

## **SUPPLEMENTAL MATERIAL**

**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'-CTCCTGCAAAGAAAAGTC-3' specific for the mutation. The SA allele was genotyped using the following primers: 5'-CGCATACGCACCCAGGCC-3' and 5'-ACCACAGCCGGAGGAACCTT-3' specific for the mutation. The wild-type S1176 allele was genotyped using the following primers: 5'-AGGTTCCCTCCGGCTGTGGTAGTTA-3' and 5'-GGTGTCTGGGACTC ACTGTCAAAG-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'-AGAACGGACAC TCTTTGAAGTCTC-30' and 5'-CATTCAAACCATAGTTTAGGTTTGTG 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<sup>1</sup>. 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<sup>1</sup>. After euthanasia, a latex-carbon black mixture liquid was injected into the heart to perfuse the

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 reprobbed 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<sup>2</sup>. Briefly, the femoral artery was catheterized for blood pressure and blood gas measurements. Platinum H<sub>2</sub>-sensitive electrodes were inserted through a burr hole into the parietal cortex. Reference Ag-AgCl electrodes were attached to the base of the tail. H<sub>2</sub> (2.5% in air) was added to anesthetic gaseous mixture via the respirator for 60 seconds and the washout H<sub>2</sub>-curves were recorded for CBF calculations. Absolute values of CBF (mL×100 g<sup>-1</sup>×min<sup>-1</sup>) 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% N<sub>2</sub>O. 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 (Docol 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<sub>4</sub> and BH<sub>2</sub>**

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<sup>3</sup>.

**Glucose and insulin tolerance tests**

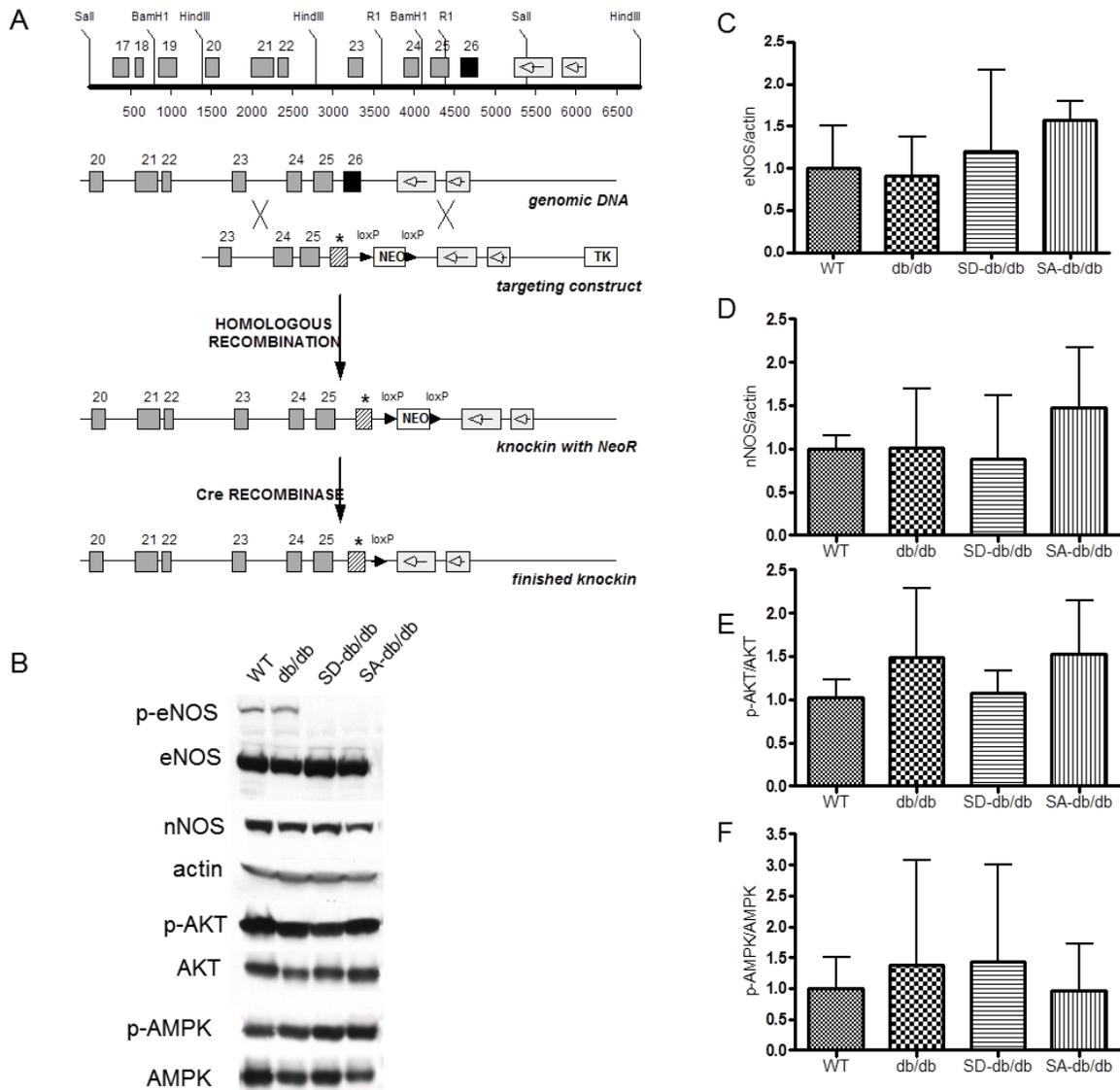
Mice were fasted 16 hours for glucose tolerance tests and 6 hours for insulin tolerance tests. Glucose (0.5 g/kg of 5% dextrose) or insulin (0.3 U/kg, Humulin-R) were administered intraperitoneally. Blood samples were drawn from the tail vein and glucose blood levels were measured at 0, 15, 30, 60, and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

**SUPPLEMENTARY TABLE I****Lipid profile, CBF, blood gases and heart rate**

	<b>WT</b>	<b>db/db</b>	<b>SD-db/db</b>	<b>SA-db/db</b>
Total cholesterol (mg/dL)	61.4 ± 7.4	154.7 ± 11.2*	189.6 ± 19.8*	138.2 ± 36.4*
LDL cholesterol (mg/dL)	6.2 ± 1.7	54.7 ± 13.5*	51.6 ± 6.6*	28.4 ± 12.6
HDL cholesterol (mg/dL)	51.9 ± 7.6	91.4 ± 6.8	131.2 ± 15.6*	90.0 ± 38.2
Total triglyceride (mg/dL)	43.0 ± 11.6	70.4 ± 18.8	50.8 ± 21.6	49.5 ± 8.2
CBF, cortex (ml/100g /min)	94.7 ± 22.7	82.7 ± 20.5	83.0 ± 23.0	84.7 ± 23.2
pH	7.33 ± 0.06	7.41 ± 0.06	7.34 ± 0.05	7.37 ± 0.05
pCO <sub>2</sub> (mm Hg)	39.4 ± 1.2	40.2 ± 1.2	39.4 ± 1.0	40.3 ± 1.3
pO <sub>2</sub> (mm Hg)	145.1 ± 12.3	144.0 ± 16.9	145.0 ± 9.1	143.0 ± 7.2
Heart rate (beats/min)	561 ± 64	582 ± 34 <sup>#</sup>	557 ± 30	556 ± 53

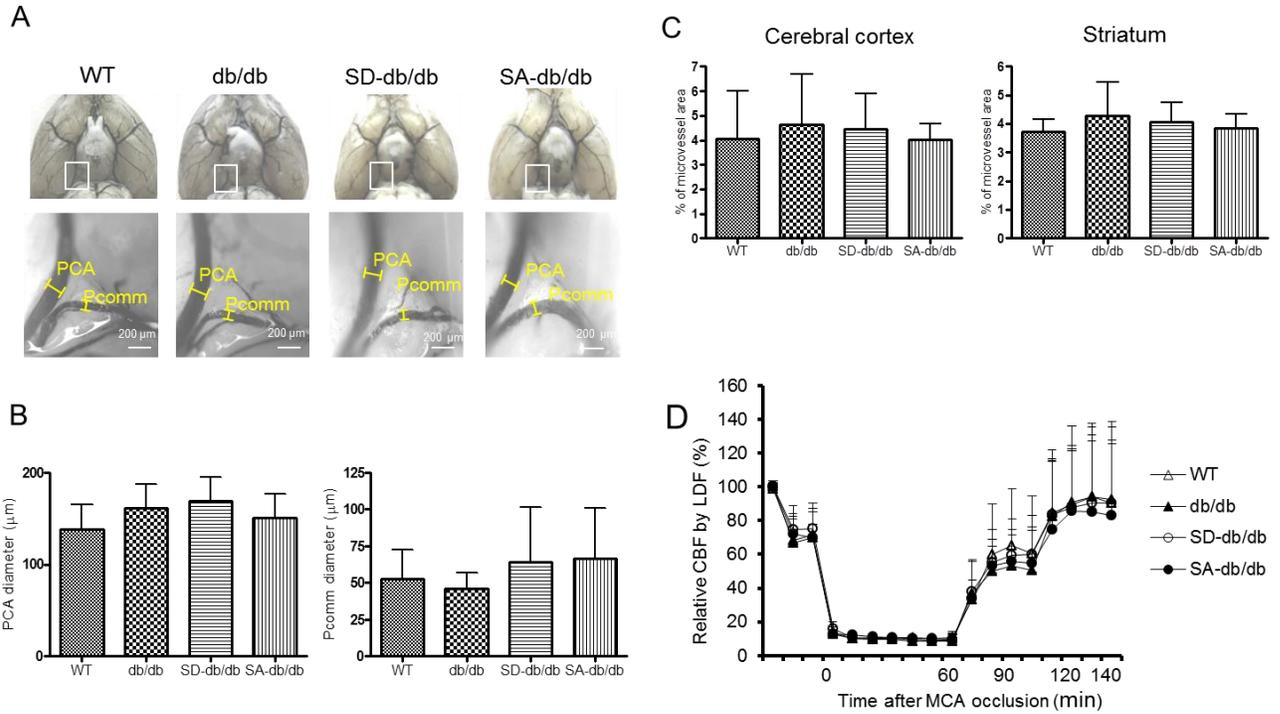
Total cholesterol, LDL-cholesterol, HDL-cholesterol, total triglyceride in serum (WT, n=8; db/db, n=5; SD-db/db, n=4; SA-db/db, n=4, \* p<0.05 vs. WT). CBF, blood gases (n=3 for each group), heart rate (n=15 for each group). #P<0.05 db/db vs. SD-db/db mice.

## SUPPLEMENTARY FIGURES



### 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).



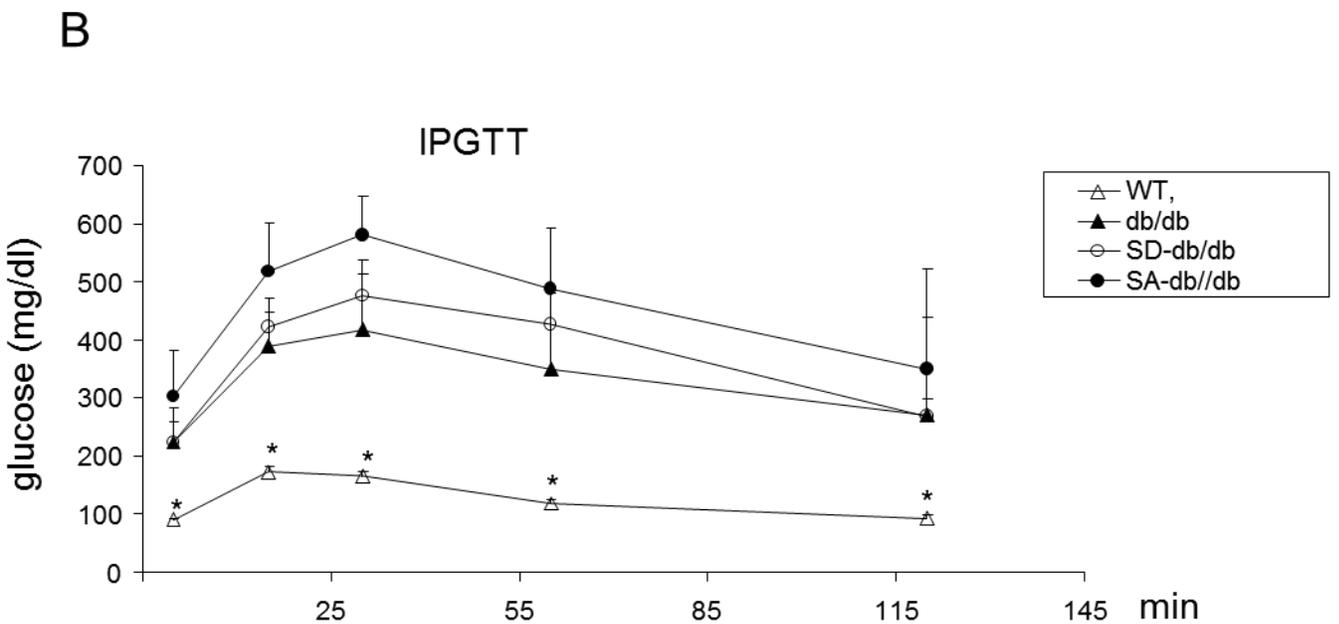
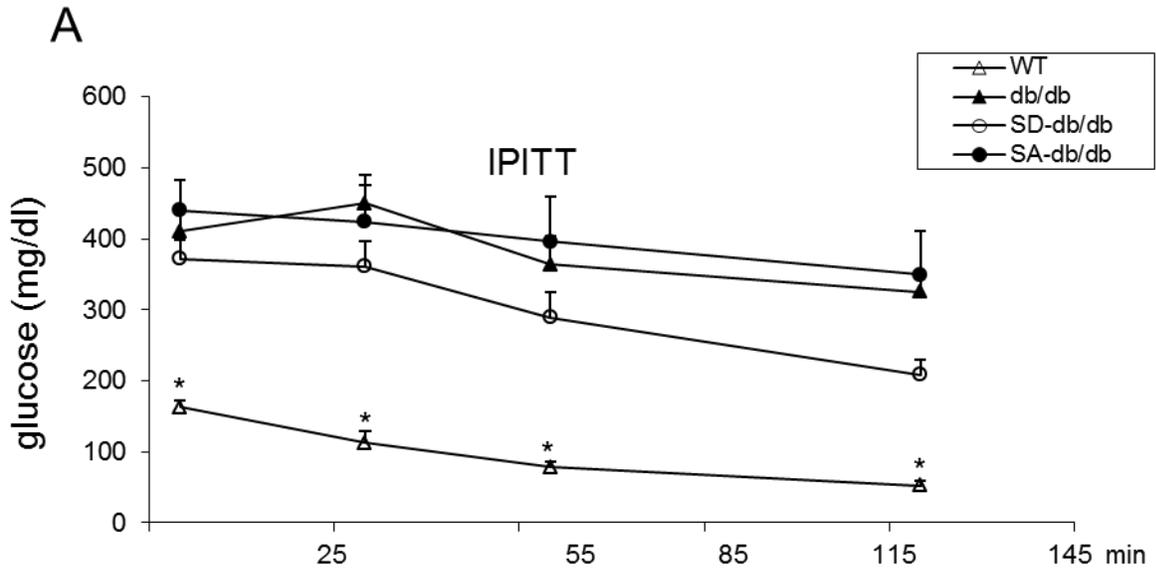
## Supplementary Figure II. Anatomy of cerebrovasculature and CBF

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**

1. Hayakawa K, Pham LD, Katusic ZS, Arai K, Lo EH. Astrocytic high-mobility group box 1 promotes endothelial progenitor cell-mediated neurovascular remodeling during stroke recovery. *Proc Natl Acad Sci U S A*. 2012;109:7505-10.
2. Wang X, Liu J, Zhu H, Tejima E, Tsuji K, Murata Y, et al. Effects of neuroglobin overexpression on acute brain injury and long-term outcomes after focal cerebral ischemia. *Stroke*. 2008;39:1869-74.
3. d'Uscio LV, Milstien S, Richardson D, Smith L, Katusic ZS. Long-term vitamin C treatment increases vascular tetrahydrobiopterin levels and nitric oxide synthase activity. *Circ Res*. 2003;92:88-95.