-induced mitogenesis and morphogenesis of cerebral capillary
endothelial cells. The role of EETs as intercellular and intracellular mediators involved in cell proliferation and angiogenesis in the central nervous system has never been systematically defined before the present study.

In this report we define the actions of P450-derived EETs, exogenously added or released from astrocytes, on proliferation and differentiation of cerebral capillary endothelial cells. We demonstrate that EETs function as angiogenic regulators for astrocyte-mediated capillary network formation.

**Materials and Methods**

**Cell Cultures**

We prepared primary cultures of astrocytes from hippocampi of postnatal 3-day-old rat pups as described previously.\(^6\) Cerebral capillary endothelial cells were prepared from brains of 4-week-old rats as described previously\(^6\) with some modifications, as outlined in Figure 1. Briefly, the cerebral cortex was dissected and homogenized in ice-cold HEPES-buffered salt solution with 0.9% glucose. Vascular tissues were separated from the rest of brain tissue by centrifugation in a HEPES-buffered salt solution containing 15% dextran. The vascular tissue was filtered through a 150-mesh screen to remove large vessels. The elutes were loaded on a glass bead column. Capillaries adhering to the beads were released by sharply shaking the beads in buffer. The microvessel pellet was digested with collagenase (500 \(\mu\)g/mL) in RPMI-1640 (Biowhitaker) containing 10% fetal bovine serum (FBS) for 15 minutes at room temperature. The formation of single cell suspension was monitored under a phase-contrast microscope. After centrifugation, the cell pellet was resuspended in a \(L\)-valine–free medium (Life Technologies), which inhibits growth of other cell types but not endothelial cells,\(^6,17\) and plated in T25 flasks precoated with fibronectin at 5 \(\mu\)g/cm\(^2\). Cells were incubated at 37°C in a 95%/5% mixture of atmospheric air and CO\(_2\). After 3 days, the medium was changed to microvascular endothelial cell growth (MV) medium formulated to promote endothelial cell growth. This medium is made from serum-free endothelial cell basal medium-2 (EBM-2) supplemented with vascular endothelial growth factor (VEGF), epidermal growth factor, fibroblast growth factor, insulin-like growth factor, ascorbic acid, hydrocortisone, heparin, FBS, and antibiotics, according to the manufacturer’s instructions (Clonetics). Confluent first passage of endothelial cells was used for our experiments.

**Coculture of Astrocytes and Cerebral Capillary Endothelial Cells**

To separate microglial cells from astrocytes, cultures were shaken for 60 minutes (at 225 rpm) at 37°C on an orbital shaker.\(^8\) The adherent astrocyte monolayer was reseeded. Cocultures of astrocytes and endothelial cells were made as described previously.\(^6\) Briefly, confluent astrocytes were trypsinized and resuspended in Dulbecco’s modified Eagle’s medium containing 10% FBS and antibiotics. Cells were plated at 6000 cells per square centimeter on fibronectin-coated coverslips. After 24 hours of incubation, the medium was removed, and endothelial cells were plated at approximately 20,000 cells per coverslip in MV medium. Control coverslips lacking either the astrocytes or endothelial cells were incubated in the same medium as used for cocultures. After another 24 hours, some cultures were changed to EBM-2 containing 0.1% bovine serum albumin (BSA). Medium was changed every 3 days until processed for further experiments.

**Treatments**

For preparation of conditioned medium from astrocytes, subconfluent astrocyte cultures were washed with Dulbecco’s PBS, and Dulbecco’s modified Eagle’s medium with 0.1% BSA (fatty acid free) was added to cells for conditioning overnight. To inhibit P450 activity in astrocytes, 17-octadecynoic acid (17-ODYA) at 10 \(\mu\)mol/L was present during the whole time of conditioning. Other treatments for endothelial cells used were at the following concentrations: VEGF 1 nmol/L, MyrPKC-I 100 nmol/L, and genistein 100 \(\mu\)mol/L. Vehicle, either ethanol or buffer in the same volume as treatments, was used as control.

**\(^3\)H-Thymidine Incorporation**

Confluent cerebral endothelial cells in T25 flasks were detached by trypsin. After centrifugation, the cells were suspended in MV medium and plated at approximately 7500 cells per well into 24-well plates and incubated for 1 day so that they reached 80% confluence. The medium was then changed to EBM-2 medium with 0.1% BSA for 2 days to make cells quiescent. EETs at various concentrations in the presence or absence of inhibitors or VEGF were added to the medium and incubated for 18 hours. \(^3\)H-Thymidine at a concentration of 2 \(\mu\)Ci/mL was then added to pulse the cells for an additional 3 hours. Cells were washed 3 times with PBS and precipitated with ice-cold 15% trichloroacetic acid for 30 minutes at 4°C. Wells were washed gently with water and allowed to try. Cells were lysed with 1N NaOH and incubated at 37°C for 30 minutes. After neutralization with 1N HCl, the radioactivity from the sample of each well was
determined by liquid scintillation spectrometry. Results were expressed as counts per minute per well. Each experimental data point represented quadruplicate wells from at least 3 independent experiments. All values were expressed as mean±SD. Paired Student’s t tests were used to compare vehicle control and treatment. \( P<0.05 \) was considered significant.

Immunocytochemistry

Cells on coverslips or brain sections of 15-μm thickness were fixed with 4% paraformaldehyde. After they were blocked with 2% BSA in PBS, cells or sections were incubated overnight at 4°C with primary antibodies for single or double labeling. Specific primary antibodies used were platelet endothelial cell adhesion molecule-1 (PECAM-1) (1:1000, gift from Dr P. Newman), glial fibrillary acidic protein (GFAP) (1:150, Chemicon), OX-42 (1:100, Serotec), neurofilament 200 (1:200, Sigma), and NeuN (1:100, Chemicon). Secondary antibodies conjugated with FITC and TRITC (1:150, Chemicon) were incubated subsequently for 1 hour at room temperature under dark. For connexin 43 (Santa Cruz), coverslips were incubated with connexin 43 antibodies at 1:10 overnight, followed by rabbit anti-goat IgG conjugated to horseradish peroxidase at 1:200 for 4 hours, and labeling was detected by the diaminobenzidine reaction. After nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI), sections or cells were rinsed, and coverslips were placed. Images were taken with Nikon E600 equipped with epifluorescence and a digital camera. No signal was detected on control cells or sections, which were processed in the same way except that primary antibodies were omitted.

Dil-Ac-LDL Staining

After they were washed with PBS, cells were incubated with Dil-Ac-LDL (Biomedical Technologies Inc) at a concentration of 10 μg/mL in serum-free EBM-2 for 2 hours at 37°C. Cells were then washed and fixed with 4% paraformaldehyde for 15 minutes, followed by placement on coverslips or continuation of the immunocytochemistry protocol. The staining of Dil-Ac-LDL was examined under an inverted microscope.

Morphogenesis Assay on Matrigel

Briefly, endothelial cells were trypsinized and resuspended in MV medium to inactivate trypsin. After centrifugation, medium was removed, and cells were resuspended in plain EBM-2, plated at 2×10^5 cells per well into 4-well chamber slides coated with thin reconstituted basement membrane (Matrigel, Becton Dickinson), and incubated in the presence or absence of EETs (100 nmol/L or 200 nmol/L for 8,9-EET; 200 nmol/L for 11,12-EET). Some wells were incubated with MV medium. Eight to 18 hours later, morphology of endothelial cells was examined under an inverted phase-contrast microscope, and images were taken with an attached camera. The relative lengths of tube formed in control or treated conditions were measured on multiple images that covered the whole area of each well. The increase in tube formation was evaluated by the ratio of the total lengths of tubes in treated wells to those of controls. Paired Student’s t tests were used to compare vehicle control and treatment. \( P<0.05 \) was considered significant. The result was reproducible in at least 3 independent experiments.

Matrigel Implantation In Vivo

Matrigel implantation was conducted as described in detail previously. While being anesthetized with halothane, adult C57BL mice or Sprague-Dawley rats (aged 7 weeks; \( n=5 \) per group) were each injected subcutaneously near the abdominal midline with 0.5 mL Matrigel supplemented with or without 200 nmol/L 8,9- or 11,12-EETs with a 25-gauge needle. The animals were kept under halothane until the injected Matrigel rapidly formed a single, solid gel. After 7 to 9 days, animals were killed, and gels were recovered and fixed immediately after dissection in 4% paraformaldehyde. Matrigel blocks were dehydrated and embedded in paraffin. Paraffin sections at 5-μm thickness were stained with hematoxylin and eosin. The number of vascular structures containing red blood cells per \( \times40 \) microscopic view on each section was counted and expressed as mean±SD. Paired Student’s t tests were used to compare vehicle control and treatment. \( P<0.05 \) was considered significant. The result was reproducible in 3 independent experiments.

Results

Interaction of Astrocytes and Capillary Endothelial Cells in Coculture

As shown in Figure 1, we were able to isolate the capillary fraction (Figure 1a) from the cerebral cortical tissue homogenates. Endothelial cells in culture exhibited classic cobblestone or spindle-shaped morphology. The use of L-valine–free medium combined with specially formulated microvascular endothelial cell growth medium helped to ensure high purity of endothelial cells and characteristic morphology (Figure 1b, 1d, 1e) compared with results obtained with the use of RPMI 1640 with 10% FBS, which contained contaminated cell types (arrows in Figure 1c). The contaminated cells often increased in number with time and overran the endothelial cells. The confluent cerebral capillary endothelial cells were positive for Dil-Ac-LDL (Figure 1d) and immunoreactive to PECAM-1 (Figure 1e), an adhesive molecule used as a marker for endothelial cells. We used endothelial cell cultures with 93±2% purity. The contaminated cell type was mainly smooth muscle cell, which is not immunoreactive to PECAM-1 but can be labeled with antibodies against α-smooth muscle actin.

Astrocytes in culture exhibited positive immunoreactivity to GFAP. When becoming confluent, 98±1% of cells in culture were astrocytes identified by GFAP immunoreactivity (Figure 2a). A few macrophage/microglial cells were found in enriched astrocyte cultures with the use of antibodies against OX-42 (Figure 2b). Neurons were not detectable in our confluent astrocyte cultures in which antibodies against neurofilament 200 (Figure 2c) and NeuN (data not shown) were used.
It has been shown previously that astrocytes can induce formation of endothelial tubelike structures in vitro. However, the interaction of 2 cell types in coculture has not been examined in detail. We cocultured primary rat astrocytes and cerebral capillary endothelial cells over a fibronectin-coated coverslip. Endothelial cells were identified by either DiI-Ac-LDL staining or PECAM-1 immunocytochemistry. Astrocytes were detected with antibodies against GFAP. Endothelial cell tubelike structures were evident at day 2, the earliest time examined. At day 5 in coculture, tubelike networks were clearly visible under a phase-contrast microscope. As can be seen in serial microscopic photography, dramatic morphological changes were found in both cell types. Endothelial cells forming tubes were positive for DiI-Ac-LDL (arrows in Figure 3a) and PECAM-1 (arrows in Figure 3b). Astrocytes sent foot processes and impinged on endothelial tubes intermittently (arrowheads in Figure 3a and 3b). Figure 3c shows a brain section in which endothelial cells were stained with PECAM-1 and astrocytes were stained with GFAP. A similar physiological arrangement between astrocytes and endothelial cells in coculture (Figure 3a and 3b) can be seen that mimics the interaction of astrocytes and endothelial cells in vivo (Figure 3c). Neither astrocytes nor endothelial cells showed the aforementioned differentiation when they were cultured alone. Cells forming tubes (identified by DAPI staining in Figure 3e) also exhibited punctate staining for connexin 43 in their cell-cell borders, an evidence of the formation of gap junction (Figure 3d and insert). However, cells outside of the tube structures showed light cytoplasmic staining of connexin 43 (Figure 3f).

**EETs Increase ³H-Thymidine Incorporation in Cerebral Capillary Endothelial Cells**

We have shown previously that endothelial tube formation was inhibited by treating the coculture with 17-ODYA, a P450 inhibitor. However, we cannot distinguish whether astrocytic P450 or endothelial P450 was involved. To answer this question, we treated astrocytes with 17-ODYA and collected conditioned medium. Astrocyte-conditioned medium in the absence of 17-ODYA showed a significant mitogenic effect on capillary endothelial cells (Figure 4). Augmentation in ³H-thymidine incorporation in endothelial cells was reduced by the conditioned medium that was made in the presence of 17-ODYA to inhibit P450 enzymatic activity in astrocytes (Figure 4). Treating endothelial cells with conditioned medium and 17-ODYA to inhibit endothelial P450 did not block increased ³H-thymidine incorporation (Figure 4), demonstrating that it was the product of P450 in astrocyte-conditioned medium, not in endothelial cells, that was involved in promoting mitogenesis of endothelial cells. 

![Figure 3](image1.png)

**Figure 3.** Changes in morphology of astrocytes and cerebral capillary endothelial cells when cocultured. a, Formation of capillary-like structures (arrow) double labeled with DiI-Ac-LDL (red) and GFAP (green) in the coculture of astrocytes and cerebral capillary endothelial cells. b, Formation of capillary-like structures (arrow) triple labeled by PECAM-1 (green), GFAP (red), and DAPI (blue) in the coculture of astrocytes and cerebral capillary endothelial cells. Note the interaction between astrocytes and endothelial tubes (arrowheads in a and b). c, Double immunolabeling of blood vessels and astrocytes with PECAM-1 (green) and GFAP (red) in a section from normal rat cortex showing astrocytes form foot processes that impinge on vessels (arrows). d, Cells (*) forming tube identified by DAPI staining (e) in coculture exhibit punctate staining of connexin 43 on their cell-cell borders (arrow). Arrow points to area shown in the insert at higher magnification. f, Cells (*) outside tubes in the same coculture as in d show moderate to light cytoplasmic staining of connexin 43. Bar in a=25 μm for a and b; bar=20 μm in c; bar in f=10 μm for d, e, and f; bar in the insert of d=5 μm.

![Figure 4](image2.png)

**Figure 4.** ³H-Thymidine incorporation in primary cerebral capillary endothelial cells (EC) after treatment with astrocyte-conditioned media. Conditioned media were prepared as described in Materials and Methods. Inhibition of P450 activity by 17-ODYA in astrocytes (AS) significantly (P<0.01) attenuated ³H-thymidine incorporation compared with conditioned medium without 17-ODYA inhibitor. **P<0.01 vs vehicle; ###P<0.01 vs conditioned media.
The cytochrome P450 2C11 gene encodes an epoxygenase that produces all 4 regioisomers of EETs in astrocytes. When we treated endothelial cells with EETs, all 4 isomers were mitogenic in cerebral capillary endothelial cells, among which 8,9-EET was the most potent and 5,6-EET was the least potent. The dose-dependent relationship of 8,9-EET on increasing \(^{3}\)H-thymidine incorporation is shown in Figure 5a. VEGF treatment was used as a positive control of a known angiogenic mitogen for endothelial cells\(^{21}\) (Figure 5a and 5b). Both 8,9-EET and VEGF at their most effective concentrations significantly increased cell numbers (Figure 5b).

To begin to explore the pathway of EET-induced mitogenic effect, we examined \(^{3}\)H-thymidine incorporation before and after treatment with an inhibitor of protein kinase C (PKC), Myr\(^{6}\)PKC-I, at a concentration tested to inhibit PKC activity previously\(^{22}\) and an inhibitor of tyrosine kinase, genistein. As can be seen in Figure 6, whereas Myr\(^{6}\)PKC-I did not inhibit \(^{3}\)H-thymidine incorporation, genistein completely abolished the effect of EETs, suggesting involvement of a tyrosine kinase in EET-induced mitogenesis. The use of these inhibitors alone had no effect on the growth of endothelial cells (Figure 6).

**EET-Induced Morphogenesis of Endothelial Cells on Matrigel**

To further characterize the mitogenic and angiogenic effects of EETs, we performed 2 sets of experiments. First, we plated capillary endothelial cells on thin Matrigel with and without EETs. Endothelial cells plated at 20,000 cells per chamber on Matrigel in the presence of 8,9-EET in EBM-2 formed capillary-like structure when examined at 18 hours after seeding (Figure 7b). Similar capillary-like structures were also induced by 11,12-EET and 14,15-EET (data not shown). The same number of cells cultured in EBM-2 alone failed to form long cordlike structures (Figure 7a). Capillary-like structures were also formed in medium containing 5% FBS and growth factors (MV medium) (see Materials and Methods and Figure 7c), which was used as a positive control. The total length of tubes formed under different treatments was measured. Tubes formed with EET treatment or with MV medium were significantly longer than those with EBM-2 (Figure 7d).

Second, we performed a standard Matrigel plug implantation to examine whether EETs were able to induce angiogenesis in vivo. In this regard, we compared the number of functional blood vessels in Matrigel plugs supplemented with or without EETs. VEGF was used as a positive control. After 9 days of subcutaneous implantation, the Matrigel blocks were recovered and examined. Matrigel formed a gel block and was readily distinguished from surrounding tissue. Matrigel with vehicle produced little or no local reaction or angiogenic response. To confirm that the observed growth of vessels in Matrigel plugs was not artifactual, we sectioned Matrigel plugs to look for functional vessels indicated by linear structures containing red blood cells after hematoxylin and eosin staining. As clearly seen in Figure 8, there was marked angiogenesis in the plug treated with 200 nmol/L 8,9-EET (Figure 8a and 8b). The number of vessels in each high-magnification view was counted. There were significantly more vessels in the Matrigel plugs treated with EET than vehicle controls (Figure 8c).

**Discussion**

Involvement of astrocytes and astrocyte-produced EETs in increasing \(^{3}\)H-thymidine incorporation and endothelial cell morphogenesis was demonstrated in the present study with the use of primary cultures of astrocytes and cerebral capillary endothelial cells. Our findings suggest that metabolites of AA formed by P450 epoxygenase in astrocytes are involved in angiogenesis in the central nervous system.
Astrocytes have been shown to participate in angiogenesis both in vitro and in vivo. Bovine retinal microvascular endothelial cells formed capillary-like structures, which exhibited a positive DiI-Ac-LDL staining, on coculture with rat brain astrocytes. When embryonic astrocytes were transplanted into the cerebral cortex of adult rats, capillary-like structures were observed at the graft-host interface zone. This finding suggests that host endothelial cells migrate toward the activated astrocytes and undergo differentiation to capillaries. The observation, in the present study, that capillary-like structures were only formed when astrocytes were cocultured with endothelial cells supports the role of astrocytes in angiogenesis. However, it must be noted that cultured astrocytes proliferate readily, which is not typical of astrocytes in vivo, although previous studies have shown that astrocytes can divide during development in adults and certainly under pathological conditions.

We showed that astrocyte-conditioned medium was mitogenic on cerebral capillary endothelial cells. Inhibition of P450 activity by 17-ODYA in astrocytes significantly reduced the conditioned medium–induced mitogenesis on brain capillary endothelial cells, whereas blockade of P450 activity in endothelial cells had no effect, indicating that mitogenic factor(s) are released from astrocytes and that their formation

---

**Figure 7.** Endothelial cell morphogenesis on Matrigel. a, Cerebral capillary endothelial cells cultured in serum-free EBM-2 on Matrigel for 18 hours. b, Cerebral capillary endothelial cells cultured in serum-free EBM-2 medium with addition of 100 nmol/L of 8,9-EET on Matrigel for 18 hours form tubelike structures. c, Cerebral capillary endothelial cells cultured in MV medium on Matrigel for 18 hours form tubelike structures. d, The effect of EETs on endothelial cell morphogenesis on Matrigel is quantitatively expressed as the ratio between the total length of tubes formed in EET-treated or MV medium compared with that in EBM-2. EET treatment significantly (*P<0.05) increased endothelial cell tube formation on Matrigel. Data represent typical images from at least 3 independent experiments. Bar=0.2 mm.

**Figure 8.** Angiogenic effect of EETs in vivo by histological analysis of recovered Matrigel plugs. a, Presence of vessels (arrowheads) in Matrigel (MG) supplemented with EETs. Note a vessel (arrows) extended into the Matrigel from the connective tissue (CT). b, High-powered micrograph of boxed area in panel a showing a well-formed vessel (arrows) with clear red blood cells and endothelium lining. c, Quantitative analysis of numbers of vessels per ×40 view in vehicle controls and EET–supplemented Matrigel blocks. Bar=50 μm in a; bar=20 μm in b.
involves activation of astrocytic P450 epoxygenase. 17-
ODYA is a potent epoxygenase inhibitor (IC_{50}=100 mmol/L). It inhibits P-450 ω-hydroxylase activity at higher concentrations. However, no study to date has shown any effect of 17-ODYA on the activity of non-P450 enzymes. The finding that AA-induced increase in \textsuperscript{3}H-thymidine uptake was blocked by nordihydroguaiaretic acid (NDGA) or low-dose ketoconazole indicates that AA metabolites, and not AA itself, act as mediators.\textsuperscript{26} Consistent with this notion, astrocytes express the epoxygenase P450 2C11, and astrocytes readily metabolize AA into EET\textsuperscript{8,27} and release EETs into medium.\textsuperscript{28} Thus, our data in the present study suggest that a P450 2C11–catalyzed production of EETs is involved in astrocyte-induced mitogenesis.

Involvement of EETs in stimulating cell proliferation has been demonstrated in other cell types. EETs have been shown to promote cell proliferation in primary cultures of rabbit proximal tubule cells.\textsuperscript{29} It has been reported that 8,9- and 14,15-EETs stimulated thymidine incorporation when they were administrated to mesangial cells in culture.\textsuperscript{26,30,31} On the other hand, inhibitors of lipoxygenase (caffeic acid) or cyclooxygenases (indomethacin) alone had no significant effects on cell growth. Given the special anatomic location of astrocytes between neurons and vascular endothelial cells, it is very possible that astrocytes function as an intermediate cell type to signal endothelial cells in responding to neural activity. Certainly, EETs were not the only factors in the astrocyte-conditioned medium that promoted mitogenesis, as indicated by the finding that 17-ODYA cannot totally abolish conditioned medium–induced mitogenic effect. However, our data suggest that EETs increase proliferation of cerebral endothelial cells with a magnitude similar to that of VEGF at their physiological concentrations and, more importantly, that EETs alone can induce differentiation of the endothelial cells on Matrigel. The level of EET-induced increase in \textsuperscript{3}H-thymidine incorporation was approximately 2-fold under our experimental conditions, which could be due to the lipid nature of synthetic EETs susceptible to oxidative degradation since studies using a more stable sulfonamide EET derivative have shown a larger increase in \textsuperscript{3}H-thymidine incorporation on renal epithelial cells.\textsuperscript{32} The average amount of 8,9-EET in the astrocyte medium was 70 nmol/L, measured by liquid chromatographic–electrospray ionization–mass spectrometry.\textsuperscript{28} The level of exogenous 8,9-EET (100 nmol/L) we used is thus close to the actual level made by astrocytes. However, the bioactivity of some EET degradation products, such as dihydroxyeicosatrienoic acids, in our experimental settings requires further study.

Extensive work has been performed on members of the VEGF family in the last decade. To focus on VEGF with respect to capillary angiogenesis in the brain is not to preclude other growth factors that play a role in this process. Release of VEGF from astrocytes occurs only during brain development\textsuperscript{33} and in the presence of hypoxic and other insults.\textsuperscript{34–36} However, EET production in astrocytes increased after glutamate treatment,\textsuperscript{14} and P450 epoxygenase inhibition reduced the response of cerebral blood flow to neuronal excitation.\textsuperscript{37,38} Indeed, there is a positive correlation between neuronal activity and vessel density in the brain.\textsuperscript{39} Angiogenesis has been shown in adult rat brain after increased metabolic demands.\textsuperscript{40} In addition, EETs have been characterized as an endothelin-derived hyperpolarizing factor to dilate cerebral vasculature.\textsuperscript{41} In the central nervous system, the major source of EETs is considered to be astrocytes, and EETs are involved in vasodilation and functional hyperemia to excitatory neurons.\textsuperscript{41,42} In vivo angiogenesis is accompanied by vasodilation. Agents capable of inducing vasodilation, such as endothelial nitric oxide synthase, played a predominant role in VEGF-induced angiogenesis.\textsuperscript{43} We also found that EETs augmented the effect of VEGF in our system (preliminary data). Thus, EETs may represent an astrocyte-derived angiogenic mitogen in regulating physiological angiogenesis and may be involved in other growth factor–mediated angiogenesis in the brain.

EETs may significantly contribute to the proangiogenic program of capillary endothelial cells by triggering important molecules in the signal transduction cascade such as tyrosine kinase and mitogen-activated kinase.\textsuperscript{44–45} A PKC inhibitor had no effect on EET-induced increase of \textsuperscript{3}H-thymidine incorporation, indicating that PKC might not be involved. Alternatively, the result could be a reflection of enhancing EET incorporation by inhibition of PKC.\textsuperscript{46} Further experiments are required to reveal the downstream targets of EETs.

In summary, our findings provide direct evidence for a novel mechanism involved in astrocyte-mediated angiogenesis and support a key role of astrocytes in regulating blood flow to match neuronal metabolic demand. Further experiments should be undertaken to reveal the signal cascade involved, which will provide more information for understanding the mechanism of angiogenesis in brain and developing treatment for diseases with disrupted balance of blood vessel formation in the central nervous system.

Acknowledgments
This work was supported by National Institutes of Health/National Heart, Lung, and Blood Institute grant PO1 HL 59996 to Dr Harder. We thank Rachel Kraemer and Joan Anders for preparation and maintenance of astrocyte cultures and Dr Meetha Medhora for technical advice.

References


Cerebral Capillary Endothelial Cell Mitogenesis and Morphogenesis Induced by Astrocytic Epoxyeicosatrienoic Acid
Chenyang Zhang and David R. Harder

Stroke. 2002;33:2957-2964; originally published online October 17, 2002;
doi: 10.1161/01.STR.0000037787.07479.9A
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 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/33/12/2957

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/