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Characterization of cerebral microvasculature in transgenic mice with endothelium targeted over-expression of GTP-cyclohydrolase I

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Abstract

Tetrahydrobiopterin (BH_4) is a critical determinant of nitric oxide (NO) production by nitric oxide synthase (NOS) in the vascular endothelium and its biosynthesis is regulated by the enzymatic activity of GTP-cyclohydrolase I (GTPCH I). The present study was designed to determine the effects of endothelium-targeted overexpression of GTPCH I (eGCH-Tg) on murine cerebral vascular function. Endothelium targeted over-expression of GTPCH I was associated with a significant increase in levels of BH_4, as well as its oxidized product, 7,8-dihydrobiopterin (7,8-BH_2) in cerebral microvessels. Importantly, ratio of BH_4 to 7,8-BH_2, indicative of BH_4 available for eNOS activation, was significantly increased in eGCH-Tg mice. However, expression of endothelial NOS, levels of nitrate/nitrite - indicative of NO production - remained unchanged between cerebral microvessels of wild-type and eGCH-Tg mice. Furthermore, increased BH_4 biosynthesis neither affected production of superoxide anion nor expression of antioxidant proteins. Moreover, endothelium-specific GTPCH I overexpression did not alter intracellular levels of cGMP, reflective of NO signaling in cerebral microvessels. The obtained results suggest that, despite a significant increase in BH_4 bioavailability, generation of endothelial NO in cerebral microvessels remained unchanged in eGCH-Tg mice. We conclude that under physiological conditions the levels of BH_4 in the cerebral microvessels are optimal for activation of endothelial NOS and NO/cGMP signaling.

Keywords

tetrahydrobiopterin; endothelial nitric oxide synthase; GTP-cyclohydrolase I; cerebral microvessels

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1. Introduction

Tetrahydrobiopterin (BH_4) is an essential co-factor for the enzymatic activity of nitric oxide synthase (NOS) isoforms, and the rate-limiting enzyme for BH_4 production is guanosine 5'-triphosphate-cyclohydrolase I (GTPCH I) (Katusic et al., 2009). Under basal conditions in blood vessels, nitric oxide (NO) is continuously produced by endothelial NOS (eNOS). In addition to eNOS, neural NOS (nNOS) serves as a transmitter in the brain and in different nerves of the peripheral nervous system. Optimal availability of BH_4 is therefore a critical determinant of NO production by all NOS isoforms (Tzeng et al., 1995; Raman et al., 1998; Zhang et al., 2001). Furthermore, we and others have shown that the endothelium is a major source of BH_4 in the arterial wall (Tsutsui et al., 1996; Landmesser et al., 2003; d'Uscio and Katusic, 2006) while vascular smooth muscle cells do not contain detectable quantities of BH_4 (Gross and Levi, 1992).

In endothelial cells enzymatic activity of GTPCH I is regulated by immunostimulants, hormones, protein kinase C, hydrogen peroxide, fluid shear stress, and protein-protein interaction (Abou-Donia et al., 1983; Milstien et al., 1996; Katusic et al., 1998; Lapize et al., 1998; Kalivendi et al., 2005; Lam et al., 2006). While the physiological role of GTPCH I has been extensively characterized in the peripheral vasculature (Alp et al., 2003; Lam et al., 2006; Peterson et al., 2009), the role of GTPCH I in the regulation of cerebral vascular function has received much less attention. In recent studies, we reported that deficiency of GTPCH I results in reduced BH_4 bioavailability leading to eNOS uncoupling, inhibition of NO signaling and augmented oxidative stress in the cerebral microvasculature (Santhanam et al., 2012a; Santhanam et al., 2012b; Santhanam et al., 2014). Moreover, inhibition of GTPCH I with 2,4-diamino-6-hydroxypyrimidine, results in depletion of intracellular BH_4 and abolishes formation of NO in response to activation of eNOS in basilar arteries (Kinoshita et al., 1997) revealing the importance of GTPCH I activity in the endothelium of cerebral circulation. To further understand the physiological role of GTPCH I in cerebral microvasculature, the present study was designed to determine the effects of endothelium-targeted overexpression of GTPCH I (eGCH-Tg) in mice.

2. Results

2.1. Mice characteristics

Blood cell count indicated that red blood cells as well as hemoglobin and hematocrit tended to be increased but did not significantly differ between eGCH-Tg mice and their wild-type littermates (Table 1). Furthermore, because BH_4 is an essential cofactor for tyrosine hydroxylase we determined levels of catecholamine. Circulating levels of catecholamine were unaltered in eGCH-Tg mice (Table 2).

2.2. Biopterin levels

Western blot analysis confirmed that GTPCH I protein expression was significantly increased in eGCH-Tg mice cerebral microvessels ($P<0.05$; Figure 1A). In addition, BH_4 levels were increased five-fold in cerebral microvessels ($P<0.05$; Figure 1B). Levels of 7,8-BH_2, the oxidative products of BH_4, were also significantly increased eGCH-Tg mice but to

a lesser extent as compared to BH_4 ($P<0.05$; Figure 1C). The resulted BH_4 to 7,8-BH_2 ratio was significantly enhanced ($P<0.05$; Figure 1D).

2.3. Protein expression of NOS isoforms

Protein expressions of constitutive NOS such as eNOS and nNOS were unaltered in the cerebral microvessels of eGCH-Tg mice ($P>0.05$ as compared to wild-type littermates; Figure 2). Furthermore, protein expression of the inducible NOS (iNOS) enzyme was low in cerebral microvessels and did not differ between wild-type littermates and eGCH-Tg mice ($P>0.05$; Figure 2).

2.4. Production of NO

Endogenous NO production as determined by quantification of total nitrite/nitrate levels was not different between wild-type littermates and eGCH-Tg mice cerebral microvessels ($P>0.05$; Figure 3A). The production of the second messenger of NO, cyclic 3′,5′-guanosine monophosphate (cGMP), was also unchanged in the cerebral microvessels of eGCH-Tg mice ($P>0.05$; Figure 3B).

2.5. Superoxide anion and expression of antioxidant proteins

Overexpression of GTPCH I protein in cerebral microvessels did not change either production of superoxide anion nor protein expression of antioxidant enzymes including copper- and zinc-superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD), and catalase (P=n.s.; Figure 4).

2.6. Vascular function in isolated basilar arteries

Resting lumen diameter and wall thickness was not different between wild-type littermates and eGCH-Tg mice (203±2μm versus 189±7μm and 13.8±0.3μm versus 14.0±0.9μm, respectively). Endothelium-dependent relaxations to acetylcholine were significantly impaired in isolated basilar arteries of eGCH-Tg mice ($P<0.05$ as compared to wild-type littermates; Figure 5A). Incubation basilar arteries with SOD mimetic Mn(III) tetra(4-benzoic acid) porphyrin chloride (MnTBAP) significantly improved relaxations to acetylcholine in eGCH-Tg mice and abolished the difference between eGCH and wild-type littermates (Figure 5B). Importantly, relaxations to acetylcholine were studied during submaximal contractions to prostaglandin H_2/thromboxane A_2 analog 9,11-dideoxy-9α, 11α-methanoepoxy-prostaglandin $F_{2\alpha}$ (U46619; 10^{-8} – 3×10^{-8} mol/L). Contractions to U46619 were not affected by MnTBAP in littermates and eGCH-Tg mice (24±3% versus 24±1% and 25±2% versus 26±2% with and without MnTBAP, respectively).

Endothelium-independent relaxations to the NO donor diethylammonium (Z)-1-(N,N-diethylamino) diazen-1-ium-1,2-diolate (DEA-NONOate) were not different between wild-type littermates and eGCH-Tg mice basilar arteries ($P>0.05$; Figure 6A). Contractions to U46619 were not affected by high concentration of BH_4 in basilar arteries of eGCH-Tg ($P>0.05$; Figure 6B).

3. Discussion

There are several novel findings in the present study. First, endothelium-specific overexpression of GTPCH I significantly increased BH_4 levels and BH_4 to 7,8-BH_2 ratio in cerebral microvessels of transgenic mice. Second, increased local concentrations of BH_4 did not affect circulating levels of catecholamine nor local NO production in cerebral microvessels. Third, production of superoxide anion and expression of antioxidants remained unchanged in cerebral microvessels derived from eGCH-Tg mice. Our results demonstrate that endothelium-targeted overexpression of GTPCH I significantly increased BH_4 bioavailability in the cerebral microvasculature.

In the absence of GTPCH I or during conditions associated with suboptimal availability of BH_4, NOS can produce superoxide anion rather than NO in cerebral microvessels of BH_4-deficient mice (Katusic et al., 2009; Santhanam et al., 2012b). On the other hand, in the present study we showed that chronically increased levels of BH_4 did not alter production of superoxide anion or NO in the cerebral microcirculation. This is of importance because local supplementation of BH_4 in endothelial cells is relatively safe with regard to oxidative stress. Indeed, several recent studies demonstrated that supplementation of BH_4 reduces superoxide anion production and preserves NO release during oxidative stress suggesting that endothelial dysfunction can be restored by increasing local concentration of BH_4 via GTPCH I (Alp et al., 2003; Du et al., 2008). We also have shown that short-term in-vivo treatment with BH_4, which increased BH_4 levels to those levels comparable in wild-type mice, was able to prevent eNOS uncoupling and to normalize NO production in cerebral microvessels of amyloid precursor protein transgenic Tg2576 mice an accepted model of Alzheimer's disease (Santhanam et al., 2015). In contrast, overexpression of eNOS causes eNOS uncoupling and superoxide anion generation in mice because of relative BH_4 deficiency (Bendall et al., 2005). Moreover, in cell-free system superoxide anion can be generated by eNOS in the absence of BH_4 and this phenomenon could be inhibited dose-dependently by BH_4 (Wever et al., 1997). However, under conditions of high iNOS expression the presence of increased BH_4 levels can worsen conditions for cerebral ischemia due to the increased formation of peroxynitrite (Cho et al., 1999; Kidd et al., 2005). In our study, expression of iNOS was very low and we failed to observe the effects of BH_4 on iNOS again suggesting that under physiological conditions increased BH_4 levels did not affect NO production in cerebral microvessels of eGCH-Tg mice.

Norepinephrine and dopamine are primary neurotransmitters in the sympathetic nervous system while norepinephrine and epinephrine are the major peripheral catecholamines synthetized in adrenal glands. Moreover, endogenous production of catecholamines has also been detected in the blood vessel wall (Sorriento et al., 2012). GTPCH I is also abundant in catecholaminergic neurons of the brain and adrenal glands. Importantly, BH_4 is an essential cofactor for tyrosine hydroxylase, a rate-limiting enzyme in the biosynthesis of catecholamines (Nagatsu et al., 1995; Anastasiadis et al., 1996). Our study revealed that circulating levels of catecholamines were unchanged in eGCH-Tg mice indicating that selective increased levels of BH_4 in the endothelium did not affect catecholamine biosynthesis under basal conditions. This is further supported by the previously reported study showing that systolic and mean blood pressures were unchanged in mice with

endothelium-specific overexpression of GTPCH I (Du et al., 2008). These findings have important therapeutical implications. Indeed, it appears that supplementation of BH_4 designed to improve endothelial function might not exert simultaneously adverse effect on biosynthesis of catecholamines.

BH_4 has been considered to play a role as a growth or proliferation factor for various mammalian cells, including hematopoietic and endothelial cells (Ziegler et al., 1982; Marinos et al., 2001; He et al., 2011). Moreover, BH_4 has been shown to increase proliferation of erythroid cells (Tanaka et al., 1989). Regarding outcomes of blood cell count studies we did not observe a statistically significant effect of GTPCH I overexpression on erythropoiesis in the hemopoietic tissues, ultimately producing of red blood cells in mice.

To gain additional insight into the functional consequences of overexpressed GTPCH I in cerebrovascular endothelium we examined vasomotor function of basilar arteries derived from wild type mice and eGCH-Tg mice. Consistent with biochemical observations in cerebral microvessels, high concentration of BH_4 in endothelium did not potentiate endothelium-dependent relaxations to acetylcholine in basilar artery. In fact, we detected paradoxical impairment of vasodilator effect of acetylcholine. This observation is identical with our previous findings demonstrating that high concentration of endogenous BH_4 in large cerebral arteries is associated with reduced reactivity to acetylcholine (Tsutsui et al., 1996). This *in-vitro* phenomenon is caused by autoxidation of BH_4 in oxygenated Krebs solution leading to increased formation of superoxide anion and subsequent chemical inactivation of nitric oxide (Tsutsui et al., 1996). In the present study, relaxations to acetylcholine were normalized in the presence of superoxide dismutase mimetic, MnTBAP, thereby confirming that high concentration of endothelial superoxide anion is responsible for impairment of endothelium-dependent relaxations to acetylcholine. Indeed, BH_4 is one of the most potent naturally occurring reducing molecules with high capacity to reduce oxygen (Tsutsui et al., 1996). It is also important to note that superoxide anion concentration was not elevated under *in-vivo* conditions as demonstrated by our findings in cerebral microvessels which were not exposed to environment with high partial pressure of oxygen (Tsutsui et al., 1996). Moreover, vasoconstrictor effects of prostaglandin H_2/thromboxane A_2 analog, U46619, and endothelium-independent relaxations to NO donor, DEA-NONOate, were not affected by high concentration of BH_4 in endothelial cells. Thus, in agreement with the results obtained in cerebral microvessels, vasomotor function of smooth muscle cells is not affected by overexpression of GTPCH I in endothelium.

We conclude that under physiological conditions BH_4 levels are optimal for activation of eNOS. Selective supplementation of BH_4 in endothelial cells in healthy mice does not affect NO/cGMP signaling in cerebral microvessels. Moreover, overexpression of GTPCH I in endothelium does not stimulate biosynthesis of catecholamines.

4. Methods

4.1. Experimental animals

eGCH-Tg mice on C57BL/6 background were kindly provided by Dr. Keith Channon (University of Oxford, United Kingdom (Alp et al., 2003)) and were bred in our laboratory.

Mice were maintained on standard chow with free access to drinking water. Housing facilities and all experimental protocols were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic and complied with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Twelve to sixteen weeks old male wild-type littermates and eGCH-Tg mice were used for the present study. Mice were euthanized by intraperitoneal injection of Pentobarbital (250 mg/kg BW). Blood cell count was performed in whole blood by VetScan® HM5 Analyzer (Abaxis).

4.2. Catecholamine Profile

Blood was immediately transferred to a tube containing EGTA and reduced glutathione and centrifuged at 4°C for 10 minutes. Plasma samples were stored at −80°C until assayed. Samples were absorbed onto activated alumina at pH 8.6, washed and eluted with diluted acetic acid as described (Chritton et al., 1991). Norepinephrine, epinephrine and dopamine were determined from elutes by HPLC and electrochemical detection. 3,4-dihydroxybenzylamine was used as internal standard.

4.3. Isolation of cerebral microvessels

Following euthanasia, brains were removed and placed in cold (4° C) modified Krebs-Ringer solution (composition in mmol/l: NaCl 118.6; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25.1; glucose 10.1; EDTA 0.026) and large cerebral arteries were discarded. Cerebral microvessels were subsequently isolated from the brains by dextran (15% w/v) centrifugation method and retained on a 40 μm nylon filter as described (Santhanam et al., 2012b).

4.4. Measurement of biopterin levels

Cerebral microvessels were homogenized in extraction buffer as described (d'Uscio and Katusic, 2006). Biopterin levels were determined after acid oxidation (which converts both BH_4 and 7,8-BH_2 to biopterin) and base oxidation (which converts only 7,8-BH_2 to biopterin) by RP-HPLC (Beckman System Gold). BH_4 levels were calculated from the difference after acid and base oxidations.

4.5. Detection of intracellular superoxide anions

Intracellular superoxide anion levels in cerebral microvessels were quantified using a HPLC-based fluorescence detection of the oxidative product dihydroethidium (Santhanam et al., 2012a).

4.6. Western blot analysis

Cerebral microvessels were homogenized in lysis buffer containing [50 mmol/L NaCl, 50 mmol/L NaF, 50 mmol/L sodium pyrophosphate, 5 mmol/L EDTA, 5 mmol/L EGTA, 0.1 mmol/L Na_3VO_4, 1% Triton X-100, 10 mmol/L HEPES, pH 7.4, and a protease inhibitor cocktail (Sigma Chemicals, St. Louis, MO)]. Protein expressions were studied by Western blotting in lysates supernatants, and the bands were quantified by enhanced chemiluminescence (Super Signal West Pico; Thermo Scientific, Waltham, MA) as described previously (Santhanam et al., 2012a).

4.7. Determination of NO production

NO production in the cerebral microvessels were determined as total nitrite and nitrate levels using a commercially available fluorometric nitrite/nitrate assay kit (Cayman Chemical Co. Ann Arbor, MI) (Santhanam et al., 2012a).

4.8. Determination of cGMP levels

Basal levels of cGMP were determined in cerebral microvascular lysates by enzyme immunoassay according to manufacturer's instructions (Cell Bio Labs, Inc., San Diego, CA) (Santhanam et al., 2012b).

4.9. Vascular reactivity studies in isolated cerebral arteries

Basilar arteries were isolated and dissected free from connective tissues in 4°C cold Krebs solution. Then they were transferred to single vessel chamber system (Living Systems Instrumentation, Burlington, VT) filled with Krebs-Ringer solution. Proximal and distal ends of the basilar arteries were mounted and sutured on two small glass micro-cannula positioned in the vessel chamber. The axial length of the vessel was carefully adjusted longitudinally under a microscope by positioning the afferent cannula. The solutions circulating from a 250 mL reservoir at a flow rate of 50 mL/min were gassed continuously with 94% O_2 and 6% CO_2 and kept at 37°C and pH 7.4. The amplified image was transmitted to a monitor and a video dimension analyzer (Living Systems Instrumentation), allowing for recording of lumen diameter and blood vessel wall thickness (d'Uscio et al., 1997). Intravascular pressure was continuously monitored by pressure servo controller with peristaltic pump. Basilar arteries were equilibrated for 45 minutes. In order to determine the optimal transmural pressure for studies of vasomotor function in isolated basilar arteries of wild-type C57BL/6 mice, the pressure was stepwise increased by 20 mmHg from 10 to 90 mmHg and equilibrated for 30 minutes. Contractions to single concentration of stable prostaglandin H_2/thromboxane A_2 analog, 9,11-dideoxy-9α, 11αmethanoepoxy-prostaglandin $F_{2\alpha}$ (U46619; 10^{-7} mol/L; Cayman Chemical, Ann Arbor, MI), were recorded and then washed out at each pressure step (n=3-4). A transmural pressure of 30 mmHg was found to be optimal for contractions to U46619 and it was set at this level for vascular reactivity studies.

Between each protocol, the system was washed out with Krebs solution and then equilibrated for 30 minutes. Concentration-dependent contractions to U46619 (10^{-9} - 10^{-6} mol/L) were obtained in basilar arteries from wild-type littermates and eGCH-Tg mice. Endothelium-dependent relaxations to acetylcholine (10^{-9} - 10^{-5} mol/L; Sigma) were obtained during contractions to U46619 (10^{-8} – 3×10^{-8} mol/L) in the presence or absence of cell permeable superoxide dismutase (SOD) mimetic MnTBAP (10^{-5} mol/L; Enzo Life Science, Farmingdale, NY; incubation time = 30 minutes prior to contraction to U46619). We also determined endothelium-independent relaxations to NO donor DEA-NONOate (10^{-9} - 10^{-5} mol/L; Cayman Chemical).

4.10. Statistical analysis

All data are expressed as mean ± SEM, and 'n' represents the number of mice used in each group. Un-paired student *t*-test was used to determine statistical difference between two groups. The concentration-response curves of the different groups were compared by ANOVA for repeated measurements followed by Bonferroni's correction. A value of $P<0.05$ was considered statistically significant.

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Highlights

- Tetrahydrobiopterin (BH_4) levels are increased in cerebral microvessels of transgenic mice with endothelium-specific overexpression of GTP-cyclohydrolase I.
- High BH_4 levels in endothelium do not affect nitric oxide signaling in cerebral microvessels.
- High BH_4 levels in endothelium do not stimulate biosynthesis of catecholamines.
- Under physiological condition BH_4 is optimal for activation of nitric oxide synthase.
- Therapeutic increase of BH_4 is unlikely to exert adverse effects in cardiovascular system.

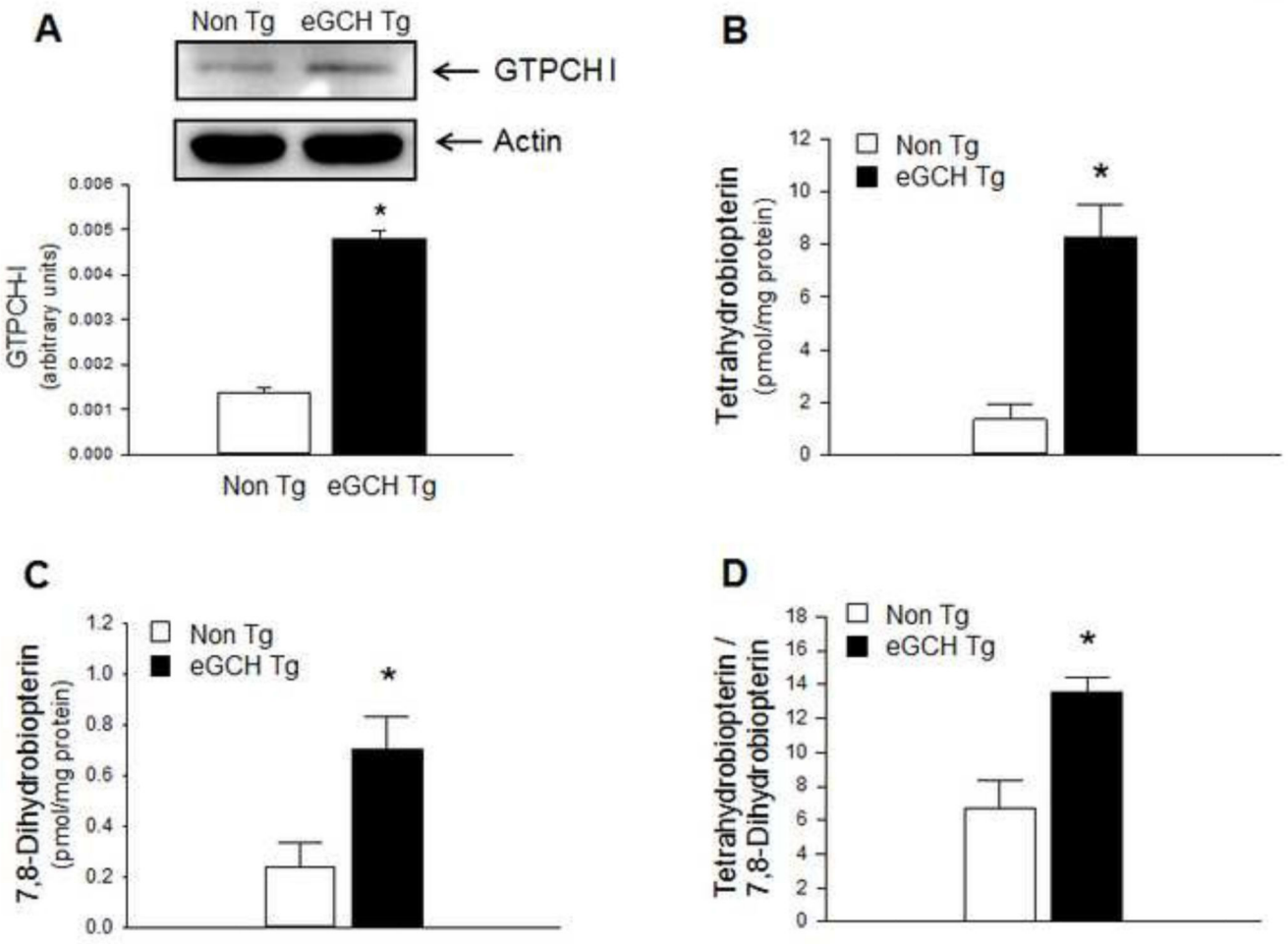

Figure 1.

(A) Protein expression studies confirmed that GTPCH I was increased in cerebral microvessels of eGCH-Tg mice (*P<0.05; n=4 independent experiments). Endothelium-targeted over-expression of GTPCH I resulted in increased levels of tetrahydrobiopterin (BH_4) (B) and 7,8-dihydrobiopterin (7,8-BH_2) (C). The ratio of BH_4 to 7,8-BH_2 (D), indicative of net BH_4 bioavailable for eNOS activation, was also significantly increased in cerebral microvessels of eGCH-Tg mice (*P<0.05 vs. non-transgenic wild-type littermates (Non-Tg); n=5-7).

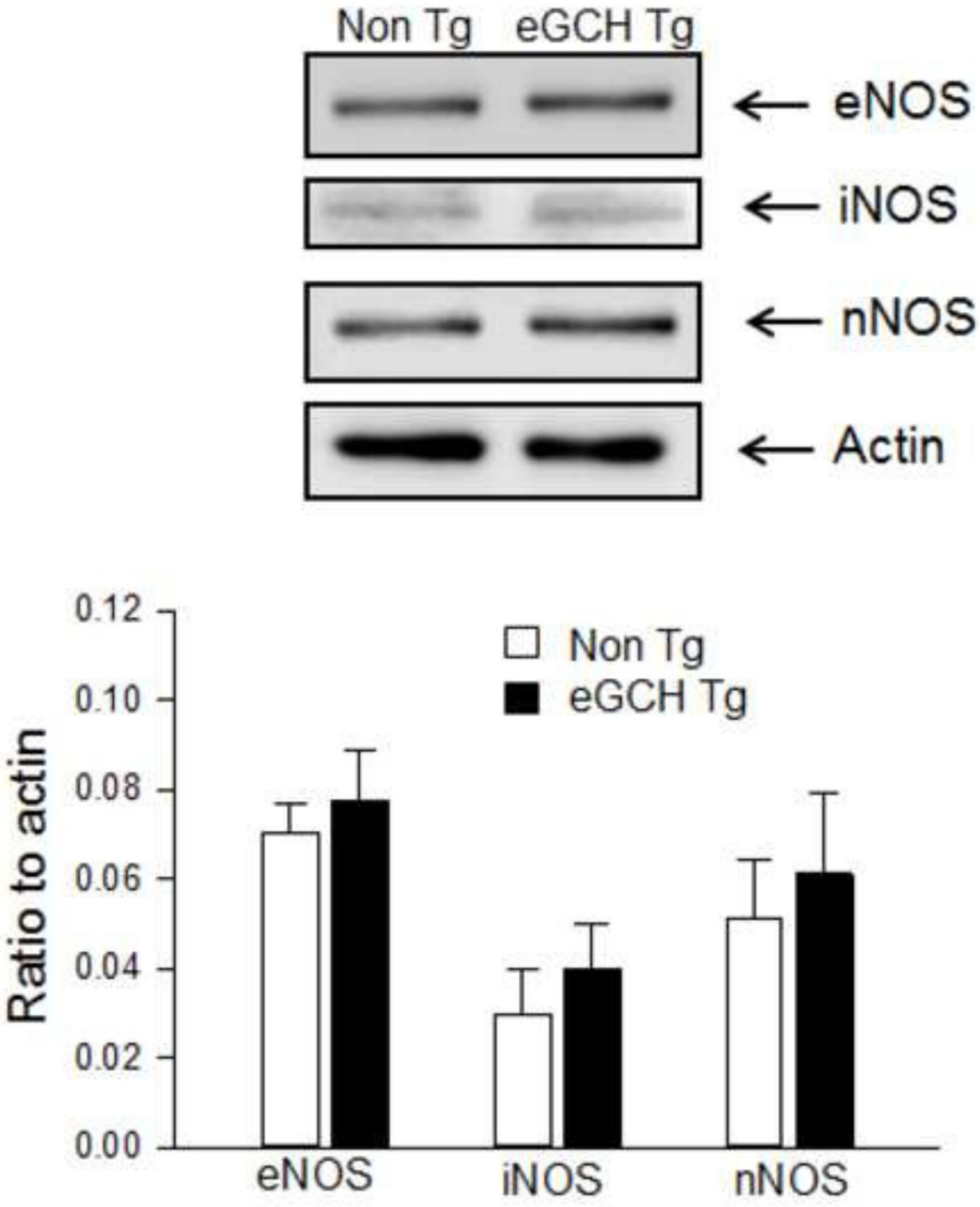

Figure 2.

Representative Western blots and densitometric analysis demonstrating no difference in expression of NOS isoforms in cerebral microvessels of non-transgenic wild-type mice (Non-Tg) and transgenic mice with endothelium-targeted over-expression of GTPCH I (eGCH-Tg) (n=4 independent experiments, n.s.).

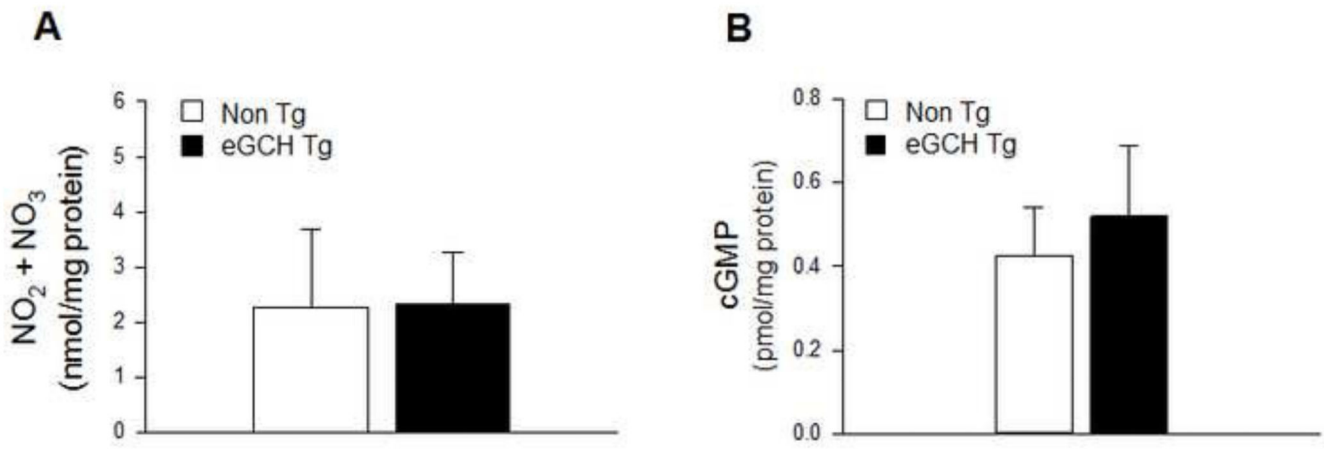

Figure 3.

(A) Levels of total NOx (NO_2 + NO_3) and (B) cGMP, indicators of NO production, were not significantly different between cerebral microvessels of wild-type mice (Non-Tg) and transgenic mice with endothelium-targeted over-expression of GTPCH I (eGCH-Tg) (n=4-7, P>0.05).

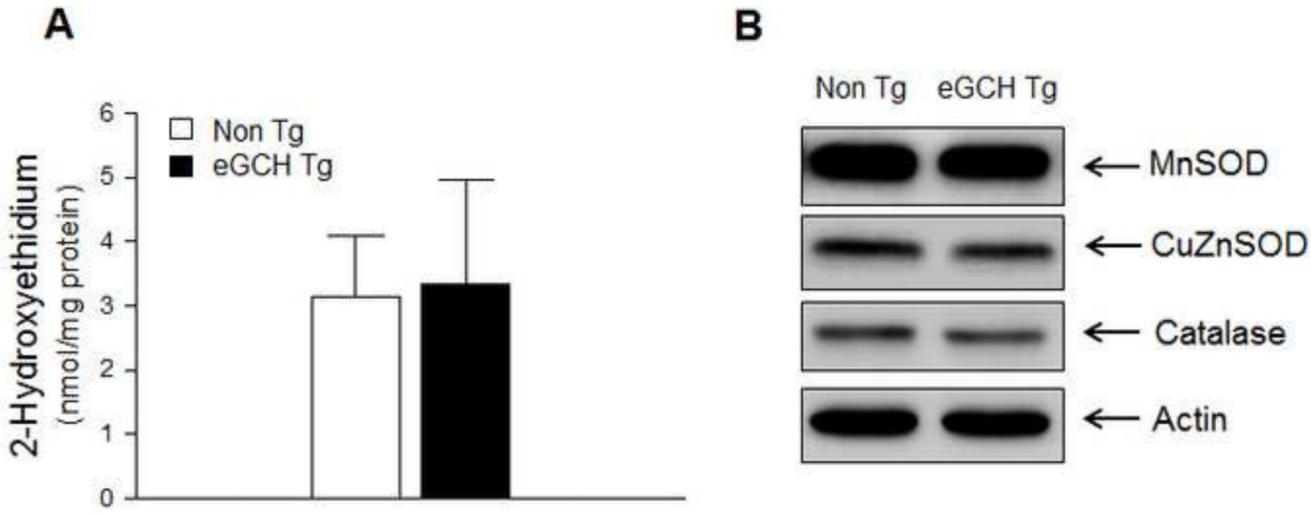

Figure 4.

Endothelium-targeted over-expression of GTPCH I (eGCH-Tg) did not affect either production of superoxide anions (A; n=7) or expression of antioxidant proteins (B; n=4 independent experiments) in mice cerebral microvessels (P>0.05).

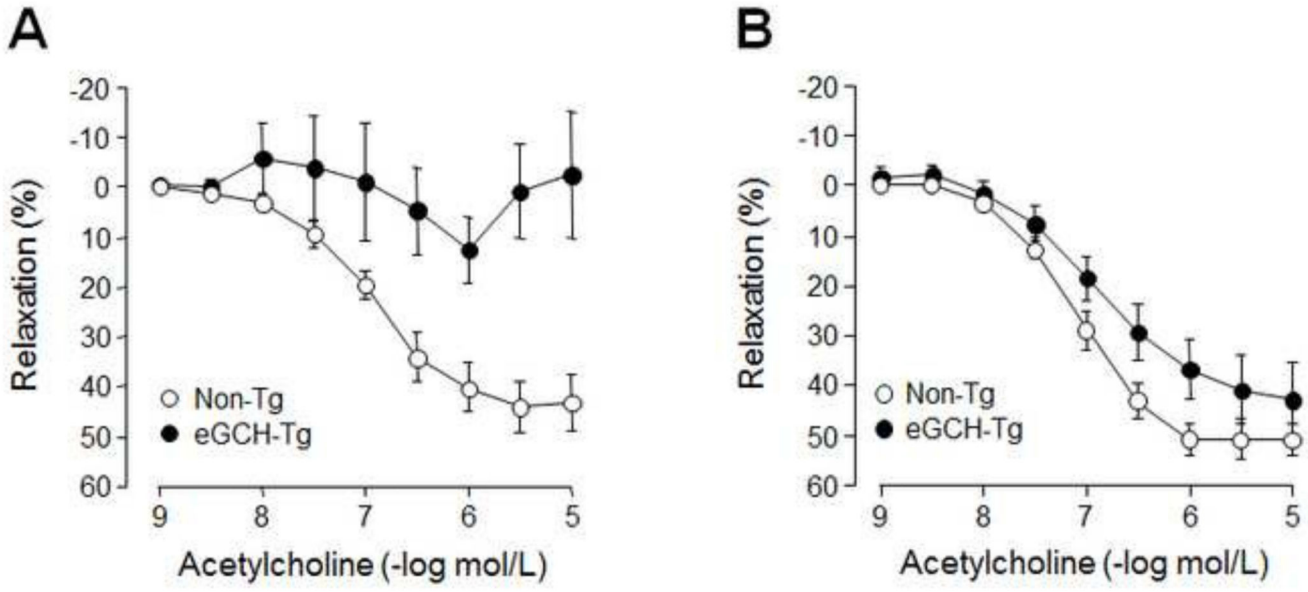

Figure 5.

Endothelium-dependent relaxations to acetylcholine in isolated basilar arteries of wild-type littermates (Non-Tg) and transgenic mice with endothelium-targeted over-expression of GTPCH I (eGCH-Tg) in the absence (A) or in the presence (B) of cell permeable superoxide dismutase mimetic Mn(III) tetra(4-benzoic acid) porphyrin chloride (MnTBAP; 10^{-5} mol/L). Please note that MnTBAP significantly improved impaired relaxations to acetylcholine in eGCH-Tg mice basilar arteries ($P<0.05$; ANOVA plus Bonferroni's; n=4-5). The relaxations were expressed as percentage of the increase in intraluminal diameter from the diameter obtained after submaximal contraction to U46619.

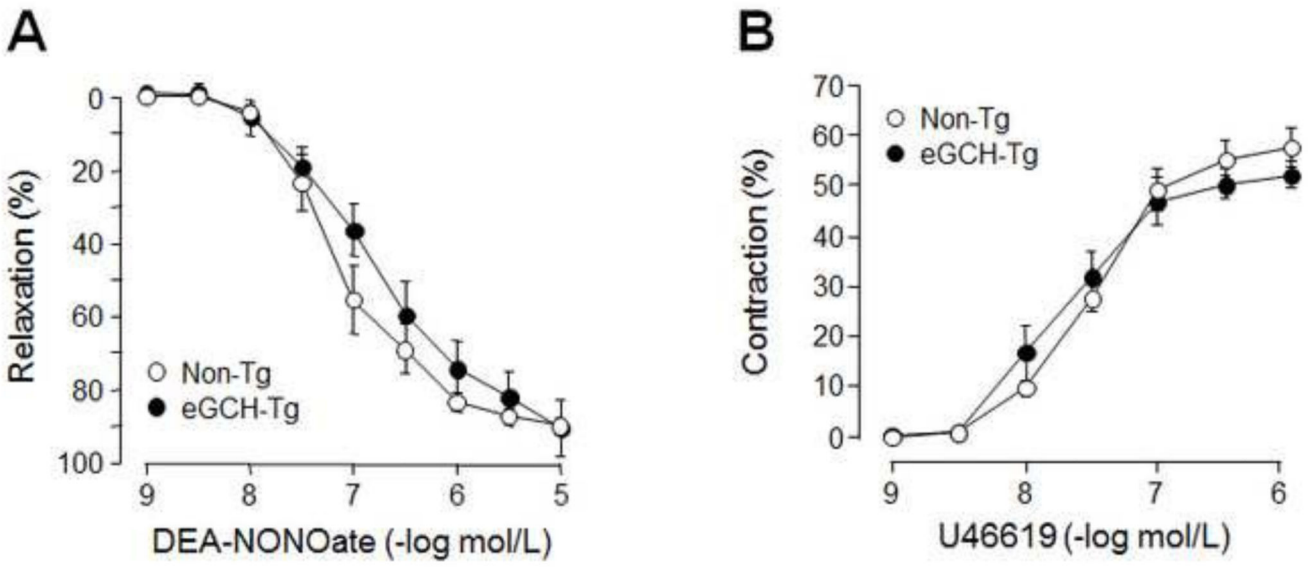

Figure 6.

(A) Endothelium-independent relaxations to NO donor DEA-NONOate and (B) concentration-dependent contractions to prostaglandin H_2/thromboxane A_2 analog U46619 in isolated basilar arteries of wild-type littermates (Non-Tg) and transgenic mice with endothelium-targeted over-expression of GTPCH I (eGCH-Tg). Please note that both of the vascular responses were not significantly different between both groups of mice (n=4-5, P>0.05; ANOVA plus Bonferroni's). The contractions to U46619 or relaxations to DEA-NONOate were expressed as percentage of the decrease in the basal intraluminal diameter or of the increase in intraluminal diameter from the diameter obtained after submaximal contraction to U46619, respectively.

Table 1

Blood cell counts in wild-type and endothelium-specific GTPCH I transgenic mice.

Parameters	Non Tg	eGCH Tg
White blood cells ($\times 10^9$/L)	11.0 ± 1.2	11.3 ± 1.4
Lymphocytes ($\times 10^9$/L)	10.0 ± 1.1	10.1 ± 1.5
Monocytes ($\times 10^9$/L)	0.2 ± 0.1	0.2 ± 0.1
Granulocytes ($\times 10^9$/L)	0.8 ± 0.3	1.0 ± 0.4
Red blood cells ($\times 10^{12}$/L)	8.9 ± 0.5	10.1 ± 0.4
Hemoglobin (g/dL)	12.7 ± 0.9	13.9 ± 0.3
Hematocrit (%)	39.5 ± 2.1	42.7 ± 1.1
Platelets ($\times 10^9$/L)	354 ± 93	535 ± 72

eGCH indicates endothelium-specific GTP-cyclohydrolase I; Tg indicates transgenic. Data are means ± SEM (n=7). P>0.05 (unpaired t-test).

Table 2

Circulating levels of catecholamine in wild-type and endothelium-specific GTPCH I transgenic mice.

Parameters	Non Tg	eGCH Tg
Dopamine (pg/mL)	214 ± 26	281 ± 80
Norepinephrine (pg/mL)	3545 ± 729	3397 ± 438
Epinephrine (pg/mL)	1517 ± 338	2017 ± 412

eGCH indicates endothelium-specific GTP-cyclohydrolase I; Tg indicates transgenic. Data are means ± SEM (n=10). P>0.05 (unpaired t-test).