Deficient eNOS Phosphorylation Is a Mechanism for Diabetic Vascular Dysfunction Contributing to Increased Stroke Size

Qian Li, MD, PhD*; Dmitriy Atochin, MD, PhD*; Satoshi Kashiwagi, MD, PhD; John Earle; Annie Wang, MD; Emiri Mandeville, MD, PhD; Kazuhide Hayakawa, Ph.D; Livius V. d’Uscio, PhD; Eng H. Lo, PhD; Zvonimir Katusic, MD, PhD; William Sessa, PhD; Paul L. Huang, MD, PhD

Background and Purpose—Phosphorylation of eNOS, an important post-translational modulator of its enzymatic activity, is reduced in diabetes mellitus. We hypothesized that modulation of eNOS phosphorylation could overcome diabetic vascular dysfunction and improves the outcome to stroke.

Methods—We used the db/db mouse model of type 2 diabetes mellitus. We mated db/db mice with eNOS knock-in mice that carry single amino acid mutations at the S1176 phosphorylation site; the phosphomimetic SD mutation (serine replaced by aspartate) shows increased eNOS enzymatic activity, whereas the unphosphorylatable SA mutation (serine replaced by alanine) shows decreased eNOS activity. We characterized the vascular anatomy, baseline physiological parameters, and vascular reactivity. We used the middle cerebral artery occlusion model of stroke and measured infarct volume and neurological deficits.

Results—db/db mice showed diminished eNOS phosphorylation at S1176. eNOS SD and SA mutations do not change the vascular anatomy at the Circle of Willis, brain capillary density, heart rate, or arterial blood gases of db/db mice. The eNOS SD mutation, but not the SA mutation, lowers blood pressure and improves vascular reactivity to acetylcholine in db/db mice. The eNOS SD mutation reduces stroke size and neurological deficit after middle cerebral artery occlusion.

Conclusions—Diminished eNOS phosphorylation is a mechanism of vascular dysfunction in db/db mice. We show here that modulation of the eNOS S1176 phosphorylation site in db/db mice is associated with improved vascular reactivity and improved outcome to stroke after middle cerebral artery occlusion. (Stroke. 2013;44:3183-3188.)

Key Words: diabetes mellitus ■ endothelial cells ■ nitric oxide

Cardiovascular disease, including stroke, is the major cause of morbidity and mortality in diabetes mellitus.1 The precise mechanisms of endothelial dysfunction in diabetes mellitus are not known. Here, we test the hypothesis that deficient endothelial nitric oxide synthase (eNOS) phosphorylation is an important cause of diabetic vascular dysfunction, and that its modulation can change the outcome to a disease model in vivo.

eNOS phosphorylation at serine 1177 (human sequence numbering), or its equivalent in other species, has been studied.2-5 This site is phosphorylated by Akt kinase,6 AMP kinase,6 and protein kinase A,7 resulting in a conformational change, enhancing electron flux through the reductase domain, and increasing NO production.8 eNOS-derived NO plays known protective roles in cerebral ischemia, including maintenance of cerebral blood flow (CBF).9 Models of cerebral ischemia show worse outcome with larger infarcts in eNOS knockout mice.10,11 To study the effects of eNOS phosphorylation, we created eNOS knock-in mice that carry single amino acid mutations at S1176, the murine site corresponding to human S1177.12 The SD mutation (serine replaced by aspartate) results in the generation of a phosphomimetic form of eNOS with increased enzymatic activity and NO generation. The SA mutation (serine replaced by alanine) results in the generation of an unphosphorylatable form of eNOS. These

Received September 25, 2012; accepted July 24, 2013.
From the Cardiovascular Research Center (Q.L., D.A., S.K., J.E., A.W., P.L.H.) and Neuroprotection Research Laboratory (E.M., K.H., E.H.L.), Massachusetts General Hospital and Harvard Medical School, Boston, MA; the Departments of Anesthesiology and Molecular, Pharmacology, and Experimental Therapeutics, Mayo Clinic, Rochester, MN (L.V.U., Z.K.); and the Department of Pharmacology and Vascular Biology and Therapeutics Program, Yale University School of Medicine, New Haven, CT (W.S.).
Guest Editor for this article was Costantino Iadecola, MD.
*Dr Li and Atochin contributed equally.

The online-only Data Supplement is available with this article at http://stroke.ahajournals.orglookup/suppl/doi:10.1161/STROKEAHA.113.002073/-/DC1.
Correspondence to Paul Huang, MD, PhD, Cardiovascular Research Center, Massachusetts General Hospital East, 149 Thirteenth St, Charlestown, MA 02129. E-mail phuang1@partners.org

© 2013 American Heart Association, Inc.

Stroke is available at http://stroke.ahajournals.org DOI: 10.1161/STROKEAHA.113.002073
mutations are located in the endogenous eNOS gene. For a mouse model of type 2 diabetes mellitus, we used the db/db mouse, which carries a point mutation in the leptin receptor gene.13,14 These animals show a phenotype similar to type 2 diabetes mellitus, with hyperglycemia and insulin resistance, as well as other metabolic abnormalities.15,16 We bred eNOS SD and SA mutant mice to db/db mice to test whether modulation of the eNOS S1176 phosphorylation site could overcome diabetic vascular dysfunction, and whether this affects stroke size in vivo.

Materials and Methods

Animals

Wild-type (WT) and db/db mice on C57BL/6 background were obtained from Jackson Laboratory, eNOS SA and SD mice were backcrossed to C57BL/6 genetic background by accelerated marker-assisted congenic breeding for 6 generations, equivalent to 10 generations of conventional breeding.17 SA and SD mice were mated with db/db mice as described in the Methods in the online-only Data Supplement. Male adult mice 8 to 17 weeks of age were used for all experiments (WT, 12.0±3.5 weeks; db/db, 12.8±1.7 weeks; SD-db/db, 13.5±3.4 weeks; SA-db/db, 13.3±2.7 weeks). All procedures were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

Biochemical and Physiological Characterization

Western blotting, cGMP assay, biopterin measurements, glucose and insulin tolerance tests, microvascular density, cerebrovascular anatomy, and CBF measurements were performed as described in the Methods in the online-only Data Supplement. Serum lipoproteins were analyzed by high performance liquid chromatography.18 Blood pressure was monitored in the carotid artery by blood pressure transducer (ADInstruments).18 Vessel reactivity studies were performed in a pressurized myograph system (Danish Myotechnologies).18

Middle Cerebral Artery Occlusion Model of Stroke

For middle cerebral artery (MCA) occlusion, a 7-0 nylon filament covered by silicon (Doccol Corp) was inserted into the internal carotid artery and advanced to the origin of the MCA for 1 hour, as described in the Methods in the online-only Data Supplement.

Determination of Infarct Size

Coronal brain sections (2 mm thick) were stained after 23 hours of reperfusion with 2% 2,3,5-triphenyltetrazolium chloride (TTC).19 Total and infarcted areas were measured on the side ipsilateral to ischemia, and total area was measured on the contralateral side. Areas were integrated over the 5 coronal sections to obtain volumes. The percentage of the infarct volume to brain size was calculated as infarct volume divided by total volume of cerebral hemispheres without the cerebellum. To correct for edema, indirect infarct volume was calculated as volume of contralateral hemisphere minus ipsilateral noninfarcted volume.

Neurological Scoring

Mice were examined for neurological deficits 24 hours after MCA occlusion as described19 with modifications. We excluded from original 5-point scale 1 point (leaning to the contralateral side) because of the inability of overweight db/db, SD-db/db, and SA-db/db mice to lean. Normal motor function was scored as 0, flexion of the contralateral torso and forearm on lifting the animal by the tail as 1, circling to the contralateral side as 2, and no spontaneous motor activity as 3. Measurements of infarct volume and neurological scoring were done by a blinded operator.

Statistics

All results are expressed as mean±SD. Statistical analysis was performed using Mann–Whitney test when 2 groups were compared, or Kruskal–Wallis test with Dunn post hoc test when >2 groups were compared. Statistical analysis for neurological deficit was performed using Kruskal–Wallis 1-way analysis of variance on ranks. Differences of P<0.05 were considered significant.

Results

eNOS Phosphorylation Is Impaired in db/db Mice

We performed Western blotting to quantitate eNOS phosphorylation in the carotid artery. As shown in Figure 1, db/db mice showed normal levels of total eNOS, whereas eNOS phosphorylation at S1176 was diminished.

SA-db/db and SD-db/db Mice

We used homologous recombination to knock in the specific SD and SA mutations into the endogenous mouse eNOS gene (Figure IA in the online-only Data Supplement). These mice were bred with db/db mice to obtain animals homozygous for the eNOS mutations and db/db alleles. Expression of eNOS and nNOS are the same in SD-db/db and SA-db/db mice as in WT and db/db mice (Figure IB–ID in the online-only Data Supplement). eNOS mutations did not change the levels of total or phosphorylated Akt or AMP kinase (Figure IB, IE, and IF in the online-only Data Supplement). The body weights of db/db (49.2±5.6 g), SD-db/db (49.3±7.4 g), and SA-db/db (45.9±6.7 g) mice were not significantly different from each other and were higher than WT mice (25.0±3.9 g).

Cerebrovascular Anatomy

We injected a latex-carbon black solution to outline the Circle of Willis and its major branches. The Circle of Willis was complete in all animals, and no redundant middle cerebral arteries were observed. We measured the diameters of the posterior cerebral arteries (PCA) and posterior communicating (Pcomm) arteries. No significant differences were observed between WT (PCA, 138.1±27.0 μm; Pcomm, 52.9±19.8 μm; Pcomm, 52.9±19.8 μm;
n=4), db/db (PCA, 161.6±26.6 μm; Pcomm, 45.9±11.0 μm; n=5), SA-db/db (PCA, 150.6±26.2 μm; Pcomm, 66.7±34.1 μm; n=4), and SD-db/db (PCA, 169.6±25.2 μm; Pcomm, 64.4±37.4 μm; n=6) mice (Figure IIA and IIB in the online-only Data Supplement). We measured microvascular density by lectin staining in the cerebral cortex (WT, 4.06±1.97%; db/db, 4.62±2.09%; SD-db/db, 4.44±1.45%; SA-db/db, 4.04±0.62%) and striatum (WT, 3.74±0.42%; db/db, 4.29±1.18%; SD-db/db, 4.05±0.70%; SA-db/db, 3.85±0.51%; n=3 per group). No significant differences were seen between any of the groups (Figure IIC in the online-only Data Supplement).

**Metabolic Parameters**

Glucose tolerance and insulin tolerance testing showed impaired responses in db/db mice compared with WT mice. The eNOS SA and SD mutations did not significantly affect the glucose tolerance and insulin tolerance curves of the db/db mice (Figure III in the online-only Data Supplement). Cholesterol levels were lower in WT mice compared with all other groups of mice. Triglyceride levels were not different between all groups of mice (Table I in the online-only Data Supplement).

**SD Mutation Reduces Stroke Size in db/db Mice**

db/db mice show a larger stroke after MCA occlusion than WT mice (Figure 2A and 2B). The percentage of the infarct volume to brain size of db/db mice was 26.5±5.0% compared with 18.0±4.9% in WT mice (n=12, P<0.01; Figure 2A). Cholesterol levels were lower in WT mice compared with all other groups of mice. The eNOS SA and SD mutations did not significantly affect the glucose tolerance and insulin tolerance curves of the db/db mice (Figure III in the online-only Data Supplement). The glucose tolerance and insulin tolerance curves of the db/db mice were impaired compared with WT mice (dose–response curve shifted to the right; EC50 43.4±25.3 nmol/L compared with WT mice (EC50 13.1±4.9 nmol/L; P<0.01; Figure 3A). The eNOS SD mutation improved the vascular reactivity of db/db mice, shifting the ACh dose–response curve back to the left toward the WT curve. The EC50 for the WT mice was 10.2±5.9 nmol/L (P<0.01 compared with db/db and SA-db/db mice). In contrast, the dose–response curve of SA-db/db mice was similar to the db/db mice (EC50 30.0±12.3 nmol/L). In response to SNP (Figure 3B), the EC50 for the WT mice was 8.0±4.8 nmol/L) was significantly lower than SD-db/db mice (25.8±13.8 nmol/L; P<0.05), and not different between SD-db/db mice and db/db mice (18.6±2.8 nmol/L; P=0.51) or SA-db/db mice (15.5±1.6 nmol/L; P=0.24). Together, these results show that the eNOS SD phosphomimetic mutation improves vascular reactivity in db/db mice, normalizing the EC50 for ACh, despite reduced sensitivity of smooth muscle cells of db/db mice to NO.

**SD Mutation Normalized Blood Pressure and Vascular Reactivity in db/db Mice**

db/db mice are hypertensive compared with WT mice. The blood pressure was significantly lower in WT-db/db mice, but not in SA-db/db mice compared with db/db mice (Figure 2D). Carotid artery vasodilation in response to ACh was impaired in db/db mice (dose–response curve shifted to the right; EC50 13.1±4.9 nmol/L; P<0.01; Figure 3A). The eNOS SD mutation improved the vascular reactivity of db/db mice, shifting the ACh dose–response curve back to the left toward the WT curve. The EC50 for the WT mice was 10.2±5.9 nmol/L (P<0.01 compared with db/db and SA-db/db mice). In contrast, the dose–response curve of SA-db/db mice was similar to the db/db mice (EC50 30.0±12.3 nmol/L). In response to SNP (Figure 3B), the EC50 for the WT mice was 8.0±4.8 nmol/L) was significantly lower than SD-db/db mice (25.8±13.8 nmol/L; P<0.05), and not different between SD-db/db mice and db/db mice (18.6±2.8 nmol/L; P=0.51) or SA-db/db mice (15.5±1.6 nmol/L; P=0.24). Together, these results show that the eNOS SD phosphomimetic mutation improves vascular reactivity in db/db mice, normalizing the EC50 for ACh, despite reduced sensitivity of smooth muscle cells of db/db mice to NO.

Figure 2. Cerebral infarct volumes, neurological score, infarct areas, and blood pressure. A, Indirect cerebral infarct volumes (mm³) 24 hours after reperfusion (n=15, WT and db/db; n=12, SD-db/db and SA-db/db mice; *P<0.01 vs WT and SD-db/db). B, Infarct areas (mm²) for each coronal section (*P<0.01 vs WT and #P<0.01 vs SD-db/db). C, Neurological scores (n=15, WT and db/db; n=12, SD-db/db and SA-db/db mice; *P<0.01 vs WT and SA-db/db) and SD-db/db mice; *P<0.01 vs WT and SD-db/db). D, Mean arterial blood pressure (n=15 each group). *P<0.01 vs WT and #P<0.01 vs SD-db/db. WT indicates wild-type.
cGMP Production and BH₄ Levels

CgMP production, as a reflection of basal NO production, was decreased in aortic rings from db/db mice compared with WT mice (P<0.05). SD-db/db mice showed greater cGMP production compared with db/db and SA-db/db (P<0.01) mice (Figure 4A). BH₄ levels were reduced in the aortas of db/db mice compared with WT mice (Figure 4B), whereas the oxidative product of BH₄, 7,8-BH₂, was not different among all studied groups (Figure 4C). The BH₄ to 7,8-BH₂ ratio was significantly decreased in db/db and SA-db/db mice aortas as compared with WT and SD-db/db mice (Figure 4D).

Discussion

Patients with type 2 diabetes mellitus display endothelial dysfunction, which is thought to play a key role in the mechanisms of atherogenesis. However, the molecular mechanisms of endothelial dysfunction are not known, nor has it been proven that improving endothelial function in diabetes mellitus will reduce cardiovascular events. Here, we use the leptin receptor–deficient db/db mouse, which is a commonly used model for type 2 diabetes mellitus. db/db mice develop hyperglycemia and insulin resistance and other metabolic abnormalities, including obesity and hyperlipidemia. Our results demonstrate that deficient eNOS phosphorylation is a molecular mechanism of vascular dysfunction in db/db mice, and that modulation of eNOS phosphorylation corrects both vascular dysfunction and increased stroke size.

Several lines of evidence suggest that phosphorylation of eNOS at S1176 is essential to the link between metabolism and vascular dysfunction. First, eNOS phosphorylation is diminished in diabetes mellitus, hypercholesterolemia, and atherosclerosis. Second, estrogens, statins, and PPARα and PPARγ agonists increase eNOS S1176 phosphorylation. Third, vasculoprotective signaling molecules such as insulin, IGF-1, vascular endothelial growth factor, adiponectin, and leptin increase S1176 phosphorylation. These agonists act through multiple kinases to converge on eNOS phosphorylation, suggesting that this is a common integration point that underlies endothelial dysfunction from various causes.

To study the effects of phosphorylation, we used single amino acid eNOS mutations at S1176. In the SD mutation, serine is replaced by aspartate, which has a negatively charged carboxyl group. The negative charge and the size of the side chain are similar to the negatively charged phosphate group of phospho-eNOS, hence the designation of the mutation as phosphomimetic. In contrast, the SA mutation replaces serine with alanine, of which the methyl side chain is unphosphorylatable. These mutations have been characterized in vitro and are known to affect eNOS enzymatic activity, with the SD mutation showing increased NO production at rest and the SA mutation showing decreased NO production. Both mutations are sensitive to calcium stimulation, and the SA mutation is not a null mutation.

Figure 3. Effect of endothelial nitric oxide synthase (eNOS) mutations on vascular reactivity. A, Dose–response curves to ACh. WT (n=18); db/db (n=17); SD-db/db (n=6); SA-db/db (n=6). *P<0.05 WT vs db/db; #P<0.05 SD-db/db vs WT and SA-db/db. B, Dose–response curves to SNP. WT (n=12); db/db (n=17); SD-db/db (n=6); SA-db/db (n=5). *P<0.05 WT vs db/db; †P<0.05 WT vs SD-db/db; #P<0.05 SD-db/db vs SA-db/db. WT indicates wild-type.

Figure 4. cGMP and biopterin levels. A, cGMP levels in aorta: WT (n=8); db/db (n=6); SD-db/db (n=6); and SA-db/db (n=4) mice. *P<0.01, db/db vs WT; #P<0.01, WT vs SD-db/db vs SA-db/db. B, BH₄ levels. C, BH₄ levels. D, BH₄/BH₂ ratio. Measurements were made in aorta of WT (n=7), db/db (n=5), SD-db/db (n=6), and SA-db/db (n=7) mice. *P<0.05, db/db and SA-db/db vs WT and SD-db/db. WT indicates wild-type.
In this study, we used eNOS SD and SA knock-in mice in which the endogenous eNOS gene carries these single amino acid mutations. We bred these mice to db/db mice to study vascular function and outcome to stroke. In the MCA occlusion model of stroke, the quantitative end point is the infarct volume. A meaningful comparison presupposes that the territories at risk are comparable, and that the effects of the filament occlusion are the same. We sought to address these issues in several ways. First, we assessed the effects of the eNOS mutations on cerebrovascular anatomy relevant to the outcome of the stroke model. We performed carbon black injections to visualize the vessels and to ensure that continuity of the Circle of Willis was not affected. We did not observe redundant MCA or changes in the sizes of the PCA or Pcomm arteries that would affect outcome to filament occlusion. We performed lectin staining to quantitate capillary density and ensure that there were no detectable differences that may affect the stroke volume. Second, we assessed absolute CBF in the animals by hydrogen clearance. Third, we calculated infarct volume in several ways: direct infarct volume, indirect infarct volume, which accounts for ipsilateral edema, and percent infarct volume to brain size, which accounts for differences in brain size. Using all of these measures, the SD mutation reduced the infarct size of db/db mice, whereas the SA mutation did not. Fourth, we verified by laser Doppler flowmetry that the filament caused the same CBF reductions in the core ischemic zone.

Improvements in CBF in the ischemic penumbra are the most likely mechanism for the eNOS SD mutation to reduce infarct size in db/db mice. In support of this, we found that the impaired vascular reactivity in db/db mice is improved by the SD mutation but not the SA mutation.

We wish to point out potential limitations of our study. In addition to vascular effects, eNOS-derived NO is known to inhibit platelet aggregation and adhesion and block leukocyte–endothelial interactions. Although we demonstrate that the SD mutation improves vascular reactivity, we did not examine whether the SD mutation also acts through alterations of hemostasis, inflammation, or other effects. These systemic effects, either in the central nervous system or other organ systems, may also affect the outcome to cerebral ischemia. Thus, the effects of eNOS phosphorylation may not be exclusively vascular.

eNOS has been reported to affect insulin sensitivity, and high-fat–fed eNOS knockout mice display insulin resistance. We performed glucose tolerance tests, insulin tolerance tests, and measured lipid profiles. The eNOS SD and SA mutations do not significantly affect these metabolic parameters in db/db mice. We measured BH4, which is important to prevent eNOS uncoupling. The ratio of BH4/BH2 is significantly reduced in db/db mice compared with WT mice, but it is the same in SD-db/db mice as WT mice. This could occur by upregulation of GTP cyclohydrolase I expression, the rate-limiting step in BH4 production. It is still possible that there are other metabolic effects of eNOS mutations that could alter the outcome to cerebral ischemia. Alternatively, the eNOS mutations and the db/db mutation may both influence stroke size, but through mechanisms unique to one or the other that do not overlap.

We confirmed the functional significance of the reduced cerebral infarct size by neurological scoring using a system tailored for mice modified for body habitus. Like the Bederson score developed for use in rats, it includes forelimb flexion and circling behavior, but it differs because it does not include lateral push, which is a less reliable indicator in overweight mice, and it does include absence of spontaneous activity as an indicator of severe neurological functional deficit.

We previously reported that eNOS knockout mice that carry mutant bovine eNOS S1179 transgenes (bovine numbering corresponding to S1177 in humans and S1176 in mice) could be used to study the effects of those mutations on an eNOS-null background. The current study differs from the previous report in several important ways. First, here we are using knock-in mice in which the endogenous eNOS gene is mutated, rather than transgenic mice. Thus, effects attributable to expression level, transgene copy number, and site of integration are avoided. Second, we are assessing the effects of the eNOS mutations on the phenotype of the db/db mice in the stroke model, not the effects of the mutations by themselves.

Because NO needs to be generated in the proper subcellular location and with precise timing, targeting phosphorylation of the endogenous eNOS enzyme offers advantages over pharmacological replacement of NO with nitrate donors or genetic overexpression of endothelial or other NOS isoforms by gene therapy. The appropriate targets for modulation of NO production by eNOS may be the kinases or phosphatases that regulate eNOS phosphorylation. In addition to eNOS phosphorylation, Akt kinase, AMPK, and PKA clearly have other substrates and effects that may impact cell survival.

Conclusions
db/db mice show diminished eNOS phosphorylation, greater stroke size after MCA occlusion, hypertension, and impaired vascular reactivity. The phosphomimetic eNOS SD mutation improves NO production, reduces stroke size, corrects hypertension, and improves vascular reactivity. These results demonstrate that modulation of the eNOS phosphorylation site in db/db mice has beneficial effects on physiology and outcome to a stroke model.

Acknowledgments
We are grateful to Helen Swanson for technical assistance.

Sources of Funding
This work was supported by National Institutes of Health grants R01 HL57818 and NS33335 to P.L.H. and American Heart Association Scientist Development grant 0835344 N to D.N.A.

Disclosures
None.

References


Deficient eNOS Phosphorylation Is a Mechanism for Diabetic Vascular Dysfunction Contributing to Increased Stroke Size
Qian Li, Dmitriy Atochin, Satoshi Kashiwagi, John Earle, Annie Wang, Emiri Mandeville, Kazuhide Hayakawa, Livius V. d'Uscio, Eng H. Lo, Zvonimir Katusic, William Sessa and Paul L. Huang

Stroke. 2013;44:3183-3188; originally published online August 29, 2013;
doi: 10.1161/STROKEAHA.113.002073

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/44/11/3183

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2013/08/29/STROKEAHA.113.002073.DC1

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/
Deficient eNOS phosphorylation is a mechanism for diabetic vascular dysfunction contributing to increased stroke size
SUPPLEMENTARY METHODS

Generation of SD-db/db and SA-db/db mice

Within exon 26 of mouse eNOS gene, we replaced the codon for S1176 (TCT) with the codon for alanine (GCT) for the SA mutation, or the codon for aspartate (GAT) for the SD mutation. We generated targeting constructs in which this region is flanked by homologous regions on either side. The constructs contain a neomycin resistance gene (to allow positive selection) flanked by lox P sites, facilitating its removal in one step by Cre recombinase. The constructs also contain a thymidine kinase gene at one end to allow negative selection; the TK gene is not incorporated into the genome by homologous recombination. Although the eNOS gene does not have a typical polyadenylation site (AATAAA), it does have a potential alternative site (CATAAA). We avoided placing the neomycin resistance gene near this site. Using J1 embryonic stem cells, we generated chimeric mice with germ line transmission of the mutations. We mated these mice with EIIa-Cre transgenic mice to allow Cre recombinase to delete the neomycin resistance gene between the loxP sites. This leaves one copy of the loxP site and the SA or SD mutation. eNOS SA and SD mice were backcrossed by the marker assisted congenic method for six generations (equivalent to ten generations of conventional backcrossing) to the C57 BL/6 genetic background.

The SD allele was genotyped using the following primers: 5’-AGAGATAGCGTGACAAGG-3’ and 5’-CTCCTGCAAAAGAAAAAGTC-3’ specific for the mutation. The SA allele was genotyped using the following primers: 5’-CGCATAACCAAGGC-3’ and 5’-ACCACAGCCGAGGAAACCTT-3’ specific for the mutation. The wild-type S1176 allele was genotyped using the following primers: 5’-AGGTTCCCTCGGTGCTGTTAGTTA-3’ and 5’-GGTGTCTGGGACTC ACTGTCGAGG-3’. By breeding heterozygous db/db mice with homozygous SA or SD knockin mice, we obtained F1 animals heterozygous for the SA or SD mutation. By mating F1 animals that carry the db allele and the mutant SA or SD allele, we obtained the animals in the F2 generation. F2 animals heterozygous for db/db but homozygous for the SA or SD mutation were used to breed mice homozygous for both db/db and the SA or SD mutation. The primers used for db/db genotyping were: 5’-AGAAACCAGACAC TCTTTGAAGTCTC-30’ and 5’- CATCCAAACCAGTTAGTTTGTG T-3’. The PCR product was further digested with Rsa-1 restriction enzyme and the digested samples were examined on 3% Nusieve Gel.

Microvessel density measurement

Brain endothelial cells were labeled by lectin-tomato (Vector laboratory) before sacrifice. Three brain coronal sections from the lectin-infused brain sections, 1.5 mm, 2.5 mm, and 3.5 mm from frontal pole were chosen. For each section, two areas in the striatum and cerebral cortex were photographed using a 20x objective. The percent area of positively stained microvessels was calculated in a blinded fashion using Image J software.

Cerebrovascular anatomy

Cerebrovascular anatomy was assessed by intra-cardiac carbon black perfusion. Brain endothelial cells were labeled by lectin-tomato (Vector laboratory) before sacrifice. Three brain coronal sections from the lectin-infused brain sections, 1.5 mm, 2.5 mm, and 3.5 mm from frontal pole were chosen. For each section, two areas in the striatum and cerebral cortex were photographed using a 20x objective. The percent area of positively stained microvessels was calculated in a blinded fashion using Image J software.
cerebrovascular arterial system. Mice were kept on ice for 10 min, after which the brain was removed and kept in 4% paraformaldehyde.

**Western blotting**

The carotid artery was dissected from its origin on the aorta to its bifurcation, and treated with tissue lysis buffer (10mM Tris-HCl, pH8; 1mM EDTA, pH8; 10% SDS, protease inhibitor cocktail). Protein samples were electrophoresed in a 7.5% Tris-HCl polyacrylamide gel and transferred to PVDF membrane. The membrane was blotted with 5% non-fat dry milk in Tris-buffered saline-0.1% Tween-20, and incubated with primary antibody in 4°C overnight. The membrane was washed and then incubated with secondary antibody conjugated with horseradish peroxidase. Detection was carried out using an ECL Plus Western Blotting Detection kit. The membrane was stripped and reprobed with additional primary antibodies. Quantitative densitometry was performed using Image J. Antibodies directed against human S1177 phospho-eNOS and total eNOS were obtained from BD Transduction Laboratories. Antibodies directed against p-Akt, Akt, p-AMPK, AMPK and nNOS were purchased from Cell Signaling Technology.

**Hydrogen clearance measurement of CBF**

Hydrogen clearance was used to measure resting CBF as described. Briefly, the femoral artery was catheterized for blood pressure and blood gas measurements. Platinum H2-sensitive electrodes were inserted through a burr hole into the parietal cortex. Reference Ag-AgCl electrodes were attached to the base of the tail. H2 (2.5% in air) was added to anesthetic gaseous mixture via the respirator for 60 seconds and the washout H2-curves were recorded for CBF calculations. Absolute values of CBF (mL×100 g−1×min−1) were calculated by the initial slope method.

**MCAO model of stroke details**

Mice were anesthetized with 1.5% of isoflurane in 30% oxygen and 70% N2O. A fiberoptic probe (Perimed) was affixed to the skull over the brain area supplied by MCA for relative CBF measurements by laser Doppler flowmetry. Baseline CBF values were measured before carotid artery ligation and MCA occlusion and considered to be 100% flow. MCA occlusion was caused by inserting a 7-0 nylon filament covered by silicon (Doccol Corp) into the internal carotid artery and advancing it to the origin of the MCA. Ischemia was confirmed by reduction in CBF to less than 20% of control values. The filament was withdrawn after one hour occlusion. Reperfusion was confirmed by laser Doppler flowmetry after withdrawal. The mouse was sacrificed and the brain was collected at 23 hours after reperfusion for TTC analysis. Mice that did not survive 23 hours after reperfusion were excluded from statistical evaluation. Mortality was 17% (3 from 18 mice) for WT, 25% (5 from 20) for db/db, 20% (3 from 15) for SD-db/db and 29% (5 from 17) for SA-db/db mice.

**cGMP measurement**

The thoracic aorta was dissected and incubated in Dulbecco’s minimal essential medium containing 0.1% BSA for 3 hours. The aorta was treated with 200 μM IBMX for 10 minutes and snap frozen in liquid nitrogen. cGMP was extracted with 6% TCA, washed with water-saturated ether, and dried with vacuum centrifuge. cGMP was measured using a cGMP EIA kit (GE Healthcare) and normalized for tissue weight.
Measurement of tissue BH$_4$ and BH$_2$

Whole aortas were homogenized in extraction buffer containing 50 mM Tris (pH 7.4), 1 mM dithiothreitol, and 1 mM EDTA at 4°C and centrifuged at 10,000g (8 min at 4°C). Biopterin levels were determined by HPLC (Beckman Coulter, Fullerton, CA) as described previously$^3$.

Glucose and insulin tolerance tests

Mice were fasted 16 hours for glucose tolerance tests and 6 hours for insulin tolerance tests. Glucose (0.5 g/kg of 5% dextrose) or insulin (0.3 U/kg, Humulin-R) were administered intraperitoneally. Blood samples were drawn from the tail vein and glucose blood levels were measured at 0, 15, 30, 60, and 120 min using a BREEZE®2 Blood Glucose Meter (Bayer).
SUPPLEMENTARY TABLE I

Lipid profile, CBF, blood gases and heart rate

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>db/db</th>
<th>SD-db/db</th>
<th>SA-db/db</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>61.4 ± 7.4</td>
<td>154.7 ± 11.2*</td>
<td>189.6 ± 19.8*</td>
<td>138.2 ± 36.4*</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>6.2 ± 1.7</td>
<td>54.7 ± 13.5*</td>
<td>51.6 ± 6.6*</td>
<td>28.4 ± 12.6</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>51.9 ± 7.6</td>
<td>91.4 ± 6.8</td>
<td>131.2 ± 15.6*</td>
<td>90.0 ± 38.2</td>
</tr>
<tr>
<td>Total triglyceride (mg/dL)</td>
<td>43.0 ± 11.6</td>
<td>70.4 ± 18.8</td>
<td>50.8 ± 21.6</td>
<td>49.5 ± 8.2</td>
</tr>
<tr>
<td>CBF, cortex (ml/100g/min)</td>
<td>94.7 ± 22.7</td>
<td>82.7 ± 20.5</td>
<td>83.0 ± 23.0</td>
<td>84.7 ± 23.2</td>
</tr>
<tr>
<td>pH</td>
<td>7.33 ± 0.06</td>
<td>7.41 ± 0.06</td>
<td>7.34 ± 0.05</td>
<td>7.37 ± 0.05</td>
</tr>
<tr>
<td>pCO₂ (mm Hg)</td>
<td>39.4 ± 1.2</td>
<td>40.2 ± 1.2</td>
<td>39.4 ± 1.0</td>
<td>40.3 ± 1.3</td>
</tr>
<tr>
<td>pO₂ (mm Hg)</td>
<td>145.1 ± 12.3</td>
<td>144.0 ± 16.9</td>
<td>145.0 ± 9.1</td>
<td>143.0 ± 7.2</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>561 ± 64</td>
<td>582 ± 34*</td>
<td>557 ± 30</td>
<td>556 ± 53</td>
</tr>
</tbody>
</table>

Total cholesterol, LDL-cholesterol, HDL-cholesterol, total triglyceride in serum (WT, n=8; db/db, n=5; SD-db/db, n=4; SA-db/db, n=4, * p<0.05 vs. WT). CBF, blood gases (n=3 for each group), heart rate (n=15 for each group). #P<0.05 db/db vs. SD-db/db mice.
Supplementary Figure I. Generation and characterization of SD-db/db and SA-db/db mice
A. Knock-in construct. Top, genomic DNA with eNOS exons 17 to 26, is shown. The construct includes the mutation indicated with an asterisk (*) in exon 26, a neomycin resistance gene (NEO) flanked by lox P sites (black triangles), and a thymidine kinase gene (TK). Homologous recombination between the genomic DNA and the targeting construct (indicated by crossed lines) replaces the region surrounding exon 26 with the mutated exon, as well as the NEO gene flanked by lox P sites. Treatment with Cre recombinase by mating the chimeric mice with EIIa-Cre mice results in excision of the NEO gene, and one residual lox P site. B. Western blot analysis of brain protein of WT, db/db, SA-db/db and SD-db/db mice (n=4 for each group). Brain tissue was isolated from mice and electrophoresed on SDS-PAGE. C-F, The average expression levels of eNOS/actin (C), nNOS/actin (D), p-Akt/Akt (E) and p-AMPK/AMPK (F).
Supplementary Figure II. Anatomy of cerebrovasculature and CBF

A. Representative images of cerebrovasculature of WT, db/db, SD-db/db and SA-db/db mice after intracardiac carbon black perfusion. Upper panels show representative images of ventral brain surface and lower panels show representative higher magnifications of the posterior Circle of Willis showing the PCA and Pcomm arteries from WT, db/db, SD-db/db and SA-db/db mice.

B. The representative diameter of PCA (left panel) and Pcomm (right panel) in WT (n=4), db/db (n=6), SD-db/db (n=6) and SA-db/db (n=5) mice.

C. Microvascular density measured by lectin staining in cerebral cortex (left panel) and striatum (right panel). n=3 for each group.

D. CBF measured in the core ischemic region by LDF, during 1 hour of MCA occlusion and 60 minutes of reperfusion. There were no significant differences between WT (n=15), db/db (n=15), SD-db/db (n=12) and SA-db/db (n=12) mice.
Supplementary Figure III. Insulin and glucose tolerance tests

A. Insulin tolerance test. Time course of blood glucose levels in WT, db/db, SA-db/db and SD-db/db mice after intraperitoneal injection of insulin (*p<0.05 WT vs. db/db, SD-db/db and SA-db/db mice).

B. Glucose tolerance test. Time course of glucose levels after intraperitoneal injection of glucose (*p<0.05 WT vs. db/db, SD-db/db and SA-db/db mice).
SUPPLEMENTARY REFERENCES

