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Endothelial nitric oxide synthase transgenic models of endothelial dysfunction

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Abstract

Endothelial production of nitric oxide is critical to the regulation of vascular responses, including vascular tone and regional blood flow, leukocyte–endothelial interactions, platelet adhesion and aggregation, and vascular smooth muscle cell proliferation. A relative deficiency in the amount of bioavailable vascular NO results in endothelial dysfunction, with conditions that are conducive to the development of atherosclerosis: thrombosis, inflammation, neointimal proliferation, and vasoconstriction. This review focuses on mouse models of endothelial dysfunction caused by direct genetic modification of the endothelial nitric oxide synthase (eNOS) gene. We first describe the cardiovascular phenotypes of eNOS knockout mice, which are a model of total eNOS gene deficiency and thus the ultimate model of endothelial dysfunction. We then describe S1177A and S1177D eNOS mutant mice as mouse models with altered eNOS phosphorylation and therefore varying degrees of endothelial dysfunction. These include transgenic mice that carry the eNOS S1177A and S1177D transgenes, as well as knockin mice in which the endogenous eNOS gene has been mutated to carry the S1177A and S1177D mutations. Together, eNOS knockout mice and eNOS S1177 mutant mice are useful tools to study the effects of total genetic deficiency of eNOS as well as varying degrees of endothelial dysfunction caused by eNOS S1177 phosphorylation.

Keywords

Nitric oxide; Endothelium; Vascular dysfunction; Transgenic mouse; Cerebral circulation

Introduction

In this article, we describe mutant mouse models of endothelial dysfunction specifically caused by total or partial deficiency in activity of endothelial nitric oxide synthase (eNOS). As the primary interface between the blood and body tissues, the endothelium regulates several important aspects of vascular responses [1–4]. First, it regulates vascular tone and regional blood flow, as it produces factors involved in vasodilation, including nitric oxide (NO) and prostacyclin, and vasoconstriction, including endothelin. Second, it modulates adhesion and aggregation of platelets, thus affecting propensity or resistance to thrombosis. Third, the endothelium expresses surface adhesion molecules that influence interactions with leukocytes

and monocytes, affecting recruitment of inflammatory cells and atherosclerotic plaque formation. Finally, it modulates vascular smooth muscle cell proliferation in response to vascular injury. A relative deficiency in the amount of bioavailable vascular NO results in endothelial dysfunction, with conditions that are conducive to the development of atherosclerosis: thrombosis, inflammation, neointimal proliferation, and vasoconstriction.

Regulation of NO production by eNOS

Physiologic endothelial function requires both basal and stimulated production of NO by eNOS. The eNOS enzyme utilizes L-arginine as substrate for generation of NO from the terminal guanidine nitrogen, producing L-citrulline from the rest of the molecule. eNOS activity is regulated at multiple steps, including transcription; substrate availability; interactions with calcium, calmodulin, and enzymatic cofactors such as FAD, FMN, NADPH, and BH₄; protein–protein interactions with hsp90 and caveolins; and post-translational modifications. eNOS is also regulated by dimerization and precise subcellular localization to caveolae [5,6]. In many processes, the downstream target of NO is soluble guanylate cyclase (sGC). NO binds to the heme moiety of sGC, activating it and resulting in increased cyclic guanosine monophosphate (cGMP) production. cGMP mediates vascular smooth muscle relaxation [7,8], as well as effects on platelet aggregation [9]. Once generated, NO may also interact with superoxide to form peroxynitrite anion, effectively removing it from its physiologic functions (Fig. 1).

eNOS is activated by various physiological mediators, including mechanical shear stress [3, 10–12], estrogens [13], insulin [14], acetylcholine [15], and other receptor-dependent agonists [10]. eNOS is post-translationally phosphorylated at several serine (S) and threonine (T) residues, including T497, S617, S635, and S1177, using the human amino acid sequence numbering [16]. Phosphorylation of eNOS at the human S1177 residue is an important mechanism for physiologic regulation of NO production. eNOS phosphorylation at this site is increased by estrogens and statins. It is decreased in diabetes, hypertension, metabolic syndrome, and obesity, as well as by interleukin-6 (IL-6), tumor necrosis factor α (TNF α), and resistin [17,18].

The corresponding positions for the human eNOS S1177 phosphorylation site differ in other species. The possibility for confusion is compounded by the fact that there are other serine residues in close proximity. The sequence for the 1203 amino acid human eNOS gene, starting at position 1171, reads SRIRTQSFSL. Within this sequence, S1177, indicated in boldface and underlined, is the serine residue that is phosphorylated. Comparison of the sequences of the eNOS gene in other species shows that the corresponding residue is S1176 in the murine sequence and S1179 in the bovine sequence. This is relevant because when the bovine sequence is used in transgenic constructs, it is the bovine S1179 site that is modified, corresponding to the serine at the 1177 position in the human sequence. For the remainder of this paper, we will always refer to this site as S1177, using the human sequence for numbering.

Endothelial dysfunction

Endothelial dysfunction, broadly defined, occurs when the endothelium fails to serve its normal physiologic and protective functions [3,19–22]. This may occur because the endothelium is damaged or missing, as in the case of denuded endothelium in coronary arteries subjected to angioplasty or percutaneous intervention. It may also occur when the normal responses of the endothelium are altered, for example, by oxidative stress, hyperglycemia, advanced glycation products, free fatty acids, or inflammatory cytokines. Endothelial dysfunction is an important final common pathway between many cardiovascular risk factors—such as hypertension, obesity, diabetes, and dyslipidemia—and the development of atherosclerosis. Metabolic diseases such as atherosclerosis, hypertension, diabetes, obesity, hyperlipidemia,

hypercholesterolemia, and metabolic syndrome impair endothelial NO availability, resulting in endothelial dysfunction.

Endothelial dysfunction can be measured by assessing physiologic vasodilator responses [20]. Vascular dilation to mediators such as acetylcholine or increased blood flow depends on NO produced by the endothelium. In animal models, vascular reactivity can be measured in isolated vessel segments using myography of vessel rings or vessel segments. In man, endothelial function can be measured in the cardiac catheterization laboratory by monitoring the effect of intracoronary acetylcholine infusion. In the clinical setting, flow-mediated dilation (FMD) can be measured by using ultrasound to quantitate the response of brachial artery diameter to reflow after a period of vascular occlusion by a blood pressure cuff. Although they measure responses in different vascular beds, FMD correlates with the catheterization laboratory measurements of coronary circulation responses. Furthermore, FMD predicts future cardiovascular events. Thus, FMD is a commonly used method to assess endothelial function, and impaired FMD is taken to reflect endothelial dysfunction.

The mechanism of endothelial dysfunction is insufficient endothelial NO availability [19]. This can be caused by reduced eNOS gene expression, L-arginine deficiency [23], inhibition of NOS by asymmetric dimethylarginine [24], uncoupling of eNOS and tetrahydrobiopterin deficiency [25], mechanical damage of endothelium, metabolic intoxication by free fatty acids and inflammatory cytokines IL-6 and TNF α [17], alteration of endothelial intracellular PI3-Akt signaling by hyperglycemia and advanced glycation products [20], and scavenging vascular NO by superoxide anion [26]. Two important causes of endothelial dysfunction include a reduction in eNOS phosphorylation at S1177 [27,28] and rapid reaction of NO with superoxide to form peroxynitrite anion [29].

Many mouse models of human disease manifest endothelial dysfunction, including leptin receptor-deficient (db/db) mice [30], leptin-deficient (ob/ob) mice [31], and genetic knockouts of apoE [32], Akt1 [33,34], superoxide dismutase [35], sGC [36], and glutathione peroxidase [37]. Endothelium-specific overexpressions of GTP cyclohydrolase I [38] and SIRT1 [39] have been shown to ameliorate endothelial dysfunction in mouse models of atherosclerosis and diabetes. This review focuses on mouse models of endothelial dysfunction caused by direct genetic modification of the eNOS gene. We first describe the cardiovascular phenotypes of eNOS knockout mice, which are a model of total eNOS gene deficiency and thus the ultimate model of endothelial dysfunction. We then describe S1177A and S1177D mutant mice as models that demonstrate altered eNOS phosphorylation, and therefore varying degrees of endothelial dysfunction.

eNOS knockout mice

eNOS knockout mice were generated as the first model of direct endothelial dysfunction by disrupting the region that encodes for the NADPH ribose and adenine binding sites, which are essential for eNOS catalytic activity [15]. These mice demonstrate the absence of eNOS mRNA and enzymatic activity, but are fertile and have normal anatomy. Other lines of eNOS knockout mice have also been generated [40–42], and all of them show similar phenotypes.

eNOS knockout mice show the phenotypes caused by total genetic absence of eNOS. This includes lack of endothelium-derived relaxing factor (EDRF) activity and hypertension [15], increased platelet aggregation [9], leukocyte–endothelial adhesion [43], increased vascular smooth muscle cell proliferation [44], and increased propensity to thrombosis [43], stroke [45,46], and atherosclerosis [47]. eNOS knockout mice do not demonstrate ischemic preconditioning [48], and show abnormalities in mitochondrial function and biogenesis [49, 50], and insulin resistance [51,52].

It is possible that other NOS isoforms may compensate for lack of eNOS in the eNOS knockout mice. To address this question, double and triple NOS knockout mice have been generated. Mice that lack both neuronal nitric oxide synthase (nNOS) and eNOS display abnormalities in hippocampal long-term potentiation, a model for learning and memory [53]. Mice that lack all three NOS isoforms show nephrogenic diabetes insipidus [54] as well as spontaneous myocardial infarction [55].

Vascular reactivity and hypertension

eNOS knockout mice lack EDRF activity as measured by vascular relaxation in response to acetylcholine and other endothelium-dependent vasodilators, both in isolated arteries [15] and in vivo [8]. This is important because in the development of atherosclerotic vascular disease, functional impairment of endothelium-dependent relaxation occurs before morphological changes of vessels and is the first sign of endothelial dysfunction. The fact that eNOS knockout mice are hypertensive proves the essential role of endothelial NO in the physiologic regulation of blood pressure. Absence of eNOS cannot be compensated by cardiac, neuronal, or hormonal homeostatic mechanisms, and hypertension occurs despite these counterregulatory mechanisms [56,57]. eNOS knockout mice paradoxically manifest decreased blood pressure after nonspecific inhibition of NO by L-NA, indicating an important role for neuronal NOS (likely in perivascular nerves, see Fig. 2) in the maintenance of blood pressure [58].

Interactions with leukocytes, platelets, and vascular smooth muscle

eNOS knockout mice demonstrate the essential roles of endothelial NO in leukocyte–endothelial interactions, which are important for both inflammatory responses and for development of atherosclerotic plaques. Absence of eNOS increases expression of endothelial P-selectin and thereby increases leukocyte rolling and adhesion to the endothelial surface [43]. Similarly, platelet aggregation, hemostasis [9], coagulability [43], and thrombosis [59, 60] are increased in the absence of eNOS. NO-induced inhibition of platelet aggregation is mediated by sGC in platelets [7,36].

The roles of eNOS in regulating vascular smooth muscle proliferation are demonstrated by the phenotype of eNOS knockout mice after vascular injury [61]. Cuff injury of the femoral artery results in neointima formation, and this response is greatly exaggerated in eNOS knockout mice as compared with wild-type mice [60].

Together, neutrophil infiltration, platelet aggregation, and smooth muscle proliferation accelerate vascular complications and reduce tissue perfusion and endothelial dysfunction. Thus, it is not surprising that apoE/eNOS double knockout mice demonstrate accelerated atherosclerosis compared to apoE knockout mice. In addition, apoE/eNOS double knockout mice show evidence of ischemic heart disease, left ventricular dysfunction, and aortic aneurysm, making them a useful model that mimics many aspects of human atherosclerotic cardiovascular disease [62]. Thus, eNOS deficiency causes endothelial dysfunction, characterized by impaired vascular reactivity, followed by abnormal vascular tone, impaired tissue perfusion, and hypertension. Effects on platelet aggregation and adhesion, leukocyte–endothelial interactions, and vascular smooth muscle proliferation likely underlie the increased propensity to atherosclerosis seen in man when there is endothelial dysfunction, particularly in the setting of diabetes and metabolic syndrome.

Effects on cardiac function, pulmonary vasculature, and mitochondria

eNOS maintains basal vasorelaxation in the lungs and protects the lung by attenuating pulmonary hypertension. Endothelial dysfunction in eNOS knockout mice increases hypoxic pulmonary hypertension [63].

NO is produced in the heart by eNOS present in the cardiac endothelium and cardiac myocytes. eNOS knockout mice show enhanced inotropic and lusitropic responses under basal conditions [64] and in response to isoproterenol [65]. These results indicate that eNOS normally blunts the inotropic response to β -adrenergic stimulation [58]. Furthermore, eNOS deficient mice demonstrate enhanced left ventricular dysfunction and post-myocardial infarction remodeling by diminished myocyte hypertrophy [66].

eNOS is essential to mitochondrial biogenesis, as eNOS knockout mice show fewer mitochondria, decreased expression of mitochondrial genes, and less mitochondrial respiration both under basal conditions and after cold exposure and exercise [49]. Furthermore, NO produced by eNOS plays important roles in the differentiation and proliferation of brown adipocytes, which are important to thermogenesis and energy metabolism [50,67–70]. These results provide evidence that abnormalities in eNOS function may underlie the metabolic changes that occur in obesity and diabetes.

Cerebral blood flow regulation and stroke

Hypertension and impaired vasorelaxation are the primary underlying mechanisms of increased vulnerability of eNOS knockout mice to stroke injury [45,46,71]. Using the middle cerebral artery occlusion model of stroke, eNOS knockout mice show larger areas of ischemia and correspondingly smaller penumbra zone (Figs. 2,3) [46,71]. This results in worse post-stroke neurological deficits and larger cerebral infarct volumes as compared with wild-type mice (Fig. 5) [46].

eNOS plays a major role in the regulation of cerebral blood flow (CBF). Cerebral vasorelaxation to acetylcholine is impaired in eNOS knockout mice [8]. Interestingly, despite absence of eNOS and resulting hypertension, eNOS knockout mice have the same level of resting CBF as WT mice have. This is likely due to autoregulation and the compensatory action of perivascular neuronal NOS.

eNOS knockout mice have been instrumental in defining the roles of eNOS in CBF regulation during hyperbaric oxygenation [8]. The physiologic response is to protect the brain by vasoconstriction against dangerously increased reactive oxygen species and hyperbaric oxygen. Quantitative CBF measurements demonstrate that the first phase cerebral vasoconstriction response to hyperbaric oxygen is mediated by endothelial NO inactivation by superoxide anion, causing decreased blood flow. Later, the second phase vasodilator response is due to NO-dependent hyperbaric oxygen vasodilation, resulting in seizures. The second phase depends on NO produced by both eNOS and nNOS. The likely mechanism triggering NO production in this phase is increased concentration of nNOS [72] and eNOS [73] mediated by hsp-90 binding. During prolonged HBO exposure, reactive oxygen species initiate NOS stimulation, increased protein–protein interactions between hsp90 and NOS isoforms and intracellular calcium entry [74]. These data show the important role of endothelial NO production in cerebral vasoconstriction and in hyperoxic hyperemia preceding oxygen-induced seizures [75].

Ischemic preconditioning is a phenomenon in which brief ischemic events protect the brain from subsequent severe ischemic insult [76,77]. eNOS knockout mice do not show ischemic preconditioning [48], demonstrating an essential role for eNOS in the protection. Resting CBF and CBF changes during preconditioning do not mediate the difference between preconditioned WT and eNOS knockout mice. The exact mechanisms underlying the protective role of eNOS in ischemic preconditioning are still not known.

eNOS S1177 mutant mice

Transgenic S1177A and S1177D mice

Although eNOS knockout mice are an extreme example of endothelial dysfunction in that they totally lack eNOS, there are no known human examples of eNOS gene deficiency. Rather, patients who manifest endothelial dysfunction show a relative deficiency in bioavailable endothelial NO. One of the mechanisms for this is abnormalities in phosphorylation of the eNOS enzyme at the S1177 residue. Indeed, S1177 phosphorylation of eNOS is one of most important and intensively investigated regulatory mechanisms for eNOS enzymatic activity. Estrogens, statins, insulin, leptin, adiponectin, VEGF, and shear stress promote S1177 eNOS phosphorylation and activation. In contrast, eNOS phosphorylation is decreased in patients with hypertension, hyperlipidemia, diabetes, and metabolic syndrome.

eNOS S1177 is a target substrate for Akt kinase [27,28,78,79]. In addition, S1177 is known to be phosphorylated by several other kinases, including AMP kinase [80], protein kinase A (PKA) and protein kinase G (PKG) [81], and calmodulin-dependent kinase II (CaMKII) [82]. The phosphatases that regulate dephosphorylation of S1177 have not been identified. Mechanistically, increased NOS activity appears to be due to enhanced electron flux through the reductase domain and reduced calmodulin dissociation upon S1177 phosphorylation [83]. By crystallography, the carboxyl tail of eNOS normally blocks electron transfer from the reductase domain [84]. Phosphorylation of S1177 results in a negative charge, swinging the entire FMN domain to allow enhanced electron transfer through the reductase domain, activating the enzyme. Physiologically, shear stress, VEGF, and insulin activate eNOS by S1177 phosphorylation, permitting greater eNOS activation to take place at any level of calcium without substantially changing the affinity of calcium-activated calmodulin for eNOS.

Mutant forms of eNOS have been used to study the role of eNOS S1177 phosphorylation. Substitution of alanine in place of S1177 (S1177A) produces a form of eNOS that cannot be phosphorylated, since it lacks the hydroxyl group on the side chain of serine. Substitution of aspartate in place of S1177 (S1177D) mimics the increased catalytic activity caused by phosphorylation, since the aspartate side chain contains a negatively charged carboxyl group that mimics the negatively charged phosphate group of eNOS phosphorylated at S1177 [83, 85]. S1177A and S1177D mutations both are active in endothelial cells [85], but S1177A eNOS does not demonstrate Akt-dependent NO release, while S1177D shows enhanced NO production even in the absence of Akt [27,28]. S1177D eNOS shows reduced calmodulin dissociation and enhanced electron flux via the reductase domain [83].

We generated transgenic mice carrying the S1177A and S1177D mutations in an eNOS cDNA transgene [46]. We bred these mice with eNOS knockout mice to obtain mice that only express S1177A or S1177D mutant eNOS forms. The expression and subcellular localization of the S1177A and S1177D mutant forms of eNOS were documented by Western blotting and en face immunostaining of arteries. There was no difference in protein level expression and subcellular localization of eNOS in either mutant strains (Fig. 4). Unphosphorylatable S1177A transgenic mice showed attenuated vascular relaxation to acetylcholine. Using the middle cerebral artery occlusion model of stroke, S1177A mice showed increased infarct volume, as compared with S1177D and WT mice [46] (Fig. 5).

Laser speckle flowmetry assesses CBF by measuring blood flow with high spatial and temporal resolution. This provides a two dimensional determination of real-time flow deficit [86]. eNOS knockout mice developed larger cortical ischemic areas compared with wild-type and S1177D mice. The S1177A mutation did not improve cortical blood flow whereas the S1177D eNOS transgene reduces the ischemic area to the size seen in wild-type mice (Fig. 2).

S1177 transgenic mice have been useful to study the involvement of eNOS S1177 phosphorylation in maintaining pulmonary relaxation and protecting against hyperoxic pulmonary injury. Comparison of three strains of mice having different level of eNOS activity (eNOS knockout, S1177A transgenic, and S1177 D transgenic mice) shows that pulmonary vascular protection correlates directly with eNOS activity [87]. S1177A transgenic mice demonstrated profound oxygen-induced pulmonary injury, while S1177D transgenic mice were protected.

Knockin S1177A and S1177D mice

After generating S1177 transgenic mice on eNOS knockout background, we made knockin mice that carry the same mutations. We replaced the endogenous eNOS gene with a mutant gene carrying the S1177D and S1177A mutations [28]. These mice are currently being characterized. As an initial step, we bred the knockin mice with Akt1 knockout mice to test whether the mutations could overcome the effects of Akt1 deficiency [88].

Acetylcholine-induced production of cGMP was diminished in aortas of S1177A/Akt1 mutant mice and increased in S1177D/Akt1 mutant mice as compared with WT mice. Measurements of NO by nitrite production showed that the S1177D mutation maintains NO production despite deficiency of Akt1.

Wound healing experiments demonstrated a critical role of eNOS as Akt substrate for postnatal pathological angiogenesis [88]. S1177A/Akt1 mutant mice show attenuated wound healing and increased ischemic limb injury after ligation of femoral artery, while S1177D/Akt1 mutant mice demonstrated faster wound healing and resistance to ischemic injury. Recovery of blood flow, revascularization, clinical score, and recruitment of pericytes was better in S1177/Akt1 mutant mice (comparable with WT) as compared with S1177A/Akt1 mutants (comparable with Akt1 knockout mice). Taken together, these results prove that wound healing and post-ischemic angiogenesis mainly depends on S1177 eNOS phosphorylation.

The mRNA expressions of HIF1 α -responsive genes, including VEGF-A, VEGF receptor (Flk-1), and angiopoietin-like 4 protein were increased in S1177D/Akt1 mutants and wild-type mice as compared with Akt1 deficient and S1177A/Akt1 mutant mice. HIF1 α abundance in ischemic muscles of S1177D/Akt1 deficient mice was higher as compared with S1177A/Akt1 deficient mice. These results indicate that the phosphomimetic S1177D mutation was able to rescue defective postnatal angiogenesis of Akt1 knockout mice by stabilization of HIF1 α and increased production of responsive genes.

Conclusion

eNOS knockout and apoE/eNOS double knockout mice are models of endothelial dysfunction due to total absence of endothelial NO production. S1177A and S1177A/Akt1 mutant mice are models with partial endothelial dysfunction, which are more clinically relevant in that they mimic the deficiency in eNOS S1177 phosphorylation seen in man. Modulation of eNOS phosphorylation on serine 1177 is a promising approach to define the roles of its phosphorylation on endothelial dysfunction. As such, S1177 mutant mice may lead to proof of concept for eNOS phosphorylation as a target for the treatment and prevention of cardiovascular disease associated with conditions that show endothelial dysfunction, including diabetes, metabolic syndrome, and obesity.

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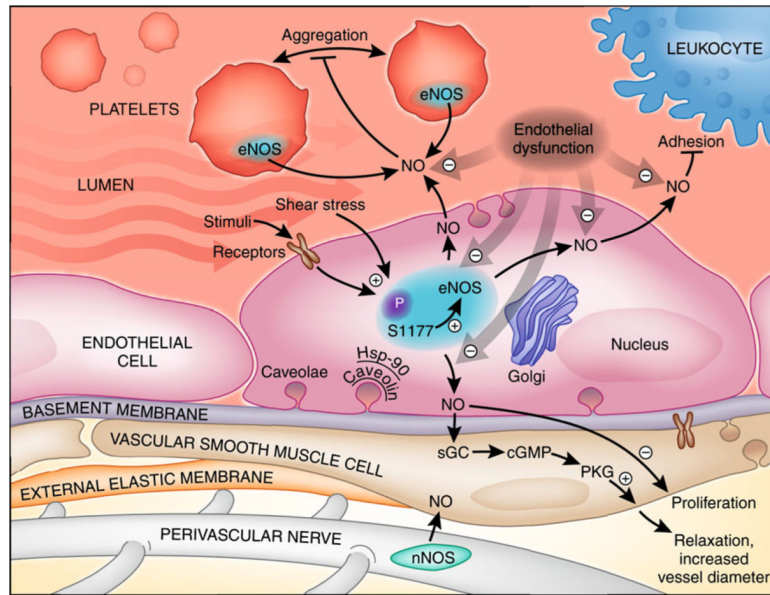


Fig. 1. Biological roles of endothelial NO: inhibition of platelets aggregation, modulation of leukocyte–endothelial interactions, modulation of smooth muscle cell proliferation, and maintenance of vascular tone. eNOS is activated by physiological and metabolic stimuli, shear stress, and receptor-dependent agonists. S1177 phosphorylation site is an important regulatory mechanism for eNOS activity. eNOS catalytic activity depends on the intracellular localization to caveolae and the protein–protein interactions with caveolins and hsp-90. NO activates cGMP, inducing PKG-dependent relaxation of smooth muscle cells and increasing luminal diameter and blood flow. *P* phosphorylation; *S1177* serine 1177, *Hsp-90* heat shock protein-90, *PKG* serine–threonine-specific protein kinase G

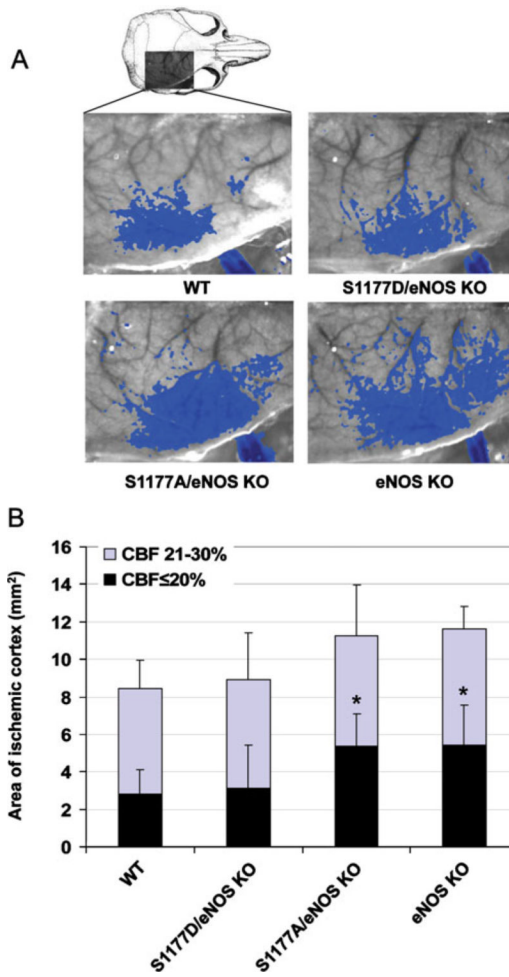


Fig. 2. Effect of eNOS mutations on CBF deficit. **a** Laser speckle contrast images in mice subjected to middle cerebral artery occlusion. Blue areas indicate regions with $\leq 30\%$ residual blood flow after 60 min of ischemia. **b** The area of cortex with $\leq 20\%$ (black) or 21–30% (blue) residual blood flow as compared with preischemic baseline. * $P < 0.05$, comparing the area with $\leq 20\%$ of residual blood flow of eNOS knockout and S1177A transgenic mice with WT and S1177D transgenic mice

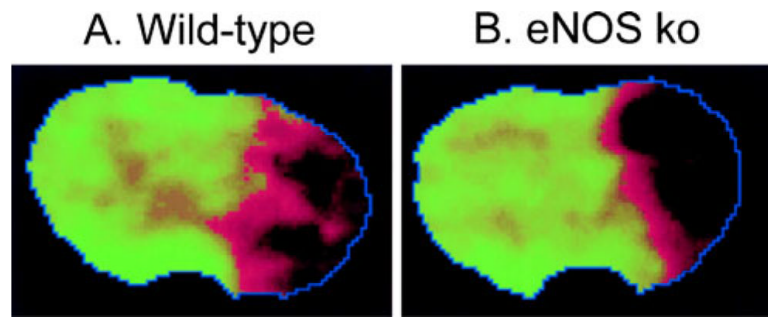


Fig. 3.
a Temporal correlation mapping image shows blood flow after focal cerebral ischemia in wild-type mouse. Normal hemodynamic status is shown as green. Regions with abnormal CBF are represented with a red-to-black sliding color scale. The core appears *black*, and the penumbra is the *reddish rim* surrounding the core. An extensive hemodynamic penumbra exist in wild-type mice 30 min after focal ischemia. **b** The hemodynamic deficit in eNOS knockout mice after focal ischemia is more severe. The core is larger, and the penumbra is correspondingly smaller compared with wild-type mice

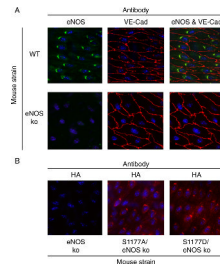


Fig. 4. Subcellular localization of eNOS S1177 mutation in transgenic mice. **a** En face immunostaining of carotid arteries shows localization of eNOS in perinuclear Golgi. VE-cadherin (VE-cad) staining shows the outlines of the endothelial membranes. **b** En face immunostaining of carotid arteries shows perinuclear location of the transgene hemagglutinin tag in the perinuclear Golgi in a pattern similar to eNOS immunostaining in wild-type mice. Original magnification $\times 600$

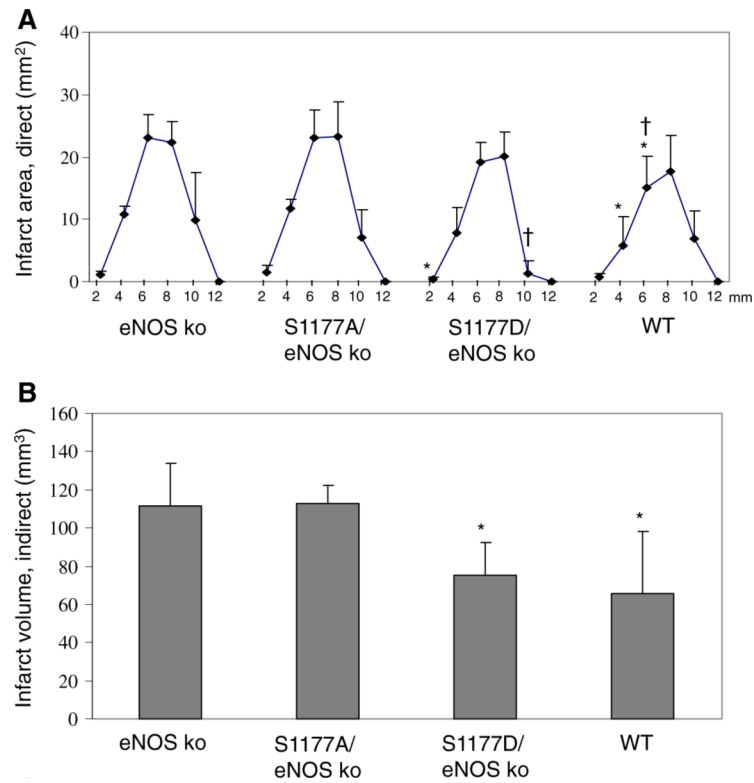


Fig. 5. Effect of S1177 mutations on cerebral infarct volume. Mice were subjected to 1 h of focal brain ischemia followed by reperfusion for 23 h. The brains were cut into coronal sections and stained using 2,3,5-triphenyltetrazolium chloride. **a** *Abscissa* shows the distance (millimeter) from the rostral surface of the brain. For each group, $n=7$ mice. * $P<0.05$ versus S1177A transgenic mice, † $P<0.05$ versus eNOS KO mice. **b** Infarct volumes were determined by the indirect method, which corrects for edema. * $P<0.05$ versus infarct volume from S1177A mutant and from eNOS knockout mice