Published in final edited form as:

Neuroscience. 2009 March 17; 159(2): 744–750. doi:10.1016/j.neuroscience.2008.12.055.

ROLE OF ENDOTHELIAL NITRIC OXIDE SYNTHETASE IN ARTERIOGENESIS AFTER STROKE IN MICE

X. CUI¹, M. CHOPP^{1,2}, A. ZACHAREK¹, C. ZHANG¹, C. ROBERTS¹, and J. CHEN^{1,*}

¹Department of Neurology, Henry Ford Health System, Detroit, MI 48202

²Department of Physics, Oakland University, Rochester, MI

Abstract

Arteriogenesis supports restored perfusion in the ischemic brain and improves long-term functional outcome after stroke. We investigate the role of endothelial nitric oxide synthetase (eNOS) and an NO donor, [(Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl) aminio] diazen-1-ium-1, 2-diolate (DETA-NONOate), in promoting arteriogenesis after stroke. Adult wild-type (WT, n=18) and eNOS-knockout (eNOS^{-/-}, n=36) mice were subjected to transient (2.5 hours) right middle cerebral artery occlusion (MCAo) and were treated with or without DETA-NONOate (0.4 mg/kg) 24 hours after MCAo. Functional evaluation was performed. Animals were sacrificed 3 days after MCAo for arterial cell culture studies, or 14 days for immunohistochemical analysis. Consistent with previous studies, eNOS^{-/-} mice exhibited a higher mortality rate (p<0.05, n=18/group) and more severe neurological functional deficit after MCAo than WT mice (p<0.05, n=12/group). Decreased arteriogenesis, was evident in eNOS^{-/-} mice compared with WT mice, as demonstrated by reduced vascular smooth muscle cell (VSMC) proliferation, arterial density and diameter in the ischemic brain. eNOS^{-/-} mice treated with DETA-NONOate had a significantly decreased mortality rate and improved functional recovery, and exhibited enhanced arteriogenesis identified by increased VSMC proliferation, and upregulated arterial density and diameter compared to eNOS^{-/-} mice after stroke (p<0.05, n=12/group). To elucidate the mechanisms underlying eNOS/NO mediated arteriogenesis, VSMC migration was measured in vitro. Arterial cell migration significantly decreased in the cultured common carotid artery (CCA) derived from eNOS^{-/-} mice 3 days after MCAo compared to WT arterial cells. DETA-NONOate-treatment significantly attenuated eNOS^{-/-}-induced decrease of arterial cell migration compared to eNOS^{-/-} control artery (p<0.05, n=6/group). Using VSMC culture, DETA-NONOate significantly increased VSMC migration, while inhibition of NOS significantly decreased VSMC migration (p<0.05, n=6/group). Our data indicated that eNOS not only promotes vascular dilation but also increases VSMC proliferation and migration, and thereby enhances arteriogenesis after stroke. Therefore, increase eNOS may play an important role in regulating of arteriogenesis after stroke.

Keywords

Endothelial Nitric Oxide Synthetase (eNOS); Nitric Oxide (NO); arteriogenesis; ischemic stroke; mice

*Correspondence to: Jieli Chen, Neurology Research, E&R Bldg., Room 3091, Henry Ford Hospital, 2799 W Grand Blvd, Detroit, MI 48202. Phone: 313-916-1991, Fax: 313-916-1318, jieli@neuro.hfh.edu.

Disclosures: There is no disclosure.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Early clinical improvement after stroke is linked to the presence of arteriolar collaterals (Christoforidis et al., 2005). Enhancement of growth of functional blood vessels is essential for the restoration of blood flow to ischemic brain and to improve functional outcome after stroke. Following arterial occlusion, blood vessels respond by sprouting new capillaries (angiogenesis) and by growing and remodeling pre-existing arterioles into physiologically relevant arteries (arteriogenesis). Arteriogenesis supports restored perfusion in the ischemic brain and promotes long-term clinical outcome in patients treated with and without thrombolysis for stroke (Wei et al., 2001, Christoforidis et al., 2005). Absence of significant collateralization increases mortality after stroke (Christoforidis et al., 2005). Therefore, enhancement of arteriogenesis in the ischemic brain appears to be an attractive therapeutic strategy in stroke (Scholz et al., 2001, Fujita and Tambara, 2004, Erdo and Buschmann, 2007). However, the mechanism underlying ischemic-induced arteriogenesis has not been fully elucidated in cerebral ischemia.

Endothelial-derived nitric oxide synthetase (eNOS) plays an important role in the post-ischemic revascularization process including endothelial cell proliferation and migration, smooth muscle cell differentiation, angiogenic processes, and arterial-venous differentiation (Rudic et al., 1998, Amano et al., 2003, Cai et al., 2004, Gertz et al., 2006, Luque Contreras et al., 2006). eNOS has been implicated in angiogenesis in ischemic brain and arteriogenesis in coronary or peripheral (hindlimb) artery occlusion models (Amano et al., 2003, Brevetti, 2003 #2761, Cai et al., 2004, Urano, 2008 #2880, Wu, 2006 #2891, Brevetti et al., 2003, Wu et al., 2006, Urano et al., 2008). Activation of eNOS promotes venous endothelial cell release of endothelial-derived nitric oxide (NO). Endothelial-derived NO has a crucial role in the regulation of vascular tone, vascular remodeling and angiogenesis (Papapetropoulos et al., 1997, Murohara et al., 1998a, Murohara et al., 1998b, Rudic et al., 1998). Recent evidence has shown that arteriogenesis is also mediated by NO, angiogenic factors and shear stress. NO-mediated increases in vascular conductance allows for greater collateral dependent blood flow to the tissue distal to occlusion (Prior et al., 2003). An NO donor, [(Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl) aminio] diazen-1-ium-1, 2-diolate (DETA-NONOate) increases angiogenesis (Jozkowicz et al., 2001, Zhang et al., 2003, Chen et al., 2004, Zacharek et al., 2006). In this study, we seek to investigate the effect of eNOS on regulation of arteriogenesis after stroke in eNOS knockout (eNOS^{-/-}) mice.

EXPERIMENTAL PROCEDURES

Middle cerebral artery occlusion (MCAo) model and experimental groups

Adult male wild-type C57BL/6J mice (WT, n=18, Charles River, Wilmington, MA) and eNOS knockout (eNOS^{-/-}) C57BL/6J mice (n=36, Jackson Laboratory, Bar Harbor, Maine) weighing 22-25 g were used in this study. All experiments were conducted in accordance with the standards and procedures of the Institutional Animal Care and Use Committee of Henry Ford Health System. Mice were anesthetized with 1% isoflurane and subjected to transient (2.5 hours) monofilament right MCAo (Chen et al., 2005a). Briefly, MCAo was induced by advancing a 6-0 surgical nylon suture (8.5-9 mm determined by body weight) with an expanded (heated) tip from the external carotid artery into the lumen of the internal carotid artery to block the origin of the MCA. Body temperature is maintained constant using a heating pad. The filament was withdrawn at 2.5 hours after MCAo and the incision was sutured. The experimental groups included: 1. WT-mice; 2. eNOS^{-/-}-mice; 3. eNOS^{-/-}-mice treated with DETA-NONOate (0.4 mg/kg, single injection via tail vein after 24h after MCAo; Alexis Biochemical, San Diego, CA, USA). The animals were divided into two sets: in the first set of mice (n = 12/group) functional tests were performed at 0, 1, 3, 7 and 14 days after MCAo. This set of mice were then sacrificed at 14 days after MCAo and used for paraffin brain sections for

immunostaining and lesion volume measurement. The second set of mice (n=6/group) were sacrificed at 3 days after MCAo for primary aortic arterial cell culture.

Behavioral tests and mortality rate

A modified neurological severity score (mNSS) and Foot-fault tests were performed before MCAo, and at 1, 3, 7, 14 days after MCAo in the first set of mice (n=12/group) by an investigator blinded to the experimental groups, as previously described (Chen et al., 2005b). The number of dead animals in each group was counted in the two set of animals (n=18/group), and the mortality rate is presented as a percentage of the total animals.

Histological assessment and infarct volume measurement

The first set of mice was sacrificed at 14 days after MCAo. Mouse brains were fixed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde, and the brains were embedded in paraffin. Using a mouse brain matrix (Activational Systems Inc., Warren, MI), the cerebral tissues were cut into seven equally spaced (1 mm) coronal blocks. A series of adjacent 6 μ m-thick sections were cut from each block and stained with hematoxylin and eosin (H&E). The seven brain sections were traced with the use of the Global Laboratory image analysis system (Data Translation). To measure infarct volume, the indirect lesion area was calculated, in which the intact area of the ipsilateral hemisphere was subtracted from the area of the contralateral hemisphere. Infarct volume is presented as a volume percentage of the lesion compared with the contralateral hemisphere (Swanson et al., 1990, Chen et al., 2001).

Immunohistochemical and double-immunofluorescence staining

Every 10th coronal section for a total 5 sections was used for artery immunohistochemical staining. Alpha smooth muscle actin [α -SMA, a marker of smooth muscle cells (SMC)] immunostaining was performed. Coronal brain sections were incubated first with antibodies against α -SMA (mouse monoclonal IgG 1:800, Dako, Carpinteria, CA, USA), at 4°C overnight and then incubated with avidin-biotin-horseradish peroxidase complex and developed in 3'3' diaminobenzidine tetrahydrochloride (DAB). Control experiments consisted of staining brain coronal tissue sections as outlined above, but the primary antibodies were omitted, as previously described (Li et al., 1998).

To specifically identify the proliferation of vascular SMCs (VSMCs), and whether the proliferating cells are co-localized with VSMCs, double immunofluorescence staining for Ki67, a marker of proliferating cells (Kee et al., 2002), with α -SMA was employed. Each coronal section was first treated with the primary anti-Ki67 antibody (Rabbit monoclonal SP6, 1:300, LabVision/NeoMarkers, Fremont, CA, USA) with FITC (Calbiochem), and was then followed by anti- α -SMA (1:800, DAKO) with Cy3 (Jackson ImmunoResearch) staining. Control experiments consisted of staining brain coronal tissue sections as outlined above, but omitted the primary antibodies, as previously described (Li et al., 1998).

Arterial diameter/density and VSMC proliferation measurements

Consequently, five sections from the standard reference coronal section were acquired. The diameter of α -SMA-immunoreactive arteries (mean diameter > 10 μ m) located both in the ischemic ipsilateral brain and leptomeninges, and the density of all α -SMA-immunoreactive arteries in the ischemic boundary zone (IBZ, see the schematic Fig. 1A) and the number of Ki67-immunoreactive VSMC in a total of 10 enlarged α -SMA-positive arteries located in the IBZ were measured by using the MCID (Imaging Research, St. Catharines, Canada) computer imaging analysis system (Zhang et al., 2002). For artery density and Ki67-reactive VSMC number measurement, eight views in each section (see Fig. 1A) were counted per mouse. Data

are presented as the number of α -SMA-immunoreactive arteries/mm² or the percentage of the Ki67-reactive VSMCs/total VSMCs.

Primary cultured artery cell migration

To elucidate whether eNOS regulates arterial cell migration, a primary arterial cell culture model was employed (Saward and Zahradka, 1997). The second set of animals including WT, eNOS^{-/-} and DETA-NONOate treatment of eNOS^{-/-} mice were sacrificed at 3 days post-MCAo and the common carotid arteries (CCA) were removed for arterial cell culture. The experimental groups include: 1) WT stroke mice CCA; 2) eNOS^{-/-} stroke mice CCA; 3) eNOS^{-/-} stroke mice CCA treated with DETA-NONOate (4 mg/kg). Briefly, the arteries were cut to 1 mm² segments and plated in Matrigel (Becton Dickinson Biosciences, Bedford, MA) in 24 wells (n = 6 wells/group) with 1 ml of DMEM containing 10% of fetal bovine serum (GIBCO, Grand Island, NY). After incubation, dishes were observed at 5 days with a phase contrast microscope and photographed at 4X magnification. The lengths of artery cell migration and arterial sprouting were measured (Zacharek et al., 2008)

VSMC migration assay

The in vitro scratch assay is a straightforward and economical method to study cell migration in vitro (Liang et al., 2007, Ma et al., 2007). This method is based on the observation that, upon creation of a new artificial gap on a confluent cell monolayer, the cells on the edge of the newly created gap move toward the opening to close the “scratch” until new cell–cell contacts are again established. To test whether eNOS regulates VSMC migration, the scratch assay was employed. Firstly, rat VSMCs (ATCC, CRL-2018) were cultured in 10% DMEM (GIBCO, Grand Island, NY) with 1% antibiotic (Penicillin-Streptomycin, GIBCO) in a 12-well plate one or two days for the formation of cell monolayer. The confluent VSMC monolayer was wounded by manually scraping the cells in the center of each well with a cell scraper (Corning Inc. Corning, NY). The cells were washed twice with PBS and then cultured in serum-free medium and treated with (n = 6 wells/group): 1) non-treatment for control; 2) + DETA-NONOate treatment (0.1 μ M); 3) + L-NAME [N(omega)-nitro-L-arginine methyl ester, a non-specific NOS inhibitor, 5 mM, Sigma, Saint Louis, MO]; 4) DETA-NONOate (0.1 μ M) with L-NAME (5 mM). Cell migration into the scratch wound surface was monitored by microscopy at 24 hours. Quantitation was done by measuring the distance of the wound edge of the migrating cells from the start point to the cell migrated point from 3 independent experiments.

Statistical analysis

One-way ANOVA were performed to assess data within the treatment groups. Variables included functional outcome, arterial density and diameter, VSMC proliferation, artery cell and VSMC migration. If there is a significant difference, at $p < 0.05$, pair-wise comparisons were made. Running regression models (Bivariate Correlation) were used to analyze the correlation of neurological functional outcome with arteriogenesis (arterial diameter) in the ischemic brain. All data are presented as mean $\pm$ Standard Error (SE).

RESULTS

eNOS deficit increases mortality and decreases functional outcome after stroke, DETA-NONOate treatment decreases mortality and improves functional outcome after stroke in eNOS^{-/-} mice

The data show that the mortality rate in eNOS^{-/-} (6/18=33.3%) is significantly higher than WT (3/18=16.7%, $p < 0.05$). In addition, eNOS^{-/-} mice exhibited more severe neurological functional deficit than WT-mice at 3, 7 and 14 days after MCAo, respectively ($p < 0.05$, Fig.1B and C). Treatment of eNOS^{-/-}-mice with DETA-NONOate (0.4 mg/kg) significantly decreased

mortality rate (4/18=22.2%, $p<0.05$) and improved functional recovery compared with eNOS^{-/-}-mice control (Fig.1B. mNSS: $p<0.05$ at 7 days; Fig.1C. Foot-fault: $p<0.05$ at 14 days). However, the infarct volume measurement shows that there is no significant difference ($p>0.05$) in the infarct volume among the three groups of WT (18.8% $\pm$ 3.9%), eNOS^{-/-} (19.3% $\pm$ 2.6%) and eNOS^{-/-} + DETA-NONOate treatment (18.9% $\pm$ 2.1%) at 14 days after MCAo.

eNOS deficit decreases arteriogenesis after stroke

Arteriogenesis refers to the remodeling of an existing artery to increase its luminal diameter in response to increased blood flow (Buschmann et al., 2003, Heil et al., 2006). The arteriogenesis response consists of the formation of new arterioles, which presumably occurs when preexisting capillaries acquire SMC coating, and these newly formed and/or preexisting arterioles transform into channels with larger diameters (Buschmann and Schaper, 2000, van Royen et al., 2001). Previous studies have shown that eNOS regulates angiogenesis (Babaei and Stewart, 2002, Smith et al., 2002, Amano et al., 2003, Urano et al., 2008). To test whether eNOS^{-/-} mediates arteriogenesis, α -SMA immunostaining was performed on brain coronal sections, and the artery diameter and density were measured. Fig. 2A-H show that the arterial diameter (A-D) and the arterial density (E-H) in the ischemic brain in the eNOS^{-/-}-mice significantly ($p<0.05$) decreased compared with WT-mice. However, DETA-NONOate-treatment of eNOS^{-/-}-mice significantly ($p<0.05$) increased arterial diameter and density compared with eNOS^{-/-}-mice alone. In addition, the correlation coefficient analysis shows significant negative correlations between Foot-fault and artery diameter ($r=-0.71$, $p<0.05$), and mNSS and artery diameter ($r=-0.78$, $p<0.05$). These data indicate that eNOS mediates arteriogenesis, and the increasing arteriogenesis in the ischemic brain correlates with functional outcome after stroke.

eNOS deficit decreases artery VSMC proliferation

The stages of arteriogenesis consist of arteriolar thinning, followed by transformation of VSMCs from the contractile- into the proliferative- and synthetic phenotype (Scholz et al., 2000). Endothelial cells and VSMCs proliferate, and VSMCs migrate to form a neo-intima (Scholz et al., 2000). For further elucidate whether eNOS regulation of VSMC proliferation, double immunostaining of Ki67 with α -SMA was performed. The percentages of Ki67-reactive VSMCs in the α -SMC-positive arteries in the IBZ significantly decreased in eNOS^{-/-}-mice after MCAo compared with WT-mice. DETA-NONOate-treatment significantly increased the percentage of Ki67-reactive VSMCs in the artery in the IBZ compared with eNOS^{-/-}-mice alone ($p<0.05$, Fig 2I-M).

eNOS deficit decreases VSMC migration

To test whether eNOS regulates VSMC migration, the primary artery culture and the wound healing assay was performed. Fig. 3 shows the arterial cell migration observed in the primary cultured CCA derived from eNOS^{-/-}-mice significantly decreased compared with those derived from WT-mice; treatment of eNOS^{-/-}-mice with DETA-NONOate significantly increased artery cell migration compared with non-treatment eNOS^{-/-}-mice ($p<0.05$, **3A-D**) after MCAo.

In vitro, for further test whether eNOS/NO increases VSMC migration, the VSMCs were cultured and treated with or without a non-specific NOS inhibitor (L-NAME, 5 mM) for 24 hours, and the migration length of VSMC was calculated. The data showed that L-NAME treatment significantly decreased VSMC migration compared with non-treatment control. While DETA-NONOate treatment significantly attenuated the L-NAME-induced reduction of VSMC migration after 24 hours of treatment ($p<0.05$, see Fig. 3E-I).

DISCUSSION

eNOS^{-/-} mice have more injury after stroke, which includes higher mortality rate (Dere et al., 2002), more severe neurological functional deficit (Chen et al., 2005a) and larger infarcts (Huang et al., 1996) than WT mice. In the present study, we found that eNOS^{-/-} mice have a higher mortality rate and worse functional outcome than WT mice. However, the infarct volume has not significantly different compared to WT mice. A reasonable explanation for the apparent discrepancy between infarct volume, mortality and functional outcome is that the lesion volume was measured at 14 days after MCAo, while most animals with severe lesion died by 3 days after MCAo. Thus, 14 day survival animals likely biases against increased infarct size. Earlier sacrifice of these animals to allow measurement of infarct size for those animals destined to die (Huang et al., 1996).

Arteriogenesis is an important process for adapting the pre-existing circuit of vessels into functional collateral conduits for delivery of oxygen enriched blood to tissue distal to occlusion of a large, peripheral conduit artery (Seetharam et al., 2006), and it substitutes arterial collaterals for the occluded artery (Buschmann et al., 2003). The adaptive proliferation of preexisting collateral pathways (arteriogenesis) is an effective biological rescue system against detrimental effects of arterial stenosis and as a means to prevent cerebral ischemia during progressing cerebrovascular disease (Busch et al., 2003, Busch et al., 2006). eNOS activity is essential for neovascularization. The expression of eNOS is moderated partly by blood flow-induced mechanical factors, i.e., shear stress (Cai et al., 2004). However, the specific role of eNOS in arteriogenesis remains unclear. Previous studies have shown that the onset of arteriogenesis occurs as early as 24 hours (Hillmeister et al., 2008), 48 hours (Nakagawa et al., 2006) and one week (Hossmann and Buschmann, 2005, Todo et al., 2008) after stroke. In the present study, we show that eNOS^{-/-} mice have deficits in arteriogenesis at 14 days after brain ischemia. Analysis of the correlation between functional outcome with the arteriogenesis provides insight into the beneficial role of eNOS in stroke. In this study, correlation coefficient analysis shows strong negative correlations between functional outcome and arteriogenesis, which indicates that arteriogenesis is beneficial for the neurological functional recovery. eNOS deficit-induced arteriogenesis decreases were rescued by increases in NO level when given an NO donor, DETA-NONOate. Our data, and others, support the hypothesis that eNOS plays an important role in arteriogenesis after stroke. eNOS also maintains cerebral blood flow and reduces neuronal injury after stroke (Samdani et al., 1997). Overexpression of eNOS increases skeletal muscle blood flow, muscle oxygen tension, and collateral arteries (arteriogenesis) in severe rat hind limb ischemia (Brevetti et al., 2003). In normal arteriolar vessels, expression of eNOS is very low, but in growing collateral vessels, it is up-regulated and activated, which, however, returned to normal levels in mature collateral vessels, and the expression of eNOS was colocalized only in endothelial cells, either in normal or growing vessels (Cai et al., 2004, Wu et al., 2006). In response to external carotid artery ligation, mice with targeted disruption of the eNOS gene did not remodel their ipsilateral common carotid arteries, whereas wild-type mice did (Rudic et al., 1998). The genetic loss of eNOS in mice impairs vascular endothelial growth factor (VEGF) and ischemia-initiated blood flow recovery resulting in critical limb ischemia (Murohara et al., 1998b, Aicher et al., 2003). eNOS^{-/-} mice have defects in arteriogenesis and functional blood flow reserve after muscle stimulation and pericyte recruitment after ischemic; however, the defects in blood flow recovery, ischemic reserve capacity, and pericyte recruitment into the growing neovasculature can be rescued by local intramuscular delivery of an adenovirus encoding a constitutively active allele of eNOS, eNOS S1179D, but not a control virus (Yu et al., 2005). Tissue perfusion and collateral-dependent blood flow were significantly increased in eNOS-overexpressing mice (eNOS^{Stg}) compared with WT immediately after ligation. In eNOS^{-/-} mice, the tissue perfusion and collateral-dependent blood flow remained significantly decreased until day 7, suggesting delayed collateral growth (Mees et al., 2007). These findings indicate that the expression of eNOS is

closely associated with the development of collateral vessels, suggesting that eNOS plays an important role in arteriogenesis (Cai et al., 2004).

Arteriogenesis describes the remodelling of pre-existing arterio-arteriolar anastomoses to completely developed and functional arteries. In the growth processes, enlargement of vascular wall structures was proposed to be covered by proliferation and migration of existing wall cells including endothelial cells and VSMCs, and the initial hypothesis is these cells-after undergoing a (trans-)differentiation-contribute by a structural integration into the growing vessel wall (Heil and Schaper, 2004, Heil et al., 2006). Our study shows that VSMC proliferation significantly decreases in the ischemic brain in eNOS^{-/-} mice. In addition, either the primary artery cell or the cultured VSMC migration significantly decreased in eNOS inhibition group, however, the defects in VSMC migration can be attenuated by an increase of NO level observed in vivo and in vitro. These data indicate that eNOS/NO mediate VSMC proliferation and migration during the arteriogenesis.

CONCLUSION

In summary, our data indicate that endogenous eNOS and eNOS-derived NO not only exerts direct effects in promoting vascular dilation, but also increases VSMC proliferation and migration, and thereby enhances arteriogenesis after stroke.

Acknowledgments

The authors wish to thank Qinge Lu and Supata Santra (Department of Neurology, Henry Ford Health System) for technical assistance.

Grant sponsor: National Institute of Neurological Disorders and Stroke (R01 NS047682 and P01 NS23393); American Heart Association (0750048Z).

References

- Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, Zeiher AM, Dimmeler S. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nature medicine* 2003;9:1370–1376.
- Amano K, Matsubara H, Iba O, Okigaki M, Fujiyama S, Imada T, Kojima H, Nozawa Y, Kawashima S, Yokoyama M, Iwasaka T. Enhancement of ischemia-induced angiogenesis by eNOS overexpression. *Hypertension* 2003;41:156–162. [PubMed: 12511546]
- Babaei S, Stewart DJ. Overexpression of endothelial NO synthase induces angiogenesis in a co-culture model. *Cardiovasc Res* 2002;55:190–200. [PubMed: 12062722]
- Brevetti LS, Chang DS, Tang GL, Sarkar R, Messina LM. Overexpression of endothelial nitric oxide synthase increases skeletal muscle blood flow and oxygenation in severe rat hind limb ischemia. *J Vasc Surg* 2003;38:820–826. [PubMed: 14560236]
- Busch HJ, Buschmann I, Schneeloch E, Bode C, Mies G, Hossmann KA. Therapeutically induced arteriogenesis in the brain. A new approach for the prevention of cerebral ischemia with vascular stenosis. *Der Nervenarzt* 2006;77:215–220. [PubMed: 16273341]
- Busch HJ, Buschmann IR, Mies G, Bode C, Hossmann KA. Arteriogenesis in hypoperfused rat brain. *J Cereb Blood Flow Metab* 2003;23:621–628. [PubMed: 12771578]
- Buschmann I, Schaper W. The pathophysiology of the collateral circulation (arteriogenesis). *The Journal of pathology* 2000;190:338–342. [PubMed: 10685067]
- Buschmann IR, Busch HJ, Mies G, Hossmann KA. Therapeutic induction of arteriogenesis in hypoperfused rat brain via granulocyte-macrophage colony-stimulating factor. *Circulation* 2003;108:610–615. [PubMed: 12835229]
- Cai WJ, Kocsis E, Luo X, Schaper W, Schaper J. Expression of endothelial nitric oxide synthase in the vascular wall during arteriogenesis. *Molecular and cellular biochemistry* 2004;264:193–200. [PubMed: 15544048]

- Chen J, Li Y, Wang L, Lu M, Zhang X, Chopp M. Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. *Journal of the neurological sciences* 2001;189:49–57. [PubMed: 11535233]
- Chen J, Li Y, Zhang R, Katakowski M, Gautam SC, Xu Y, Lu M, Zhang Z, Chopp M. Combination therapy of stroke in rats with a nitric oxide donor and human bone marrow stromal cells enhances angiogenesis and neurogenesis. *Brain research* 2004;1005:21–28. [PubMed: 15044060]
- Chen J, Zacharek A, Zhang C, Jiang H, Li Y, Roberts C, Lu M, Kapke A, Chopp M. Endothelial nitric oxide synthase regulates brain-derived neurotrophic factor expression and neurogenesis after stroke in mice. *J Neurosci* 2005a;25:2366–2375. [PubMed: 15745963]
- Chen J, Zhang C, Jiang H, Li Y, Zhang L, Robin A, Katakowski M, Lu M, Chopp M. Atorvastatin induction of VEGF and BDNF promotes brain plasticity after stroke in mice. *J Cereb Blood Flow Metab* 2005b;25:281–290. [PubMed: 15678129]
- Christoforidis GA, Mohammad Y, Kehagias D, Avutu B, Slivka AP. Angiographic assessment of pial collaterals as a prognostic indicator following intra-arterial thrombolysis for acute ischemic stroke. *Ajnr* 2005;26:1789–1797. [PubMed: 16091531]
- Dere E, De Souza Silva MA, Topic B, Fiorillo C, Li JS, Sadile AG, Frisch C, Huston JP. Aged endothelial nitric oxide synthase knockout mice exhibit higher mortality concomitant with impaired open-field habituation and alterations in forebrain neurotransmitter levels. *Genes Brain Behav* 2002;1:204–213. [PubMed: 12882365]
- Erdo F, Buschmann IR. Arteriogenesis: a new strategy of therapeutic intervention in chronic arterial disorders. Cellular mechanism and experimental models. *Orvosi hetilap* 2007;148:633–642. [PubMed: 17403636]
- Fujita M, Tambara K. Recent insights into human coronary collateral development. *Heart (British Cardiac Society)* 2004;90:246–250. [PubMed: 14966033]
- Gertz K, Priller J, Kronenberg G, Fink KB, Winter B, Schrock H, Ji S, Milosevic M, Harms C, Bohm M, Dirnagl U, Laufs U, Endres M. Physical activity improves long-term stroke outcome via endothelial nitric oxide synthase-dependent augmentation of neovascularization and cerebral blood flow. *Circulation research* 2006;99:1132–1140. [PubMed: 17038638]
- Heil M, Eitenmuller I, Schmitz-Rixen T, Schaper W. Arteriogenesis versus angiogenesis: similarities and differences. *Journal of cellular and molecular medicine* 2006;10:45–55. [PubMed: 16563221]
- Heil M, Schaper W. Influence of mechanical, cellular, and molecular factors on collateral artery growth (arteriogenesis). *Circulation research* 2004;95:449–458. [PubMed: 15345667]
- Hillmeister P, Lehmann KE, Bondke A, Witt H, Duelsner A, Gruber C, Busch HJ, Jankowski J, Ruiz-Noppinger P, Hossmann KA, Buschmann IR. Induction of cerebral arteriogenesis leads to early-phase expression of protease inhibitors in growing collaterals of the brain. *J Cereb Blood Flow Metab* 2008;28:1811–1823. [PubMed: 18594555]
- Hossmann KA, Buschmann IR. Granulocyte-macrophage colony-stimulating factor as an arteriogenic factor in the treatment of ischaemic stroke. *Expert opinion on biological therapy* 2005;5:1547–1556. [PubMed: 16318419]
- Huang Z, Huang PL, Ma J, Meng W, Ayata C, Fishman MC, Moskowitz MA. Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine. *J Cereb Blood Flow Metab* 1996;16:981–987. [PubMed: 8784243]
- Jozkowicz A, Cooke JP, Guevara I, Huk I, Funovics P, Pachinger O, Weidinger F, Dulak J. Genetic augmentation of nitric oxide synthase increases the vascular generation of VEGF. *Cardiovasc Res* 2001;51:773–783. [PubMed: 11530111]
- Kee N, Sivalingam S, Boonstra R, Wojtowicz JM. The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. *Journal of neuroscience methods* 2002;115:97–105. [PubMed: 11897369]
- Li Y, Jiang N, Powers C, Chopp M. Neuronal damage and plasticity identified by microtubule-associated protein 2, growth-associated protein 43, and cyclin D1 immunoreactivity after focal cerebral ischemia in rats. *Stroke* 1998;29:1972–1980. [PubMed: 9731626]discussion 1980-1971
- Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature protocols* 2007;2:329–333.

- Luque Contreras D, Vargas Robles H, Romo E, Rios A, Escalante B. The role of nitric oxide in the post-ischemic revascularization process. *Pharmacology & therapeutics* 2006;112:553–563. [PubMed: 16950515]
- Ma J, Wang Q, Fei T, Han JD, Chen YG. MCP-1 mediates TGF-beta-induced angiogenesis by stimulating vascular smooth muscle cell migration. *Blood* 2007;109:987–994. [PubMed: 17032917]
- Mees B, Wagner S, Ninci E, Tribulova S, Martin S, van Haperen R, Kostin S, Heil M, de Crom R, Schaper W. Endothelial nitric oxide synthase activity is essential for vasodilation during blood flow recovery but not for arteriogenesis. *Arterioscler Thromb Vasc Biol* 2007;27:1926–1933. [PubMed: 17556651]
- Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, Kearney M, Chen D, Symes JF, Fishman MC, Huang PL, Isner JM. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest* 1998a;101:2567–2578. [PubMed: 9616228]
- Murohara T, Horowitz JR, Silver M, Tsurumi Y, Chen D, Sullivan A, Isner JM. Vascular endothelial growth factor/vascular permeability factor enhances vascular permeability via nitric oxide and prostacyclin. *Circulation* 1998b;97:99–107. [PubMed: 9443437]
- Nakagawa T, Suga S, Kawase T, Toda M. Intracarotid injection of granulocyte-macrophage colony-stimulating factor induces neuroprotection in a rat transient middle cerebral artery occlusion model. *Brain research*. 2006
- Papapetropoulos A, Garcia-Cardena G, Madri JA, Sessa WC. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J Clin Invest* 1997;100:3131–3139. [PubMed: 9399960]
- Prior BM, Lloyd PG, Ren J, Li Z, Yang HT, Laughlin MH, Terjung RL. Arteriogenesis: role of nitric oxide. *Endothelium* 2003;10:207–216. [PubMed: 14660080]
- Rudic RD, Shesely EG, Maeda N, Smithies O, Segal SS, Sessa WC. Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. *J Clin Invest* 1998;101:731–736. [PubMed: 9466966]
- Samdani AF, Dawson TM, Dawson VL. Nitric oxide synthase in models of focal ischemia. *Stroke* 1997;28:1283–1288. [PubMed: 9183363]
- Saward L, Zahradka P. Coronary artery smooth muscle in culture: migration of heterogeneous cell populations from vessel wall. *Molecular and cellular biochemistry* 1997;176:53–59. [PubMed: 9406145]
- Scholz D, Cai WJ, Schaper W. Arteriogenesis, a new concept of vascular adaptation in occlusive disease. *Angiogenesis* 2001;4:247–257. [PubMed: 12197469]
- Scholz D, Ito W, Fleming I, Deindl E, Sauer A, Wiesnet M, Busse R, Schaper J, Schaper W. Ultrastructure and molecular histology of rabbit hind-limb collateral artery growth (arteriogenesis). *Virchows Arch* 2000;436:257–270. [PubMed: 10782885]
- Seetharam D, Mineo C, Gormley AK, Gibson LL, Vongpatanasin W, Chambliss KL, Hahner LD, Cummings ML, Kitchens RL, Marcel YL, Rader DJ, Shaul PW. High-density lipoprotein promotes endothelial cell migration and reendothelialization via scavenger receptor-B type I. *Circulation research* 2006;98:63–72. [PubMed: 16339487]
- Smith RS Jr, Lin KF, Agata J, Chao L, Chao J. Human endothelial nitric oxide synthase gene delivery promotes angiogenesis in a rat model of hindlimb ischemia. *Arterioscler Thromb Vasc Biol* 2002;22:1279–1285. [PubMed: 12171788]
- Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR. A semiautomated method for measuring brain infarct volume. *J Cereb Blood Flow Metab* 1990;10:290–293. [PubMed: 1689322]
- Todo K, Kitagawa K, Sasaki T, Omura-Matsuoka E, Terasaki Y, Oyama N, Yagita Y, Hori M. Granulocyte-macrophage colony-stimulating factor enhances leptomeningeal collateral growth induced by common carotid artery occlusion. *Stroke* 2008;39:1875–1882. [PubMed: 18388343]
- Urano T, Ito Y, Akao M, Sawa T, Miyata K, Tabata M, Morisada T, Hato T, Yano M, Kadomatsu T, Yasunaga K, Shibata R, Murohara T, Akaike T, Tanihara H, Suda T, Oike Y. Angiopoietin-related growth factor enhances blood flow via activation of the ERK1/2-eNOS-NO pathway in a mouse hind-limb ischemia model. *Arterioscler Thromb Vasc Biol* 2008;28:827–834. [PubMed: 18258819]

- van Royen N, Piek JJ, Buschmann I, Hoefler I, Voskuil M, Schaper W. Stimulation of arteriogenesis; a new concept for the treatment of arterial occlusive disease. *Cardiovasc Res* 2001;49:543–553. [PubMed: 11166267]
- Wei L, Erinjeri JP, Rovainen CM, Woolsey TA. Collateral growth and angiogenesis around cortical stroke. *Stroke* 2001;32:2179–2184. [PubMed: 11546914]
- Wu XQ, Cai WJ, Luo XG. Expression of eNOS in the endothelial cells during the arteriogenesis of pig hind-limbs. *Zhong nan da xue xue bao Yi xue ban = Journal of Central South University* 2006;31:63–65.
- Yu J, deMuinck ED, Zhuang Z, Drinane M, Kauser K, Rubanyi GM, Qian HS, Murata T, Escalante B, Sessa WC. Endothelial nitric oxide synthase is critical for ischemic remodeling, mural cell recruitment, and blood flow reserve. *Proc Natl Acad Sci U S A* 2005;102:10999–11004. [PubMed: 16043715]
- Zacharek A, Chen J, Cui X, Yang Y, Chopp M. Simvastatin Increases Notch Signaling Activity and Promotes Arteriogenesis After Stroke. *Stroke*. 2008
- Zacharek A, Chen J, Zhang C, Cui X, Roberts C, Jiang H, Teng H, Chopp M. Nitric oxide regulates Angiopoietin1/Tie2 expression after stroke. *Neuroscience letters* 2006;404:28–32. [PubMed: 16762501]
- Zhang R, Wang L, Zhang L, Chen J, Zhu Z, Zhang Z, Chopp M. Nitric oxide enhances angiogenesis via the synthesis of vascular endothelial growth factor and cGMP after stroke in the rat. *Circulation research* 2003;92:308–313. [PubMed: 12595343]
- Zhang ZG, Zhang L, Tsang W, Soltanian-Zadeh H, Morris D, Zhang R, Goussev A, Powers C, Yeich T, Chopp M. Correlation of VEGF and angiopoietin expression with disruption of blood-brain barrier and angiogenesis after focal cerebral ischemia. *J Cereb Blood Flow Metab* 2002;22:379–392. [PubMed: 11919509]

Abbreviations

eNOS	endothelial nitric oxide synthetase
DETA-NONOate	[(Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl) aminio] diazen-1-ium-1, 2-diolate
αSMA	alpha smooth muscle actin
VSMC	vascular smooth muscle cell
MCAo	middle cerebral artery occlusion
NO	nitric oxide

Fig.1. Schematic image and functional tests

A: Schematic representation of a coronal brain section shows eight fields selected along the ischemic boundary zone (IBZ) for quantitative measurements of α SMA-positive artery density and Ki67-positive VSMC proliferation. **B:** modification of neurological severity score (mNSS) test. **C:** Foot-fault test.

Fig.2. eNOS^{-/-} decreases arterial density and diameter and VSMC proliferation in the ischemic brain after MCAo in mice, and treatment of eNOS^{-/-} with DETA-NONOate increases density and diameter and VSMC proliferation

A-D: αSMA-immunoreactive positive arteries present in the leptomenige of ischemic brain at 14 days after MCAo (A: WT; B: eNOS^{-/-}; C: eNOS^{-/-} + DETA-NONOate; D: Quantitative diameter data). **E-H:** αSMA-immunoreactive positive arteries present in the IBZ at 14 days after MCAo (E: WT; F: eNOS^{-/-}; G: eNOS^{-/-} + DETA-NONOate; H: Quantitative density data). **I-M:** αSMA and Ki67 double-immunofluorescent staining in the arteries in the IBZ in mice after MCAo (I: αSMA immunostaining shows VSMCs; J: Ki67 immunostaining shows the proliferated VSMC; K: DAPI staining shows all cell nuclei; L: merged image). **M:** quantitative data of VSMC proliferation in the IBZ in WT and eNOS^{-/-} and DETA-NONO-treatment eNOS^{-/-} mice after stroke. Scale bar = 100 μm. n = 12 / group.

Fig.3. eNOS^{-/-} reduced artery cell and VSMC migration after stroke, DETA-NONOate treatment enhanced artery cell and VSMC migration

A-D: Common carotid artery cell migration derived from WT (A), eNOS^{-/-} (B), eNOS^{-/-} + DETA-NONOate-treatment (C), mice and quantitative data (D) at 3 days after MCAo. **E-I:** VSMC migration in control (E), DETA-NONOate treatment (F), L-NAME treatment (G) and L-NAME+DETA-NONOate treatment (H) groups, and the quantitative data (I) after 24 hours culture. n = 6 / group.