Targeted overexpression of endothelial nitric oxide synthase in endothelial cells improves cerebrovascular reactivity in Ins2Akita-type-1 diabetic mice

Saurav B Chandra1,*, Sumathy Mohan2,*, Bridget M Ford1, Lei Huang1, Preethi Janardhanan2, Kaiwalya S Deo1, Linlin Cong1, Eric R Muir1,3 and Timothy Q Duong1,3

Abstract

Reduced bioavailability of nitric oxide due to impaired endothelial nitric oxide synthase (eNOS) activity is a leading cause of endothelial dysfunction in diabetes. Enhancing eNOS activity in diabetes is a potential therapeutic target. This study investigated basal cerebral blood flow and cerebrovascular reactivity in wild-type mice, diabetic mice (Ins2Akita+/−/C0), nondiabetic eNOS-overexpressing mice (TgeNOS), and the cross of two transgenic mice (TgeNOS-Ins2Akita+/−/C0) at six months of age. The cross was aimed at improving eNOS expression in diabetic mice. The major findings were: (i) Body weights of Ins2Akita+/−/C0 and TgeNOS-Ins2Akita+/−/C0 were significantly different from wild-type and TgeNOS mice. Blood pressure of TgeNOS mice was lower than wild-type. (ii) Basal cerebral blood flow of the TgeNOS group was significantly higher than cerebral blood flow of the other three groups. (iii) The cerebrovascular reactivity in the Ins2Akita+/−/C0 mice was significantly lower compared with wild-type and TgeNOS mice. Overexpression of eNOS rescued cerebrovascular dysfunction in diabetic animals, resulting in improved cerebrovascular reactivity. These results underscore the possible role of eNOS in vascular dysfunction in the brain of diabetic mice and support the notion that enhancing eNOS activity in diabetes is a potential therapeutic target.

Keywords

Cerebral blood flow, cerebrovascular dysfunction, MRI, hypercapnia, eNOS, arterial spin labeling

Introduction

Endothelial dysfunction is a hallmark of diabetes. Reduced bioavailability of nitric oxide (NO) as a result of impaired endothelial nitric oxide synthase (eNOS) activity is a leading cause of endothelial dysfunction in diabetes. Diabetic mice with eNOS knockout show a deleterious effect on renal microvasculature and aortic vessels. NO deficiency has been shown to precede the development of several complications of diabetes such as accelerated atherosclerosis affecting large arteries and microvascular dysfunction resulting in neuropathy and nephropathy. Enhancing eNOS activity in diabetes is a potential therapeutic target.

NO is generated from the conversion of L-arginine to L-citrulline by isoforms of NOS using tetrahydrobiopterin (BH4) as a cofactor. Among the three isoforms, eNOS is the predominant one expressed in vascular endothelial cells that constitutively generates NO. NO is...
an important mediator in the regulation of cerebral blood flow (CBF). CBF is increased by NO derived from endothelial cells or neurons. Administering NOS inhibitors narrows cerebral arteries and reduces CBF. Decreased CBF has been observed in type-1 diabetic patients. Transgenic eNOS overexpression in mice has been shown to protect against endotoxin shock, systemic hypotension, and skeletal muscle ischemic/reperfusion injury. eNOS overexpression also minimizes neointimal lesion formation and medial thickening in a model of vascular remodeling. Rats overexpressing eNOS using adenoviral gene transfer develop a short-term increase in CBF. However, the effects of eNOS overexpression in the diabetic brain have not been studied. Given that reduced NO availability is a leading cause of endothelial dysfunction in diabetes, normalizing eNOS activity by enhancing NO bioavailability through transgenic engineering could be a therapeutic option. In the present study, we investigated the effects of overexpression of eNOS on CBF and cerebrovascular reactivity (CR) in diabetes. CR was measured as the percent CBF changes associated with hypercapnic challenge (inhalation of 5% CO₂ in air). CBF and CR measurements were made using MRI on Ins2 Akita spontaneously diabetic mice, nondiabetic Tie2-specific transgenic eNOS-overexpressing (TgeNOS) mice, and the cross of these two animals (TgeNOS-Ins2Akita+/−) with the goal of enhancing eNOS production in diabetic animals. Physiological parameters (respiration rate, heart rate, mean arterial blood pressure, body weight, and blood glucose) were measured and compared across groups. We tested the hypothesis that overexpression of eNOS will rescue cerebrovascular dysfunction in diabetes.

Materials and methods

The following ARRIVE guidelines were followed in the preparation of this manuscript: Title, Abstract, Background, Objectives, and Ethical statement; Study Design; Experimental procedures; Experimental animals, Housing, and husbandry; Experimental outcomes; Statistical methods, Numbers analyzed; Interpretation and scientific implications; and Generalizability/translation and Funding. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center San Antonio in accordance with the Guide for the Care and Use of Laboratory Animals.

Breeding and genotyping

A pair of heterozygous C57BL/6J-Ins2Akita+/− (stock number 003548) from Jackson Laboratories (Bar Harbor, ME) was used for breeding. Heterozygous Ins2Akita+/− mice obtained after back crossing of these parental strains were used for experiments. The tail snips obtained from litters at the time of weaning (about three weeks after birth) were used to identify their genotype using a PCR/restriction length polymorphism assay. Purified DNA from tail snips were subjected to PCR amplification using the following primers and conditions recommended by Jackson Laboratories: the primers used for Ins2 Akita genotyping include FP: 5’-TGC TGA TGC CCT GGC CTG CT3’ (MR1093) and RP: 5’-TGG TCC CAC ATA TGC ACA TG3’. The PCR product of Akita genotype was further digested with Fnu-1 restriction enzyme and the digested samples were examined using 12% polyacrylamide gels.

A hemizygous breeding pair of TgeNOS overexpressing mice (expressing human eNOS fused with green fluorescent protein in vascular endothelial cells) on a C57BL/6J background was used for breeding experiments. Litters born for this hemizygous breeding pairs were genotyped using primers FP: 5’-GATGGAAGACTTGGTGGGAG3’ and RP: 5’-GTTCCTCCTGGATTAAAGTGAG3’ that specifically amplify the human eNOS and results were further confirmed by the presence of green fluorescent protein (Transnety, Cordova, TN). To generate Ins2Akita+/− mice that express human eNOS, hemizygous Ins2Akita mice were crossbred with hemizygous TgeNOS expressing mice for two to three generations, even though both strains were already available on a C57BL/6J background, to obtain genetic homogeneity. The litters were genotyped for both Ins2Akita+/− and hemizygous TgeNOS, and mice that were positive for both strains (hemizygous) were referred to as TgeNOS-Ins2Akita+/−.

Magnetic resonance imaging

For MRI studies, four groups of male mice aged 5–7 months with a body weight ranging between 21 and 30 g were studied: (i) wild-type (WT) C57BL/6J mice (n = 8), (ii) TgeNOS mice (n = 9), (iii) Ins2Akita (n = 9) mice, and (iv) a genetic cross between TgeNOS and Ins2Akita mice (TgeNOS-Ins2Akita+/−) (n = 8).

All animals were noninvasively monitored for respiration rate, oxygen saturation, and rectal temperature and maintained within normal physiological ranges unless otherwise perturbed by hypercapnia. MRI experiments were performed on a 7 Tesla magnet (Bruker Biospec, Billerica, MA) with a 150 Gauss/cm gradient. A small custom-made circular surface coil designed for brain imaging (inner diameter = 1.1 cm) and a circular labeling heart coil (inner diameter = 0.8 cm) was used for arterial spin labeling. Mice were anesthetized with 1.0–1.5% isoflurane.
Temperature was maintained at 37°C and respiratory rate at 80–110 bpm. CBF was acquired using the continuous arterial spin labeling technique and single-shot echo-planar imaging with field of view = 1.28 × 1.28 cm, data matrix = 64 × 64, slice thickness = 1 mm, 9 slices, labeling duration = 2.2 s, post label delay = 350 ms, repetition time = 3 s, and echo time = 8 ms. Basal CBF was acquired in air. CBF hypercapnic responses were measured using inhalation of 5 min air followed by 5 min 5% CO₂. CBF maps and hypercapnia-induced percent changes maps were calculated.16,17 Quantitative CBF and CBF changes were tabulated for the neocortices using region of interest analysis.

**Blood glucose and blood pressure measurements**

On a separate group of animals of similar age and weight as the MRI studies, blood pressure, heart rate, and nonfasting blood glucose were measured under similar experimental conditions outside the scanner (n = 5 each of the four groups). For blood pressure measurements, a common carotid artery was catheterized with PE-10 tubing and connected to a pressure transducer in a terminal surgery (Biopac System Inc., MP150, Goleta, CA). Blood glucose levels were measured using an AlphaTRAK 2® Blood Glucose Monitoring System (Abbott Laboratories, Abbott Park, IL).

**Statistical analysis**

Statistical analysis employed paired t test for CBF changes between normocapnic versus hypercapnic challenge within group and unpaired t test for CBF changes across different animal groups. Statistical significance was set at p < 0.05. Mean ± SD was used in text and table. Mean ± SEM was used for graphs.

**Results**

Physiological parameters (age, weight, respiration rate, blood glucose, blood pressure, and heart rate) are summarized in Tables 1 and 2. The mean ages were about six months and they were not statistically different amongst the four animal groups. Body weights of Ins2Akita+/− (21 ± 0.4 g) and TgeNOS-Ins2Akita+/− (23 ± 0.9 g) mice were significantly lower than that of wild-type (28 ± 0.9 g) and TgeNOS (30 ± 0.9 g) mice. Respiration rates were not statistically different amongst the four animal groups. Blood glucose values of wild-type and TgeNOS mice were normal but those of Ins2Akita+/− and TgeNOS-Ins2Akita+/− were markedly elevated compared with wild-type as expected. Blood glucose was also significantly different between Ins2Akita+/− and TgeNOS-Ins2Akita+/−. Blood pressure of TgeNOS mice was low (48 ± 9 mmHg) compared with wild-type mice (61 ± 17 mmHg) but the difference was not significant, whereas blood pressure of Ins2Akita+/− (62 ± 11 mmHg) and TgeNOS-Ins2Akita+/− (56 ± 7 mmHg) mice were similar to that of wild-type mice. Heart rates of the Ins2Akita+/− (501 ± 63 bpm) and TgeNOS-Ins2Akita+/− (558 ± 57 bpm) were lower compared with that of wild-type (601 ± 25 bpm) and TgeNOS (570 ± 13 bpm) mice.

Genotyping of wild-type, Ins2Akita+/−, and TgeNOS-Ins2Akita+/− mice are shown in Figure 1. In panel (a), the wild-type mice (lane 1) showed the wild-type allele at 140 bp, the Ins2Akita+/− mice (lane 2) and TgeNOS-Ins2Akita+/− (lane 3) both showed the mutant Ins2Akita allele at 280 bp in addition to the wild-type product at 140 bp (indicating the heterozygous genotype). In panel (b), only the TgeNOS mice (lane 3) showed expression of the TgeNOS gene at 361 bp. In panel (c), only the TgeNOS-Ins2Akita+/− mice (lane 3) showed the human eNOS product at 361 bp. These results confirmed the genotypes of mice generated from our breeding colony.

Representative multi-slice CBF images are shown in Figure 2(a). Quantitative CBF was tabulated for neocortices as shown by the overlaid region of interests. The mean basal CBF of the TgeNOS group was

Table 1. Physiological parameters (age, weight, and respiration rate).

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Age (months)</th>
<th>Weight (grams)</th>
<th>Respiration (breaths/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (8)</td>
<td>5.9 ± 0.2</td>
<td>28 ± 0.9</td>
<td>88 ± 3</td>
</tr>
<tr>
<td>TgeNOS (9)</td>
<td>6.7 ± 0.8</td>
<td>30 ± 0.9</td>
<td>80 ± 4</td>
</tr>
<tr>
<td>Ins2Akita+/− (9)</td>
<td>6.3 ± 0.2</td>
<td>21 ± 0.4</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>TgeNOS-Ins2Akita+/− (8)</td>
<td>7.4 ± 0.5</td>
<td>23 ± 0.9</td>
<td>75 ± 4</td>
</tr>
</tbody>
</table>

Ages, weights, and respiration rates from the animals used for MRI studies. Mean ± SD, *p < 0.05 from wild type. Significance was calculated with respect to controls.

Table 2. Physiological parameters (blood glucose, blood pressure, and heart rate).

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Blood glucose (mg/dL)</th>
<th>Blood pressure (mmHg)</th>
<th>Heart rate (beats/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (5)</td>
<td>215 ±8</td>
<td>61 ±17</td>
<td>601 ±25</td>
</tr>
<tr>
<td>TgeNOS (5)</td>
<td>231 ±28</td>
<td>48 ±9</td>
<td>570 ±13</td>
</tr>
<tr>
<td>Ins2Akita+/− (5)</td>
<td>675 ±164*</td>
<td>62 ±11</td>
<td>501 ±63</td>
</tr>
<tr>
<td>TgeNOS-Ins2Akita+/− (5)</td>
<td>447 ±97</td>
<td>56 ±7</td>
<td>558 ±57</td>
</tr>
</tbody>
</table>

Note: Blood glucose, blood pressure, heart rates from a separate group animals. Mean ±SD, *p < 0.001 from wild-type. *p < 0.05 between Ins2Akita+/− and TgeNOS-Ins2Akita+/−.
Figure 1. Genotyping of wild-type, \( \text{Ins2}^{\text{Akita+/-}} \) and \( \text{TgeNOS-Ins2}^{\text{Akita+/-}} \). (a) Lane 1 (wild-type mice): a single band (140 bp) of the wild-type allele. Lane 2 (\( \text{Ins2}^{\text{Akita+/-}} \) mice): wild-type (140 bp) and mutant \( \text{Ins2}^{\text{Akita+/-}} \) gene (280 bp). Lane 3 (\( \text{TgeNOS-Ins2}^{\text{Akita+/-}} \) mice): wild-type (140 bp) and mutant \( \text{Ins2}^{\text{Akita+/-}} \) gene (280 bp). (b) Only Lane 3 (\( \text{TgeNOS} \) mice) shows expression of the \( \text{TgeNOS} \) gene (361 bp). (c) Only Lane 3 (\( \text{TgeNOS-Ins2}^{\text{Akita+/-}} \) mice) shows overexpression of the \( \text{TgeNOS} \) gene (361 bp).

Figure 2. (a) Multi-slice CBF images from the anterior to the posterior region of the brain were acquired from a wild-type animal. Region of interests of the neocortices were used for quantitative analysis. (b) Group CBF values of wild-type, \( \text{Ins2}^{\text{Akita+/-}} \), \( \text{TgeNOS} \), and \( \text{TgeNOS-Ins2}^{\text{Akita+/-}} \) mice. Mean ± SEM, *\( p < 0.05 \), **\( p < 0.001 \).
The percent CBF increase of the TgeNOS-Ins2Akita mice, diabetic mice (Ins2Akita+/−) and nondiabetic eNOS-overexpressing mice (TgeNOS), and the cross mice (TgeNOS-Ins2Akita+/−) at six months of age. The major findings were: (i) body weights of Ins2Akita+/− and TgeNOS-Ins2Akita+/− were significantly different from those of the wild-type and TgeNOS mice. Only blood pressure of TgeNOS mice was lower than wild-type. (ii) Basal CBF of the TgeNOS group was significantly higher than CBF of the other three groups, whereas the basal CBF of the remaining three groups were not statistically different from each other. (iii) The CR in the Ins2Akita+/− mice was significantly lower compared with the wild-type mice, whereas that in the TgeNOS-Ins2Akita+/− was significantly higher compared with the Ins2Akita+/− and TgeNOS group, indicating normalization of CR. These findings suggest overexpression of eNOS rescued some aspects of cerebrovascular dysfunction in diabetes, resulting in improved CR in diabetic animals. These findings also underscore the role of eNOS in cerebrovascular dysfunction in the brain in diabetes, and thus enhancement of eNOS activity in diabetes could be a potential therapeutic target.

**Physiological parameters**

Body weights of Ins2Akita+/− and TgeNOS-Ins2Akita+/− were significantly lower compared with those of the wild-type and TgeNOS mice, likely due to complications of diabetes, consistent with a previous study. The body weight of TgeNOS mouse was normal, suggesting overexpression of eNOS per se did not affect the body weight, consistent with previous studies.

Blood glucose of TgeNOS-Ins2Akita+/− mice was lower than that of Ins2Akita+/− mice. It is possible that eNOS overexpression could interact with diabetes and contribute to the lower blood glucose observed in TgeNOS-Ins2Akita+/− mice, although there is no evidence of such interaction. However, TgeNOS per se did not increase blood glucose and thus this explanation is unlikely. The mechanism and the modulators that regulate blood glucose in this novel TgeNOS-Ins2Akita+/− mice are unknown. Note that there were large variations of blood glucose in Ins2Akita+/− and TgeNOS-Ins2Akita+/− mice compared with WT or TgeNOS mice. This is a result of measurement of non-fasting blood glucose which could spike-up and fluctuate dramatically in diabetic animals. Blood pressure of the TgeNOS mice was lower than that of wild-type mice, which was expected because overexpressing
eNOS (a vasodilator) would reduce vascular resistance. Blood pressure of Ins2Akita+/- mice was similar to that of wild-type mice. A previous study reported systolic blood pressure to be elevated after 12 weeks of age compared with wild-type mice. Possible explanations for this discrepancy in blood pressure could be due to: (i) the use of isoflurane, a vasodilator, which could have masked the differences between Ins2Akita+/- and wild-type mice; and/or (ii) previous studies measured blood pressure without the influence of anesthetics or under different anesthetics. Blood pressure of TgeNOS-Ins2Akita+/- mice was not different from wild-type mice. There is no published blood pressure data on TgeNOS-Ins2Akita+/- mice with which to compare. An additional confound is that isoflurane may upregulate NO production. However, the anesthesia settings were identical in all the animal groups, enabling the comparisons.

**Basal CBF**

Basal CBF of Ins2Akita+/- was not significantly different from that of wild-type mice. To our knowledge, there are no previous reports of CBF in Ins2Akita+/- mice. However, decreases in retinal blood flow have been observed at 6 and 7.5 months and choroidal blood flow at 2.5 and 7.5 months in Ins2Akita+/- mice. The brain is one of the organs well protected from most systemic disorders, and thus it likely has compensatory mechanisms to maintain normal CBF under basal (nonchallenged) conditions. In type-1 diabetic patients, reduced local CBF has been reported. A possible explanation of this discrepancy could be due to difference in severity of the disease or species differences. It is possible that older Ins2Akita+/- mice would show reduced basal CBF and future studies will need to investigate these animals at different age groups.

Basal CBF of TgeNOS mice was, however, significantly higher than basal CBF of the other groups. This is expected as NO has vasodilatory effects and overexpressing eNOS in TgeNOS mice is expected to increase CBF, consistent with a previous report. Basal CBF of TgeNOS-Ins2Akita+/- was not significantly different from that of wild-type. It is likely that the genetic cross resulted in less eNOS production, insufficient to elevate CBF as observed in the TgeNOS mice.

**Cerebrovascular reactivity**

Carbon dioxide is a potent vasodilator. Hypercapnic challenge with 5% CO₂ is commonly used to evaluate CR and cerebrovascular reserve in MRI. As expected, CBF significantly increased during hypercapnia compared with baseline in all animal groups. In addition, isoflurane, also a vasodilator, could have caused vasodilation at baseline and thus attenuated the hypercapnic response herein. Ins2Akita+/- mice showed significantly reduced hypercapnic response compared with wild-type mice, suggesting compromised cerebrovascular function. Compromised CR has been reported in diabetic patients. These findings support the notion that there is endothelial dysfunction in diabetes. TgeNOS mice showed reduced hypercapnic response compared with wild-type mice but did not reach statistical significance. We expected that overexpressing eNOS, a vasodilator, would dilate vessels at baseline and thus CO₂ would have less vasodilatory effects, thereby resulting in a smaller hypercapnia-induced CBF increase. TgeNOS-Ins2Akita+/- mice had a significantly higher hypercapnic response compared with Ins2Akita+/- mice (p < 0.01), but similar to wild-type mice, suggesting the overexpression of eNOS rescued some aspects of cerebrovascular dysfunction in diabetes. This finding demonstrates the positive influence of eNOS overexpression on vascular tone on diabetes.

**Limitations and future perspectives**

A limitation of this study is that we did not measure eNOS expression or NO production in our animals, and thus we could not validate the mechanism of action, although available data from this and a previous study suggest that cross animals have observable effects from eNOS overexpression. Future studies will need to make such measurements. We studied only 6-month old animals, and future studies will investigate these animals at different age groups. There are also alternative approaches to increase eNOS activity, such as by diet supplementation of L-arginine, sepiapterin, and salsalate, warranting further investigation. Future studies could include investigating neurovascular coupling associated with evoked functional stimulation. We predict functional neurovascular coupling would be perturbed in TgeNOS and Ins2Akita+/- mice, and less so in TgeNOS-Ins2Akita+/- animals.

**Conclusions**

These findings support the hypothesis that overexpression of eNOS rescues CR in diabetes. The brain likely has compensatory mechanisms to maintain normal CBF under basal (nonchallenged) conditions but could not compensate under challenged conditions. These findings underscore the role of eNOS in cerebrovascular dysfunction in diabetes and suggest that restoring eNOS activity by enhancing NO bioavailability is a promising treatment strategy that warrants further investigation. Future studies will need to measure eNOS activity and NO production to validate mechanism of action.
Funding
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported in part by the NIH (NS045879 to TD and DK096119 to SM), MERIT Award from the Department of Veterans Affairs, and a Translational Resource Technology Grant and a Pilot Grant from the Clinical Translational Science Award (parent grant UL1RR025767).

Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' contributions
SBC, SM, ERM, and TQD designed the study and developed the methodology; SBC, BMF, LH, PJ, KSD, and LC collected the data; SBC, KSD, SM, and BMF performed the analysis; and SBC, SM, and TQD wrote the manuscript.

References


