Published in final edited form as:
J Neurochem. 2012 September ; 122(6): 1211–1218. doi:10.1111/j.1471-4159.2012.07872.x.

Uncoupling of eNOS causes superoxide anion production and impairs NO signaling in the cerebral microvessels of hph-1 mice

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Abstract

In the present study, we used the GTP cyclohydrolase I-deficient mice, ie., hph-1 mice, to test the hypothesis that the loss of tetrahydrobiopterin (BH_4) in cerebral microvessels causes endothelial nitric oxide synthase (eNOS) uncoupling resulting in increased superoxide anion production and inhibition of endothelial nitric oxide (NO) signaling. Both homozygous mutant (hph-$1^{-/-}$) and heterozygous mutant (hph-$1^{+/-}$ mice) demonstrated reduction in GTP cyclohydrolase I activity and reduced bioavailability of BH_4. In the cerebral microvessels of hph-$1^{+/-}$ and hph-$1^{-/-}$ mice, increased superoxide anion production was inhibited by supplementation of BH_4 or NOS inhibitor - L-NAME, indicative of eNOS uncoupling. Expression of 3-nitrotyrosine was significantly increased, while NO production and cGMP levels were significantly reduced. Expressions of antioxidant enzymes namely CuZnSOD, MnSOD and catalase were not affected by uncoupling of eNOS. Reduced levels of BH_4, increased superoxide anion production as well as inhibition of NO signaling were not different between the microvessels of male and female mice. The results of our study are the first to demonstrate that, regardless of gender, reduced BH_4 bioavailability causes eNOS uncoupling, increases superoxide anion production, inhibits eNOS/cGMP signaling, and imposes significant oxidative stress in the cerebral microvasculature.

Keywords

oxidative stress; endothelial dysfunction; cerebral microvasculature; GTP cyclohydrolase I

Introduction

Under physiological conditions, reduction of molecular oxygen results in electron transfer required for oxidation of L-arginine at the reductase domain of endothelial nitric oxide synthase (eNOS). This electron transfer (from NADPH) is facilitated by essential co-factor of eNOS, tetrahydrobiopterin (BH_4). When concentration of BH_4 becomes suboptimal, electron transfer does not couple with production of NO. Under these conditions, eNOS becomes uncoupled to generate superoxide rather than NO (Alp & Channon 2004, Katusic *et al.* 2009, Moens & Kass 2006, Vasquez-Vivar 2009). Oxidative stress, characterized by elevated production of superoxide anions and/or impaired activation of antioxidant enzymes, has emerged as a primary mediator of endothelial dysfunction observed in various cerebrovascular disorders (Chrissobolis & Faraci 2008). While numerous studies, including ours, have examined the role of eNOS uncoupling in oxidative stress in the peripheral vasculature (Forstermann & Li 2011, Landmesser *et al.* 2003, Meininger *et al.* 2000, Schulz

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et al. 2008, Stroes *et al.* 1997), effects of eNOS uncoupling on cerebral microvasculature has not been studied.

The hyperphenylalaninemic mutant, hph-1 mice, reported to be deficient in GTP cyclohydrolase I, the rate-limiting enzyme in BH_4 biosynthesis (Bode *et al.* 1988, Canevari *et al.* 1999, McDonald *et al.* 1988, Shimoji *et al.* 1999), has been widely used to study the effects of eNOS uncoupling on vascular function (Cosentino *et al.* 2001, d'Uscio *et al.* 2011, Khoo *et al.* 2005). In fact, Cosentino and colleagues reported that reactive oxygen species derived from uncoupling of eNOS mediate endothelium-dependent relaxations in aorta of hph-1 mice (Cosentino *et al.* 2001). However, in a recent study on hph-1 mice, we demonstrated that aorta and mesenteric arteries varied in response to oxidative stress caused by eNOS uncoupling (d'Uscio *et al.* 2011), suggestive of differential tolerance to oxidative stress induced by eNOS uncoupling between large conduit and small resistance arteries (d'Uscio *et al.* 2011).

In the present study, we aimed to determine the effects of GTP cyclohydrolase I deficiency on cerebral microvasculature. We hypothesized that, irrespective of gender, the cerebral microvasculature is highly sensitive to loss of BH_4, thereby exhibiting augmented superoxide anion production, loss of NO and oxidative stress.

Methods

Mice

Three months old male and female, homozygous mutant (hph-$1^{-/-}$) and heterozygous mutant (hph-$1^{+/-}$) mice and sex-matched wild-type (C57BL/6) littermates were maintained on standard chow with free access to drinking water. Male hph-$1^{-/-}$ mice (on C57BL/6 background) provided to us by Dr. Keith M. Channon (University of Oxford, Oxford, UK) were bred with commercially available female C57BL/6 mice (Jackson Laboratories). Genotyping was performed as previously reported (Khoo *et al.* 2004). Housing facilities and all the experimental protocols were performed as per the guidelines laid by the Institutional Animal Care and Use Committee of the Mayo Clinic College of Medicine. Mice were killed by injecting an overdose of pentobarbital. In some experiments, female wild-type, hph-$1^{-/-}$ and hph-$1^{+/-}$ mice were injected subcutaneously with 100 μmol/kg (b.w.) of BH_4 [(6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride; Schircks Laboratories, Jona, Switzerland]. Three hours later, mice were killed and brain was isolated and studied.

Blood vessel isolation

Brain was removed and placed in cold (4° C) modified Krebs-Ringer bicarbonate solution (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). Under a surgical microscope, anterior and posterior cerebral, middle cerebral and basilar arteries were separated away from the brain. Cerebral microvessels were subsequently isolated from the brain by Dextran centrifugation, as described earlier (Austin *et al.* 2010). Briefly, brain was homogenized in ice cold PBS with Dounce homogenizer and centrifuged at 2000g at 4°C. The supernatant was discarded and the pellet was suspended and layered over 15% Dextran (in PBS) (Sigma, St. Louis, MO) and centrifuged at 4000g for 20 minutes at 4°C. The non-vascular fraction remained suspended on the top and was removed, while the pellet was re-suspended in 1% bovine serum albumin (BSA) and was then passed through a 40 μm nylon mesh (BD Falcon). Microvessels retained on the mesh were washed with BSA/PBS and collected by centrifugation at 1000g for 10 minutes at 4°C.

Measurement of biopterin levels and GTP cyclohydrolase I activity

Cerebral microvessels were homogenized in extraction buffer containing 50 mmol/l Tris (pH 7.4), 1 mmol/L dithiothreitol, and 1 mmol/L EDTA at 4°C and were centrifuged at 10,000g (8 min at 4°C). Biopterin levels were determined after differential oxidation in acid (which converts both BH_4 and its oxidized derivative 7,8-BH_2 to biopterin) and base (which converts only 7,8-BH_2 to biopterin) conditions by reversed-phase HPLC, as reported previously (d'Uscio *et al.* 2003, d'Uscio *et al.* 2011).

For determination of GTP cyclohydrolase I activity, cerebral microvessel homogenates were filtered using a Sephadex G25M column (GE Healthcare) to remove endogenous neopterin, BH_4 and phenylalanine. An incubation solution of 0.1 mmol/l Tris-HCl (pH 7.4), 100 μmol/l PMSF, 10 mg/ml BSA and 10 μl of GTP was added to 100 μl homogenates and incubated for 2 hours at 37°C. Reaction was terminated at the end of incubation by the addition of 1 mol/l HCl. An iodine solution (1% I_2/2%KI, 1:1 wt/vol) was added and the samples were incubated for 1 hour at room temperature in the dark. 10 μl of ascorbic acid (20%) was subsequently added followed by 1 mol/l NaOH. Neopterin triphosphate thus formed was dephosphorylated by incubation with alkaline phosphatase (250 mU/μl, Fisher) for one hour at 37°C. Samples were then centrifuged at 11,000 rpm for 10 minutes. Neopterin was then measured by reversed-phase HPLC with fluorescence detection, as reported earlier (d'Uscio *et al.* 2003, d'Uscio *et al.* 2011).

Detection of intracellular superoxide anion

Intracellular superoxide anion levels in cerebral microvessels were quantified using a HPLC-based fluorescence detection of the oxidation of dihydroethidium. Briefly, microvessels were incubated in Krebs-HEPES buffer containing 50 μmol/L of dihydroethidium (Molecular Probes) at 37°C for 15 minutes. In some experiments, blood vessels were either incubated with L-NAME (30 μmol/L) for 30 minutes prior to addition of dihydroethidium (d'Uscio *et al.* 2010, d'Uscio *et al.* 2011). The samples were washed to remove the free dihydroethidium and incubated in Krebs-HEPES buffer for one hour at 37°C. The blood vessels were homogenized in cold methanol and centrifuged at 9,000g. The supernatant was analyzed by fluorescence detection by HPLC (Beckman Coulter) in 37% acetonitrile in 0.1% trifluoroacetic acid aqueous solution. Data were quantified using 2-hydroxyethidium standard from the reaction between dihydroethidium and Fremy's salt and normalized against tissue protein levels (d'Uscio *et al.* 2010, d'Uscio *et al.* 2011).

In some experiments, female mice were injected subcutaneously with 100 μmol/kg BH_4. The dose and duration of BH_4 treatment has been previously demonstrated to elevate BH_4 levels as well as cGMP concentration in the brain (Canevari *et al.* 1999). Three hours later, brains were isolated and superoxide anion production were measured in the absence and presence of L-NAME (30 μmol/l).

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 protease inhibitor cocktail (Sigma)]. The experimental methods for protein expression studies are published elsewhere (Santhanam *et al.* 2007). Monoclonal antibodies against eNOS (1:500), nNOS (1:500), phospho S1177eNOS (1:500) [BD Transduction], catalase (1:1000), β-actin (1:5000) [Sigma], 3-nitrotyrosine [Stressgen] and polyclonal antibodies (1:1000 dilution) against CuZn superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD), extracellular superoxide dismutase (EC-SOD) [Stressgen], Neuron Specific

Nuclear Protein [NeuN, Millipore] were used and bands were visualized by enhanced chemiluminescence (Super Signal West Pico Chemiluminescence, Thermo Scientific, IL).

Measurement of nitrite/nitrate

Nitric oxide in the cerebral arteries and cerebral microvessels were measured as total nitrite/nitrate (NO_3 + NO_2) using a commercially available fluorometric nitrite/nitrate assay kit according to manufacturer's instructions (Cayman Chemical Co.) (Austin *et al.* 2010).

Measurement of cGMP levels

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 protease inhibitor cocktail (Sigma)]. Basal cGMP content in the lysates were determined by enzyme immunoassay according to manufacturer's instructions (Cell Bio Labs, Inc., San Diego, CA).

Statistical analysis

Data are represented as mean ± SEM, ‘n’ represents the number of mice used in each group. Un-paired students ‘t’ test was used to determine statistical difference between two groups, and multiple comparisons were performed by one-way ANOVA followed by Bonferroni's post-hoc test. A value of $P<0.05$ was considered statistically significant.

Results

Characterization of cerebral microvessels

Expression of vascular and neuronal markers was determined in cerebral microvessels. Expressions of neuronal NOS and NeuN could be detected only in the brain, whereas microvessels did not express these neuronal markers (Figure 1). Expression of eNOS could be detected in the microvessels as well as in brain, while inducible NOS was predominantly expressed in brain (Figure 1).

Bioavailability of BH_4 in GTP cyclohydrolase I-deficient cerebral microvessels

Enzymatic activity of GTP cyclohydrolase I, the rate-limiting enzyme in BH_4 biosynthesis, was significantly reduced in cerebral microvessels of hph-$1^{-/-}$ mice. Interestingly, GTP cyclohydrolase I activity was also significantly reduced in cerebral microvessels of hph-$1^{+/-}$ mice as compared to wild-type controls (Figure 2A). There were no differences in GTP cyclohydrolase I activity between male and female mice (Figure 2A).

In wild-type mice, levels of BH_4 in the cerebral microvessels were not significantly different between male and female animals (Figure 2B). We observed significant attenuation of BH_4 in the hph$1^{-/-}$ mice of both gender (Figure 2B). Consistent with reduced GTP cyclohydrolase I activity in hph-$1^{+/-}$ mice irrespective of gender, BH_4 levels were also significantly reduced, and the attenuated values were comparable to those from hph-$1^{-/-}$ mice (Figure 2B). The levels of 7,8- BH_2 in the cerebral microvasculature of hph-$1^{-/-}$ and hph-$1^{+/-}$ mice were similar to 7,8-BH_2 levels in wild-type mice (Figure 2C). However, the ratio of BH_4 to 7,8-BH_2 were significantly decreased in cerebral microvessels of hph-$1^{-/-}$ and hph-$1^{+/-}$ mice, and this decrease in ratio of BH_4 to 7,8-BH_2 ratio was independent of gender (Figure 2D).

Protein expression studies on the cerebral microvasculature of hph-$1^{-/-}$ and hph-$1^{+/-}$ mice demonstrated that expressions of eNOS remained unchanged (Figure 3).

Endothelial NOS uncoupling and superoxide anion production

In the cerebral microvasculature of hph-$1^{-/-}$ mice, levels of superoxide anion were significantly increased in male as well as female mice. In addition, we observed a similar increase in superoxide anion production in the cerebral microvessels of hph-$1^{+/-}$ mice (Figure 4A).

The increased production of superoxide anions in hph-$1^{-/-}$ mice and hph-$1^{+/-}$ mice were abolished by NOS inhibitor L-NAME (30 μmol/l, Figure 4A), confirming the role of NOS in increased superoxide generation. Furthermore, superoxide anion production in wild-type mice was not affected by L-NAME, demonstrating selectivity of L-NAME in reversing superoxide generated by eNOS uncoupling. In addition, treatment with BH_4 abolished the increase in superoxide anion production observed in cerebral microvessels of both hph-$1^{-/-}$ and hph-$1^{+/-}$ mice (Figure 4B). Subsequent treatment with L-NAME did not have any further effect on the superoxide anion production (Figure 4B).

To further confirm the selective contribution of eNOS uncoupling toward increased superoxide anion production in the cerebral microvasculature of hph-$1^{-/-}$ and hph-$1^{+/-}$ mice, we studied the expression of antioxidants proteins. Genetic inactivation of GTP cyclohydrolase I did not affect protein expression of CuZnSOD, MnSOD, and catalase (Figure 5) in the cerebral microvasculature. Protein expressions of extracellular SOD (ECSOD) or glutathione peroxidase1 could not be detected in the cerebral microvessels (data not shown).

Effect of eNOS uncoupling on NO signaling

The levels of 3-nitrotyrosine, marker of peroxynitrite formation, was significantly increased in the cerebral microvessels of both hph-$1^{-/-}$ and hph-$1^{+/-}$ mice in comparison to their sex-matched wild-type control mice (Figure 6).

Levels of nitrite/nitrate, an indicator of NO production, tended to be higher in female mice as compared to male wild-type mice (Figure 7). In comparison to wild-type controls, levels of nitrite/nitrate were significantly reduced in cerebral microvessels of hph-$1^{-/-}$ mice. Similar to the reduction seen in hph-$1^{-/-}$ mice, levels of nitrite/nitrate were significantly reduced in cerebral microvessels of hph-$1^{+/-}$ mice (Figure 7).

Consistent with reduced NO production, basal levels of cGMP were significantly reduced in cerebral microvessels of male and female hph-$1^{-/-}$ mice (Figure 8). Similar reductions in basal levels of cGMP were also observed in hph-$1^{+/-}$ mice, irrespective of gender (Figure 8).

Discussion

We report several novel findings: a) significant reductions in BH_4 levels were detected in the cerebral microvessels of homozygous or heterozygous hph-1 mice, b) reduced bioavailability of BH_4 in cerebral microvessels of hph-1 mice resulted in increased production of superoxide anion, c) the increased superoxide anion production was selectively inhibited by either supplementation with BH_4 or treatment with NOS inhibitor - L-NAME, demonstrating that eNOS is the source of superoxide anion, d) uncoupling of eNOS did not alter expression of antioxidant proteins in the cerebral microvessels, e) 3-nitrotyrosine formation was significantly increased in the cerebral microvessels of hph-1 mice, and f) NO production and basal cGMP levels were significantly reduced, indicative of endothelial dysfunction in BH_4-deficient cerebral microvessels. Finally, no major difference was detected between the cerebral microvessels of male and female hph-1 mice. To the best of our knowledge, this is the first study to determine the effects of GTP cyclohydrolase I and BH_4 deficiency on cerebral microvasculature.

We observed similar reductions in GTP cyclohydrolase I activity between hph-$1^{-/-}$ and hph-$1^{+/-}$ mice. Consistent with reduced GTP cyclohydrolase I activity, we also detected similar reductions in levels of BH_4 between hph-$1^{-/-}$ and hph-$1^{+/-}$ mice. While we do not have an exact explanation for this observation, we also detected similar reductions in BH_4 levels in small mesenteric arteries from hph-$1^{-/-}$ and hph-$1^{+/-}$ mice (d'Uscio LV, Smith LA, Katusic ZS, unpublished observations). These results suggest that mutation in single gene copy is sufficient to significantly reduce levels of BH_4 in small resistant arteries in cerebral and peripheral circulation. Interestingly, and in contrast to BH_4, deficiency of GTP cyclohydrolase I activity did not reduce the levels of 7,8-BH_2 in the cerebral microvessels. This may be explained by potential oxidation of BH_4 to 7,8-BH_2 by peroxynitrite (Milstien & Katusic 1999), thereby increasing 7,8-BH_2 to levels comparable with 7,8-BH_2 levels detected in wild-type mice. As a result, ratio of BH_4 to 7,8-BH_2 was significantly reduced in vessels obtained from hph-1 mice.

We previously reported that eNOS is uncoupled in peripheral and conduit resistance arteries derived from hph-1 mice (d'Uscio *et al.* 2011). Conceptually, results of the present study regarding metabolism of BH_4 in cerebral microvessels are consistent with findings obtained on peripheral blood vessels. However, eNOS-derived production of superoxide anion appears to be significantly higher in cerebral microvessels as compared to previously detected superoxide anion formation in aorta and mesenteric arteries. The exact reasons for higher production of superoxide anion by uncoupled eNOS in cerebral circulation are unclear. Interestingly, despite relatively high levels of superoxide anion in cerebral microvessels, we did not detect any alteration in antioxidant capacity or expression of eNOS, thereby suggesting that in the cerebral microvessels, adaptive responses to oxidative stress are absent. We ruled out the adaptive up-regulation of superoxide dismutase(s) and catalase, the enzymes that were previously reported to be increased in peripheral resistant vessels of hph-1 mice (d'Uscio *et al.* 2011). However, our studies do not rule out involvement of other antioxidants, including glutathione, in eliciting adaptive responses to oxidative stress. Furthermore, our conclusion is in agreement with reported inability of cerebral arterioles (but not aorta or large cerebral arteries) to develop adaptive response to increased superoxide anion production caused by deficiency of MnSOD (Andresen *et al.* 2004, Faraci *et al.* 2006b). Similar to our findings, availability of NO in cerebral arterioles was reduced as a result of increased production of superoxide anion in MnSOD-deficient mice (Faraci *et al.* 2006b). In addition, peroxynitrite formation, demonstrated by increased 3-nitrotyrosine levels provide additional evidence supporting the concept that oxidative stress is present in BH_4-deficient cerebral microvasculature. In aggregate, our results suggest that eNOS uncoupling has more pronounced detrimental effect on availability of NO and formation of cyclic GMP in cerebral than in peripheral circulation (d'Uscio *et al.* 2011). Moreover, we provide evidence that uncoupling of eNOS causes oxidative stress in the cerebral microvessels.

Our findings may help to explain observations reported by number of previous studies demonstrating higher susceptibility of cerebral circulation to endothelial dysfunction under pathological conditions including hypertension (Didion *et al.* 2002, Didion *et al.* 2000, Faraci *et al.* 2006a), diabetes (Kitayama *et al.* 2006), hyperhomocysteinemia (Dayal *et al.* 2004), feeding with high fat diet (Beyer *et al.* 2008) as well as aging (Brown *et al.* 2007, Modrick *et al.* 2009). Of note, all of these conditions are associated with increased risk of stroke, dementia and Alzheimer's disease. Indeed, our previous study demonstrated that eNOS activity and production of NO in cerebral microvessels modulate expression and processing of amyloid precursor protein (Austin *et al.* 2010). In addition, our studies may also have important implications for understanding the pathogenesis of cerebral vasospasm. In fact, recent studies documented uncoupling of eNOS during development of cerebral vasospasm induced by subarachnoid hemorrhage (Sabri *et al.* 2011a), and demonstrated the

ability of simvastatin to re-couple eNOS and alleviate cerebral vasospasm and neuronal injury (Sabri *et al.* 2011b). Further studies are obviously required to establish exact contribution and relevance of eNOS uncoupling in pathogenesis of endothelial dysfunction in the cerebral circulation.

In summary, our findings support the concept that under conditions of GTP cyclohydrolase I deficiency in cerebral microvasculature, eNOS becomes uncoupled to generate superoxide anions thereby resulting in oxidative stress and impaired NO/cGMP signaling. We believe that the results of the present study regarding high production of eNOS-derived superoxide anion in cerebral microvessels of hph-1 mice may help to explain susceptibility of cerebral circulation to endothelial dysfunction.

Acknowledgments

No conflicts of interest, financial or otherwise, are declared by the author(s). This study was funded in part by American Heart Association Scientist Development Grant (08-35436N to AVS), National Institutes of Health Grants (HL 91867, HL111062 to ZSK), and the Mayo Foundation.

List of abbreviations used

7,8-BH_2	7,8-dihydrobiopterin
BH_4	tetrahydrobiopterin
cGMP	3,5-cyclic guanosine monophosphate
CuZnSOD	Copper and Zinc superoxide dismutase
ECSOD	extracellular superoxide dismutase
eNOS	endothelial nitric oxide synthase
hph-1	hyperphenylalaninemic
iNOS	inducible nitric oxide synthase
L-NAME	L- N^G-nitro arginine-methyl ester
MnSOD	Manganese superoxide dismutase
nNOS	neuronal nitric oxide synthase
NO	nitric oxide

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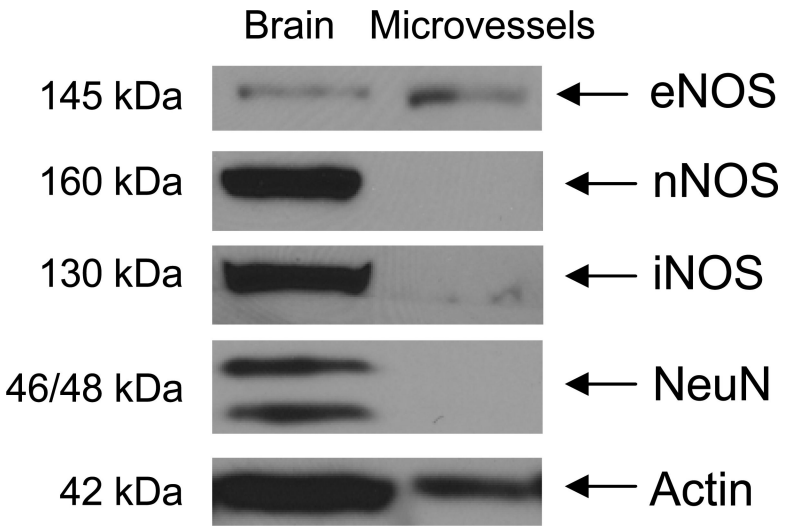


Figure 1.
Protein expression studies demonstrating expression of NO synthase in the microvessels and brain. Expression of neuronal markers, nNOS and NeuN were not detected in purified microvessels, while nNOS and NeuN could be detected in the brain. Protein expression of iNOS was significantly lower in purified microvessels as compared to brain. Protein expression studies were performed by SDS-PAGE on 25 μg total protein in brain and microvessels, and data are representative of expression from three different wild-type (C57BL/6) mice.

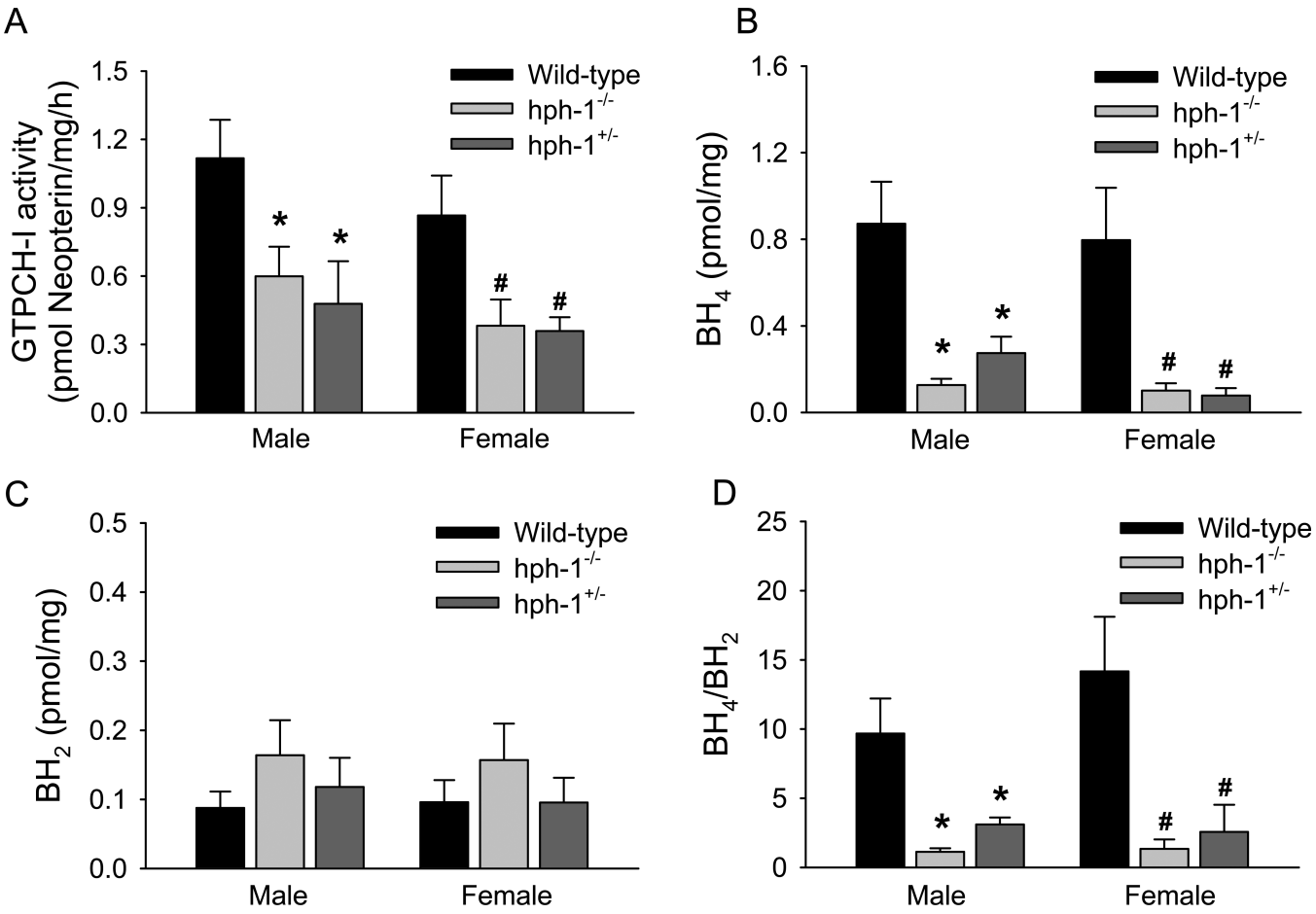


Figure 2.

Enzymatic activity of GTP cyclohydrolase I (A) and levels of tetrahydrobiopterin (BH_4, B) were significantly decreased in the cerebral microvessels of hph-$1^{-/-}$ and hph-$1^{+/-}$ mice as compared to their sex-matched controls (* $P<0.05$, n=6 in comparison to wild-type male mice; $^{\#}P<0.05$, n=8 in comparison to wild-type female mice). Levels of 7,8-dihydrobiopterin (BH_2), oxidized product of BH_4 did not significantly increase in the cerebral microvessels of either hph-$1^{-/-}$ or hph-$1^{+/-}$ mice (C). In the cerebral microvessels of both male and female mice, ratio of BH_4 to BH_2, indicator of BH_4 bioavailable for eNOS activation, were significantly decreased in the cerebral microvessels of hph-$1^{-/-}$ and hph-$1^{+/-}$ mice (* $P<0.05$, n=6 in comparison to wild-type male mice; $^{\#}P<0.05$, n=8 in comparison to wild-type female mice).

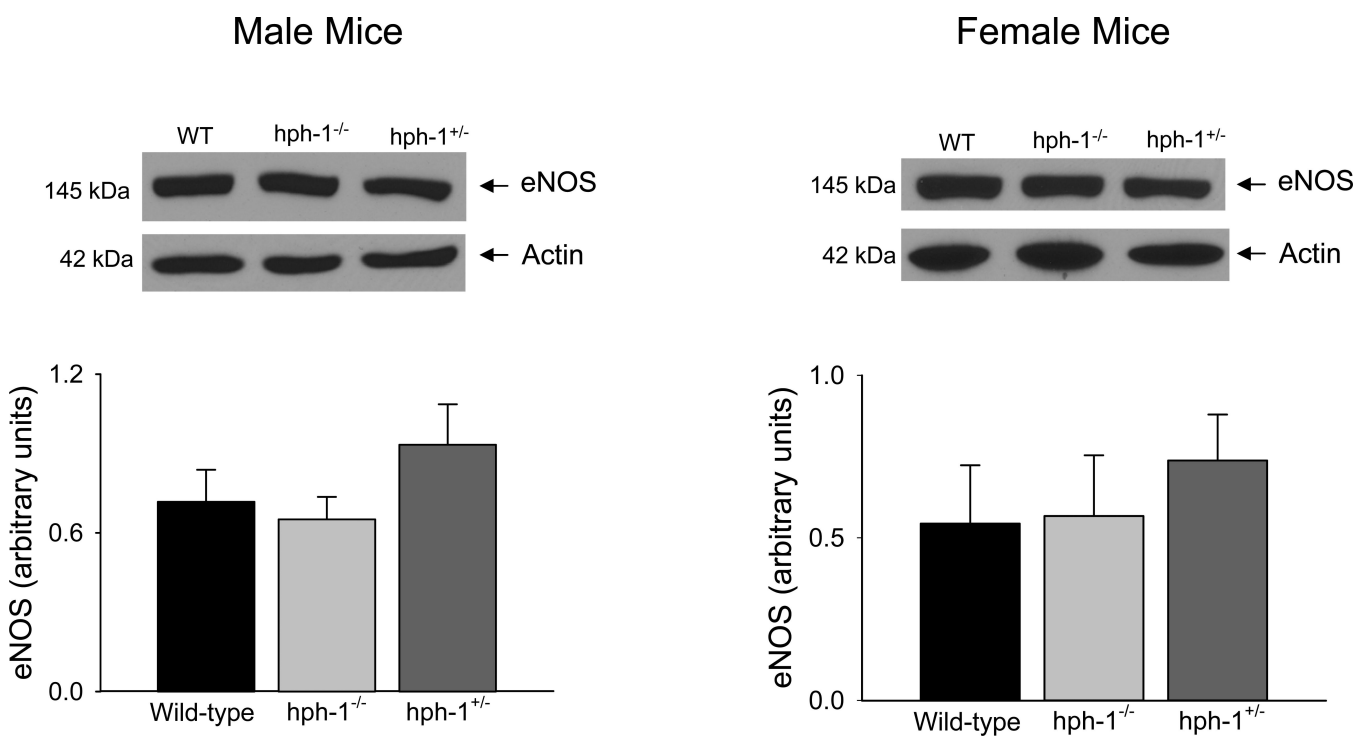
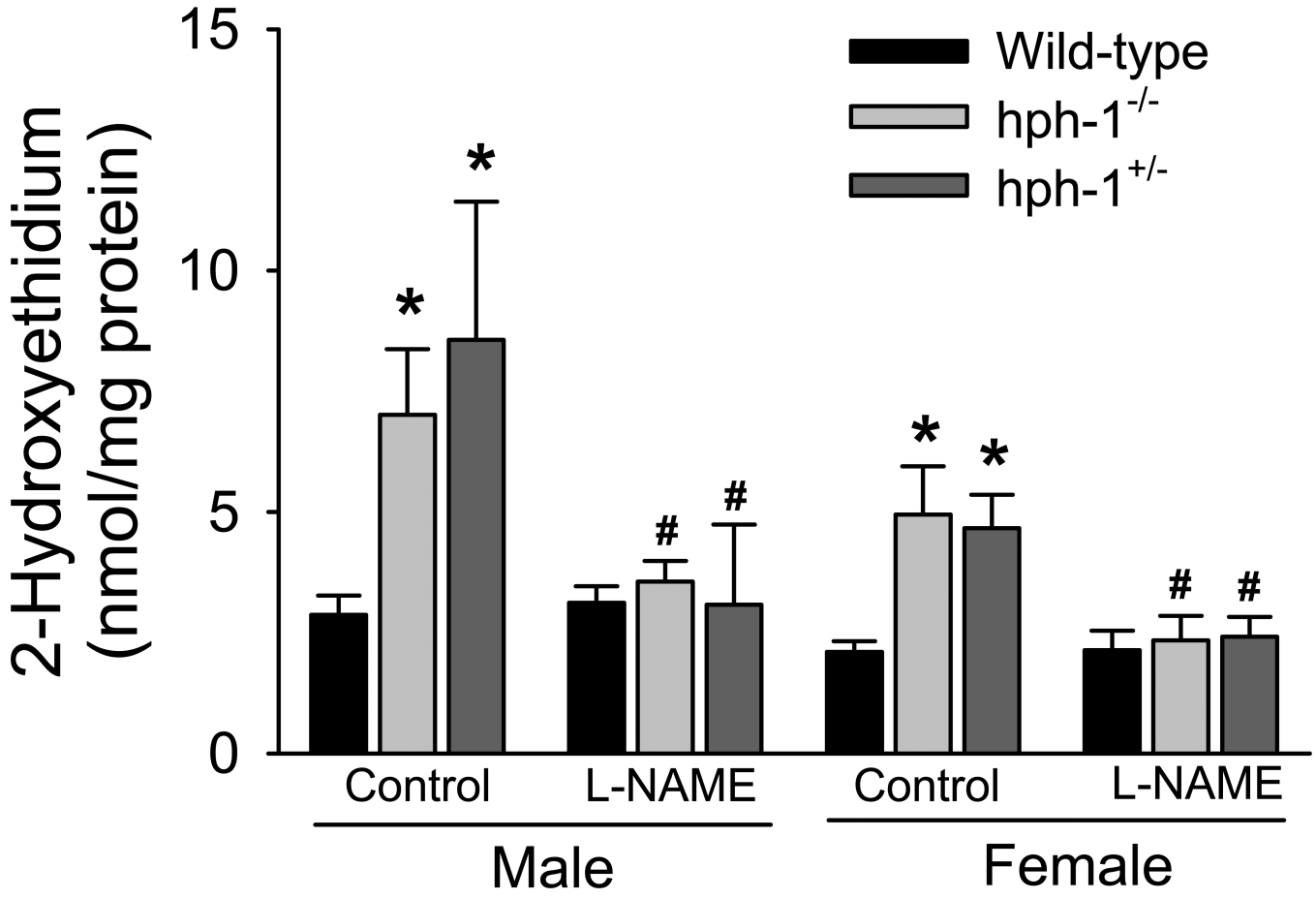


Figure 3.

Representative Western blots and densitometric analysis demonstrating expression of eNOS in the cerebral microvessels of male and female, hph-$1^{-/-}$ and hph-$1^{+/-}$ mice. Expression of eNOS did not differ between these three experimental groups (n=6).

A



B

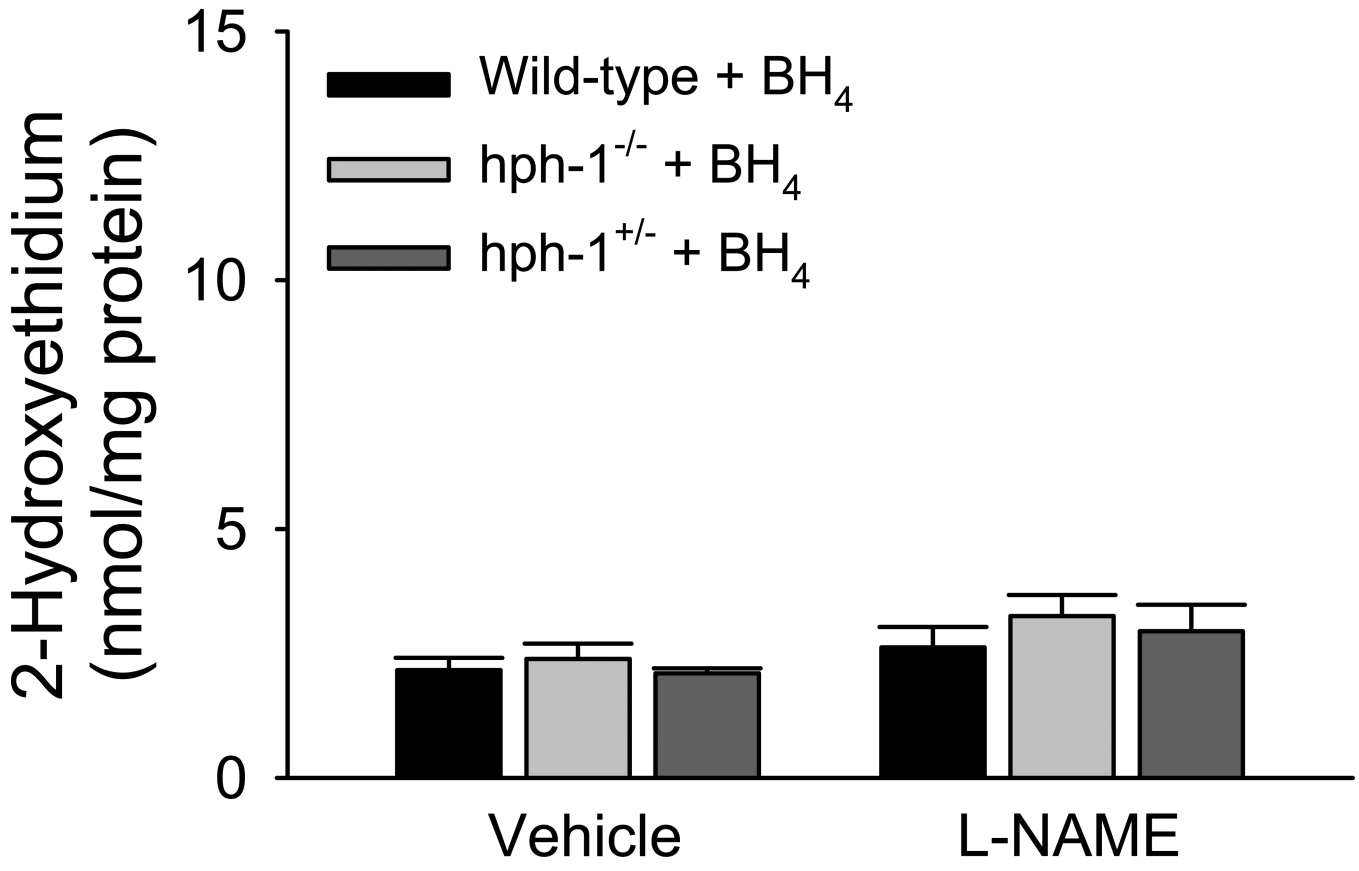


Figure 4.

(A) Superoxide anion production in the cerebral microvessels of hph-$1^{-/-}$ and hph-$1^{+/-}$ mice. Basal superoxide production between wild-type male and female mice was not statistically significant. Superoxide anion production remained significantly increased in male and female cerebral microvessels in both hph-$1^{-/-}$ and hph-$1^{+/-}$ groups (* $P<0.05$, in comparison to their wild-type controls, n=8). Treatment with L-NAME (30 μmol/l) significantly reduced the superoxide anion production in cerebral microvessels of both hph-$1^{-/-}$ and hph-$1^{+/-}$ mice ($^{\#}$ $P<0.05$ in L-NAME treated group as compared to corresponding sex-matched vehicle (Krebs-HEPES buffer) treatment, n=8). (B) Superoxide anion production in microvessels of female wild-type, hph-$1^{-/-}$ and hph-$1^{+/-}$mice injected with BH_4 (100 μmol/kg, 3hours). Treatment with BH_4 abolished the increase in superoxide anion production observed in cerebral microvessels of hph-$1^{-/-}$and hph-$1^{+/-}$ mice. Treatment with L-NAME (30 μmol/l) did not have any further effect on the superoxide anion production (P=ns, n=4).

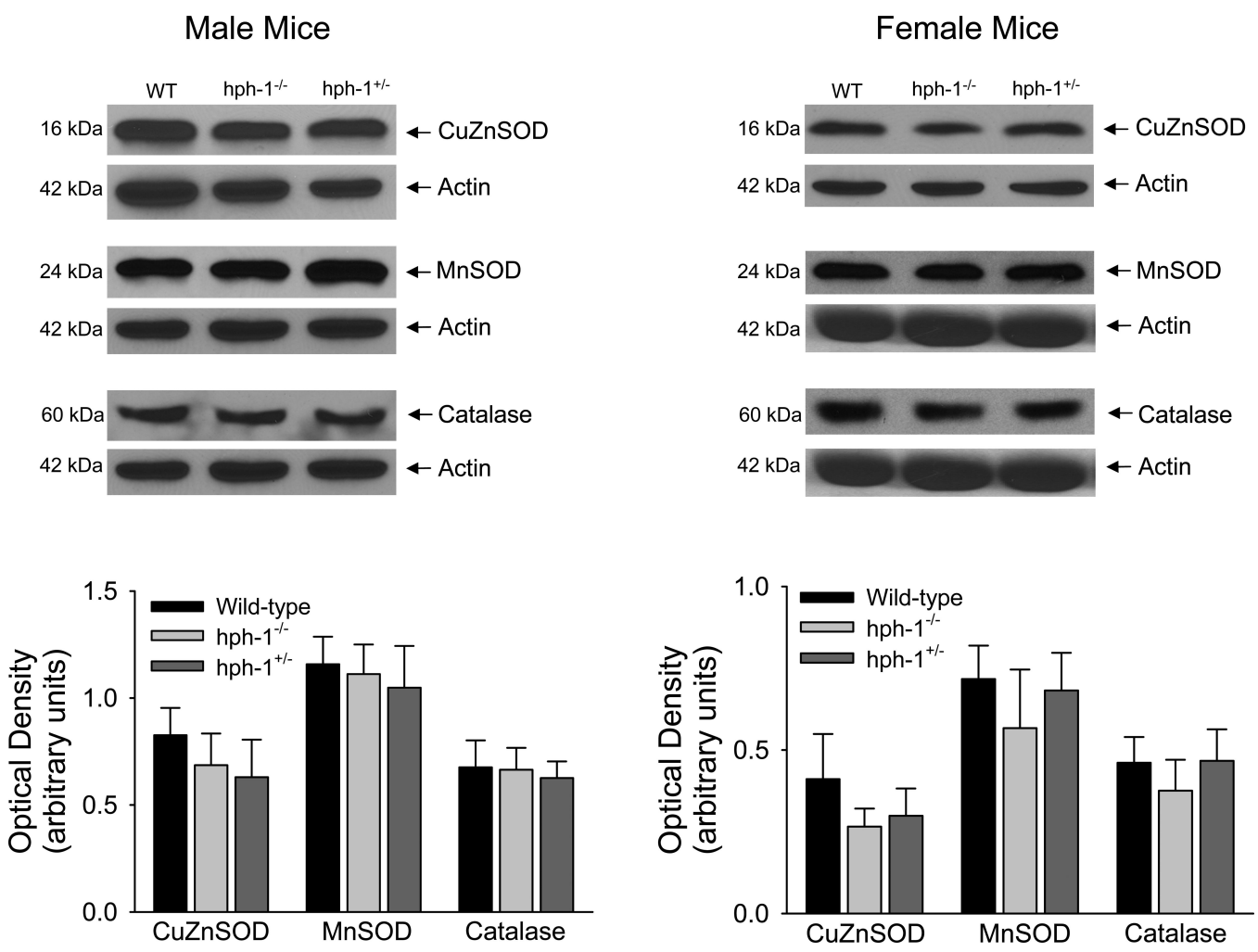


Figure 5.

Representative Western blots and densitometric analysis demonstrating expression of antioxidant proteins in the cerebral microvessels of both male and female, hph-1$^{-/-}$ and hph-1$^{+/-}$ mice. Expressions of CuZn superoxide dismutase (CuZnSOD), MnSOD and catalase did not differ in cerebral microvessels of hph-1$^{-/-}$ and hph-1$^{+/-}$ mice, as compared to their sex-matched wild-type controls (n=6).

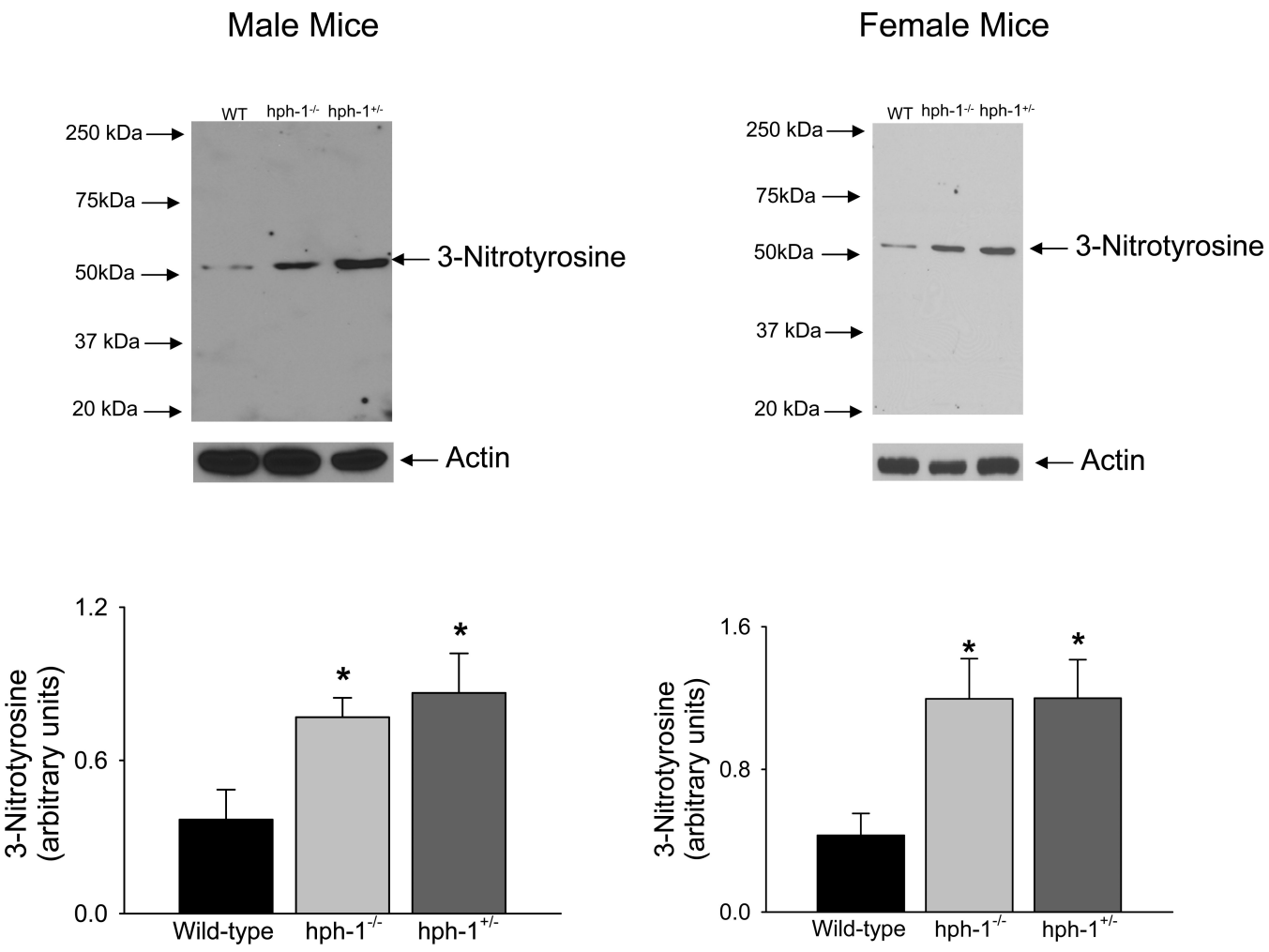


Figure 6.

Expression of 3-nitrotyrosine in the cerebral microvessels of both male and female, hph-1$^{-/-}$ and hph-1$^{+/-}$ mice. Densitometric analysis showed that 3-nitrotyrosine expression was significantly increased in both hph1$^{-/-}$ and hph1$^{+/-}$ mice (*P<0.05, n=4).

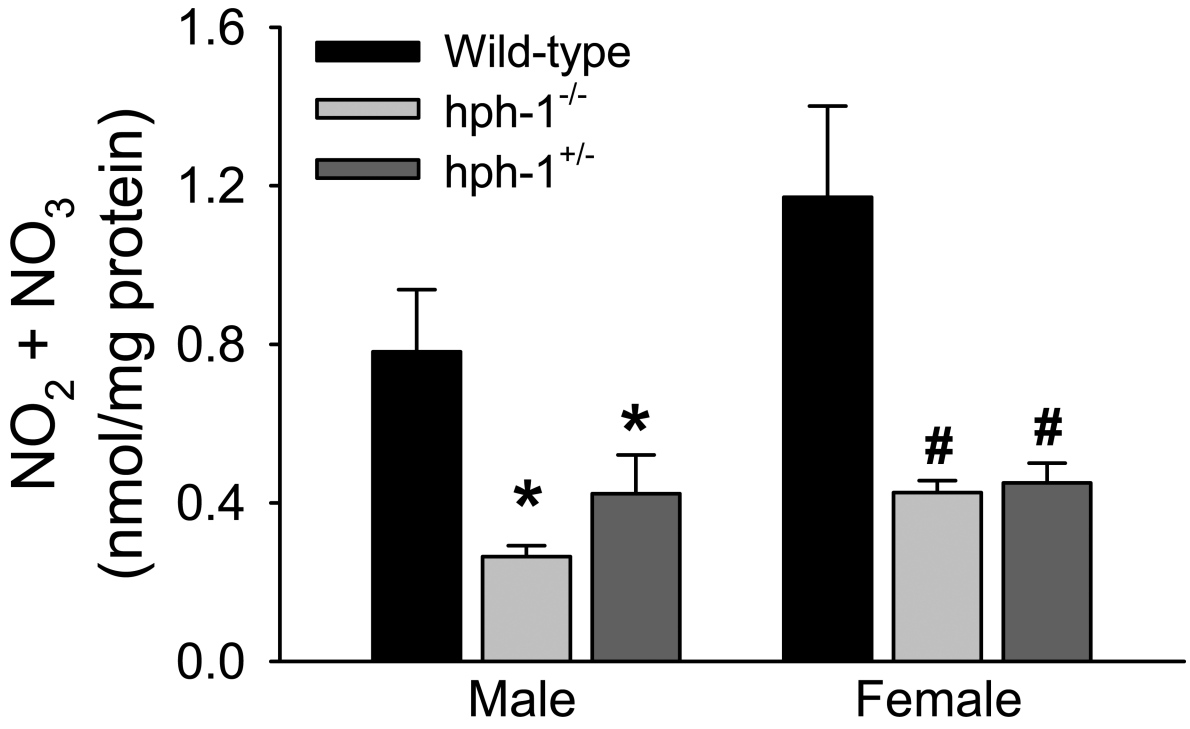


Figure 7.
Levels of nitrite/nitrate, indicator of NO production, was significantly reduced in cerebral microvessels of hph-$1^{-/-}$ and hph-$1^{+/-}$ mice as compared to their sex-matched wild-type control mice (* $P<0.05$, n=6 in comparison to wild-type male mice; $^{\#}P<0.05$, n=6 in comparison to wild-type female mice).

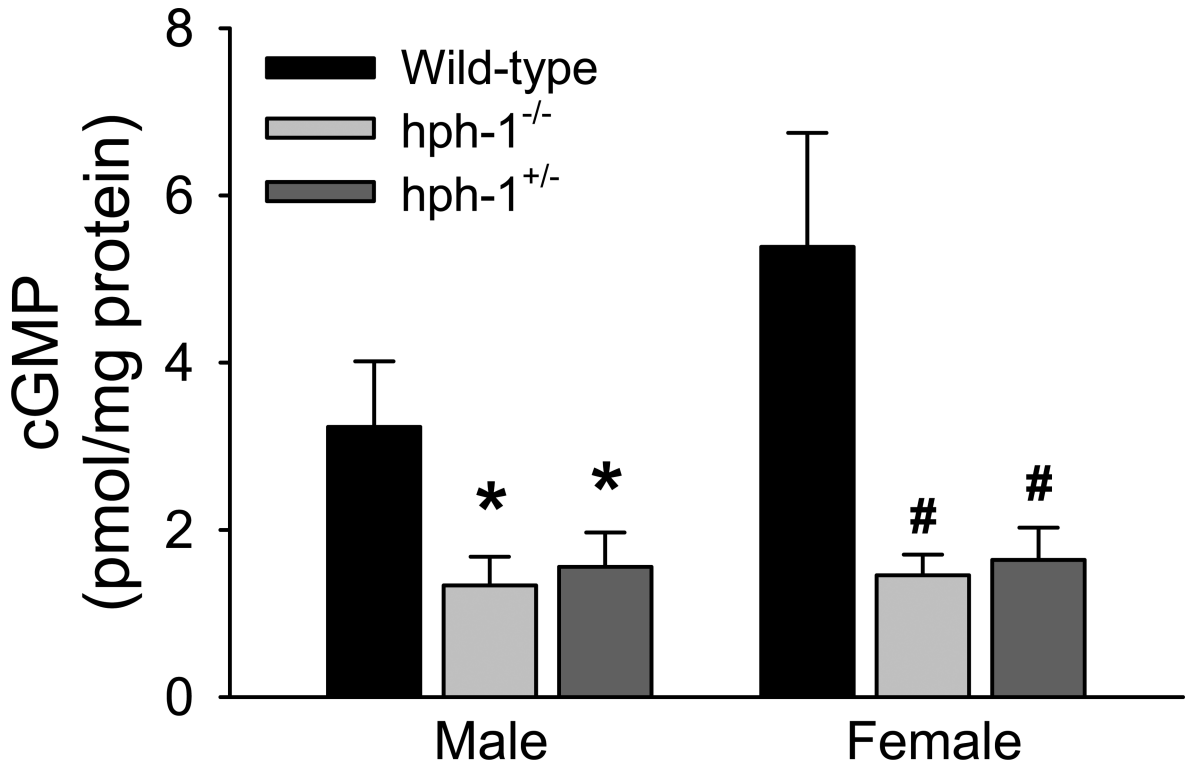


Figure 8.

Bar diagram depicting significantly reduced levels of cGMP, second messenger of NO, in cerebral microvessels of hph-$1^{-/-}$ and hph-$1^{+/-}$ mice as compared to their sex-matched wild-type control mice (* $P<0.05$, n=6 in comparison to wild-type male mice; $^{\#}P<0.05$, n=6 in comparison to wild-type female mice).