Published in final edited form as:

J Cardiovasc Pharmacol. 2014 December ; 64(6): 514–521. doi:10.1097/FJC.000000000000145.

Erythropoietin prevents endothelial dysfunction in GTP-cyclohydrolase I-deficient hph1 mice

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Abstract

In the present study, we used the mutant hph-1 mouse model, that has deficiency in GTP-cyclohydrolase I (GTPCH I) activity, to test the hypothesis that erythropoietin (EPO) protects aortic wall from oxidative stress induced by uncoupling of endothelial nitric oxide synthase (eNOS). Both GTPCH I activity and tetrahydrobiopterin (BH₄) levels were reduced in hph-1 mice while 7,8-dihydrobiopterin (7,8-BH₂) levels were significantly increased. Furthermore, BH₄ deficiency caused increased production of superoxide anion and hydrogen peroxide in the aorta thus resulting in impairment of endothelium-dependent relaxations to acetylcholine. Treatment of hph1 mice with recombinant human EPO (1000 U/kg, s.c. for three days) significantly decreased superoxide anion production by eNOS and improved BH₄ to 7,8-BH₂ ratio in aortas. EPO also significantly decreased production of hydrogen peroxide and improved endothelium-dependent relaxations in aortas of hph1 mice. In addition, EPO treatment increased protein expressions of copper-/zinc-superoxide dismutase, manganese-superoxide dismutase, and catalase in the aorta of hph1 mice. Our findings demonstrate that treatment with EPO prevented oxidative stress and endothelial dysfunction caused by eNOS uncoupling. Increased vascular expressions of antioxidants appear to be an important molecular mechanism underlying vascular protection by EPO during chronic BH₄ deficiency.

Keywords

Nitric oxide; endothelial cells; tetrahydrobiopterin; mouse aorta; erythropoietin

Introduction

Endothelium-derived NO plays a key regulatory role in vascular homeostasis.¹ Tetrahydrobiopterin (BH₄) is an essential cofactor for endothelial nitric oxide synthase (eNOS) and it is synthesized from guanosine-5'-triphosphate (GTP) by enzymatic activity of GTP-cyclohydrolase I (GTPCH I).² Depletion of intracellular BH₄ reduces formation of NO in endothelial cells and supplementation of BH₄ has been shown to improve vascular

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Conflict of Interest: None

endothelial function.³ Several studies also have reported that BH₄ deficiency could be caused by either increased oxidation of BH₄ leading to formation of 7,8-dihydrobiopterin (7,8-BH₂) or by decreased enzymatic activity of GTPCH I or both.^{4, 5} Suboptimal concentration of BH₄ causes eNOS uncoupling and increase in eNOS-derived superoxide anion production.^{6, 7}

Erythropoietin (EPO) is the primary regulator of erythropoiesis.⁸ Therapy with recombinant human EPO has been shown to be efficient and safe in improving the management of the anemia associated with chronic renal failure.⁹ Moreover, recent evidences indicate that EPO receptors are also widely distributed in the cardiovascular system, including endothelial, smooth muscle, cardiac, and other cell types consistent with non-hematopoietic effects of EPO.¹⁰⁻¹² Indeed, EPO has potentially beneficial effects on endothelial cells including nitric oxide (NO) production, anti-apoptotic effects, mitogenic and angiogenic activities.¹³⁻¹⁸ In contrast, EPO causes hypertension and increased medial thickness of injured carotid arteries in eNOS-deficient mice indicating that the vascular protective effects of EPO are critically dependent on activation of eNOS.¹⁶

Our studies as well as those of others have shown that endothelial dysfunction and eNOS uncoupling is present in hyperphenylalaninemic mutant (hph1) mice, currently used as a mouse model of GTPCH I deficiency.¹⁹⁻²¹ Uncoupling of eNOS is associated with increased superoxide anion production and impaired NO signaling in the aorta of hph-1 mice.²⁰ Importantly, increased antioxidant capacity is one of the key mechanisms in prevention of eNOS uncoupling in hph-1 mice.²² Moreover, several studies reported that EPO increases expression and/or activity of copper-/zinc-superoxide dismutase (CuZnSOD) indicating that EPO possess tissue protective properties against oxidative stress.²³⁻²⁵ Whether EPO prevents detrimental consequences of eNOS uncoupling including superoxide anion production in hph1 mice is unclear. Therefore, the present study was designed to determine the molecular mechanisms underlying effects of EPO on vascular endothelial function in GTPCH I-deficient hph1 mice.

Methods

Experimental Animals

Wild-type littermates and homozygous hph1 mice were generated from in-house breeding and were genotyped using PCR analysis.²⁰ Male mice used in studies were maintained on standard chow with free access to drinking water. At 16-20 weeks of age wild-type and hph1 mice were treated for 3 days with either PBS or EPO (recombinant human EPO alpha, 1000 U/kg body weight, s.c.; Amgen, Thousand Oaks, CA) once daily. The dose of EPO was selected based on previous studies.^{16, 26-28} After treatments, mice were euthanized with overdose of pentobarbital (200-250 mg/kg BW, i.p.), and aortas were carefully removed and dissected free from connective tissue in cold (4°C) modified Krebs-Ringer solution (in mmol/L: NaCl 118.6; KCl 4.7; CaCl₂ 2.5; MgSO₄ 1.2; KH₂PO₄ 1.2; NaHCO₃ 25.1; glucose 10.1; EDTA 0.026). Housing facilities and 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.

Blood Pressure

A tail-cuff method (Harvard Apparatus Ltd., Kent, England) was used for measurements of systolic blood pressure in quiescent mice before and after 3 days treatment with EPO.²⁰

Measurements of Biopterin Levels

Biopterin levels were determined in aortas after differential oxidation in acid (which converts both BH₄ and 7,8-BH₂ to biopterin) and base (which converts only 7,8-BH₂ to biopterin) conditions by reversed-phase HPLC/fluorescence (Beckman Coulter, Inc.) as described previously.²⁹ BH₄ content was calculated from the difference in biopterin levels after acid and base oxidations, and the results were normalized against protein levels.

Measurement of GTP-Cyclohydrolase I Activity

Enzymatic activity of GTP-cyclohydrolase I (GTPCH I) were determined in lungs by the quantification of neopterin formation using reversed-phase HPLC/fluorescence (Beckman Coulter, Inc.), and the results were normalized against protein levels.²⁰

Vasomotor Reactivity Studies

Isometric force of aortic rings was recorded in organ bath filled with Krebs-Ringer solution as described²⁹ and the data were acquired using LabChart Pro software (ADInstruments, Colorado Springs, CO). Aortas were progressively stretched to their optimal passive tension as assessed by response to 80 mM KCl. The optimal points on length/tension curve were 1.48±0.2g (wild-type), 1.47±0.2g (wild-type + EPO), 1.43±0.3g (hph1), and 1.47±0.3g (hph1 + EPO; n=7). Concentration-dependent response curves to acetylcholine (10⁻⁹ - 10⁻⁵ M), and diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (DEA-NONOate; 10⁻¹⁰ - 10⁻⁵ M) were cumulatively obtained during submaximal contractions to phenylephrine. Concentrations of phenylephrine (1-3×10⁻⁷ M) were selected in order to obtain the same submaximal contraction in aortic rings from both wild-type (32±3%) and hph1 mice (28±3%) and those treated with EPO (29±3% and 26±4%, respectively).

Measurement of cGMP Levels

10-15 mm long segment of aorta was incubated for 60 in MEM (containing 0.1% BSA, 100 U/mL penicillin and 100 µg/mL streptomycin) at 37°C in CO₂ incubator in the presence of 3-isobutyl-1-methylxanthine (100 µmol/L) to inhibit the degradation of cyclic nucleotides by phosphodiesterases.²⁰ In some of aortas catalase (1200 U/mL) was added and incubated for 60 minutes prior to 3-isobutyl-1-methylxanthine. Aortas were then quickly frozen in liquid N₂ and stored at -80°C until assayed. A ELISA kit (Cell Biolabs Inc., San Diego, CA) was used for cGMP measurements in aortas, and the results were normalized against protein levels (BioRad, Hercules, CA).

Measurement of Superoxide Anion

Levels of superoxide anion was quantified using HPLC/fluorescence assay that employs dihydroethidium as a probe as described previously.³⁰ In brief, 15 mm long aortas were incubated in Krebs-HEPES buffer (pH 7.4) at 37°C in CO₂ incubator in the absence or presence of NOS inhibitor N^ω-nitro-L-arginine methyl ester (L-NAME, 3×10⁻⁴ M) for 60

min prior addition of dihydroethidium (50 μ M; Molecular Probes).²⁰ After 15 min the samples were washed of dihydroethidium and incubated in Krebs-HEPES buffer for additional one hour. Then the samples were homogenized in 100% methanol and centrifuged at 10,000 rpm. Supernatants were subjected to HPLC analysis, and the pellets were dissolved in 2% sodium dodecyl sulfate for protein quantification.

Measurement of Hydrogen Peroxide

H₂O₂ content was quantified in aortas using Amplex[®] Red assay kit (Life Technologies, Grand Island, NY). Aortas were homogenized in buffer solution provided by the kit and centrifuged at 10,000 rpm. H₂O₂ content was analyzed in supernatants according manufacturer instruction, and the results were normalized against protein levels.²⁰

Western Blot Analysis

Whole mouse aortas were homogenized in lysis buffer as described.³¹ Equal amounts of protein (50 μ g) were separated by SDS-PAGE and transferred to nitrocellulose membrane (Amersham). Primary antibodies against eNOS (BD Biosciences); CuZnSOD, manganese-superoxide dismutase (MnSOD), extracellular-superoxide dismutase (EcSOD; Enzo); catalase, (Sigma); and glutathione peroxidase 1 (GPx1; AbFrontier). For loading controls, blots were reprobed with β -actin (Sigma). The bands were visualized using a commercially available ECL (Amersham), and the densitometry was determined using NIH-Image.

Statistical Analysis

All results are expressed as means $\pm$ SEM and “n” indicates the number of animals from which tissues were harvested. Relaxations (expressed as percentage of contraction) were determined for each individual concentration-response curve by non-linear regression analysis. The concentration-response curves of the different groups were compared by ANOVA for repeated measurements followed by Bonferroni's correction. Wild-type and hph1 mice treated with or without EPO were compared by ANOVA with Bonferroni's. In addition, groups of treated wild-type and hph1 mice without or with L-NAME were compared by two-way ANOVA for multiple comparisons. A value of $P < 0.05$ was considered significant.

Results

Blood Pressure

Deficiency of BH₄ did not affect systolic blood pressure in hph-1 mice (Table 1). Furthermore, EPO treatment for 3 days did not increase SBP in hph1 mice and their wild-type littermates (Table 1). In addition, diastolic blood pressure was also unchanged in EPO-treated wild-type and hph1 mice (data not shown; n=6).

Vascular Biopterin Metabolism

BH₄ levels were significantly reduced in hph1 mice aortas as compared to their wild-type littermates ($P < 0.05$; Figure 1A). However, we also observed an increased oxidation of BH₄ to 7,8-BH₂ in hph1 mice ($P < 0.05$; Figure 1B) resulting in a decrease of BH₄ to 7,8-BH₂

ratio ($P<0.05$; Figure 1C). EPO treatment for 3 days significantly increased BH_4 levels in wild-type aortas ($P<0.05$) while it has no effect in *hph1* mice (Figure 1A). In contrast, EPO decreased 7,8- BH_2 levels and thus improved BH_4 to 7,8- BH_2 ratio in *hph1* mice aortas ($P<0.05$; Figures 1C and 1D, respectively).

In previous studies, we showed that enzymatic activity of GTPCH I is decreased in *hph1* mice aortas.²⁰ We confirmed that GTPCH I activity was very low in the lungs of *hph1* mice (0.42 ± 0.06 pmol/mg/hr; $P<0.05$ vs. wild-type mice: 3.90 ± 0.82 pmol/mg/hr; $n=10$). Treatment with EPO significantly increased GTPCH I activity in wild-type mice (6.79 ± 1.14 pmol/mg/hr; $P<0.05$; $n=10$) but not in *hph1* mice lungs (0.76 ± 0.13 pmol/mg/hr; $n=10$).

Endothelial Function

Vascular reactivity studies of isolated aortas revealed that endothelium-dependent relaxations to acetylcholine were significantly impaired in *hph1* mice ($P<0.05$ vs. wild-type mice; Figure 2A). Treatment with EPO for 3 days significantly improved relaxations to acetylcholine in *hph1* mice ($P<0.05$ vs. control *hph1* mice; Figure 2A). In contrast, endothelium-dependent relaxations were unchanged by EPO in wild-type littermates (Figure 2A). On the other hand, endothelium-independent relaxations to the NO-donor DEA-NONOate were not different between wild-type and *hph1* mice and those treated with EPO (Figure 2B).

Vascular Superoxide Anion

BH_4 deficiency caused an increase in superoxide anion production in *hph1* mice aortas ($P<0.05$; Figure 3A). Treatment with EPO significantly reduced superoxide anion levels in *hph1* mice ($P<0.05$), while in wild-type mice superoxide levels tended to be decreased by EPO (Figure 3A). Furthermore, incubation of aortas with NOS inhibitor L-NAME prevented increase in superoxide anion only in *hph1* mice ($P<0.05$; Figure 3A).

Vascular H_2O_2 Content

We also observed increased H_2O_2 levels in aortas of *hph1* mice ($P<0.05$; Figure 3B). Interestingly, EPO decreased H_2O_2 levels in *hph1* mice aortas while it did not affect in wild-type mice ($P<0.05$; Figure 3B).

Vascular cGMP levels

Basal cGMP levels were significantly increased in *hph1* mice aortas ($P<0.05$; Figure 4A). Treatment with EPO normalized cGMP levels in aortas of *hph1* mice (Figure 4A; $P<0.05$). Furthermore, EPO tended to increase cGMP levels in wild-type mice; this increase did not attain statistical significance. Incubation of aortas with catalase significantly decreased cGMP levels in the aorta of *hph1* mice ($P<0.05$; Figure 4B).

eNOS Protein Expression

Western blot analysis showed that protein expression of eNOS did not differ between wild-type littermates and *hph1* mice and those treated with or without EPO (Figure 5).

Protein Expressions of SOD Isoforms

Western blot analyses indicated that protein expressions of CuZnSOD, MnSOD and EcSOD did not differ between wild-type and hph1 mice aortas (Figure 6). Treatment with EPO significantly increased protein expression of CuZnSOD in both wild-type and hph1 mice ($P<0.05$; Figure 6A) while EPO increased MnSOD expression only in hph1 mice aortas ($P<0.05$; Figure 6B). In contrast, expression of EcSOD was unaffected by EPO in either wild-type or hph1 mice (Figure 6C).

Protein Expressions of Catalase and GPx1

While protein expression of catalase tended to be increased in aortas of hph1 mice, EPO treatment significantly augmented catalase expression in both wild-type mice and hph1 mice aortas ($P<0.05$; Figure 7A). In contrast, protein expression of GPx1 was not different between wild-type and hph1 mice aortas and those treated with EPO (Figure 7B).

Discussion

There are several novel findings in this present study. First, EPO prevented oxidation of BH₄ levels and improved the BH₄ to 7,8-BH₂ ratio in hph1 mice. Second, treatment with EPO prevented superoxide anion and H₂O₂ production caused by eNOS uncoupling and significantly improved endothelium-dependent relaxations to acetylcholine in hph1 mice aortas. Third, EPO inhibited H₂O₂-induced increase in cGMP levels during BH₄ deficiency. Fourth, EPO treatment increased protein expressions of CuZnSOD, MnSOD, and catalase in aortas of hph1 mice. Thus, it appears that the vascular protective effects of EPO were caused by the increased antioxidant capacity which in turn prevented oxidative stress induced by eNOS uncoupling.

It has been proposed that reduced bioavailability of vascular BH₄ may result in impairment of NO-mediated endothelium-dependent vasodilatation, an initiating event in the pathogenesis of atherosclerosis.³ Indeed, there are several reports showing that in vivo administration of BH₄ ameliorates impaired endothelium-dependent vasodilatation in diabetes, hypercholesterolemia, atherosclerosis, and smokers.³²⁻³⁵ We and others have previously shown that BH₄ deficiency due to the decreased biosynthesis of BH₄ via GTPCH I caused eNOS uncoupling and increased superoxide anion production in hph1 mice aortas.¹⁹⁻²¹ However, BH₄ itself is also a molecular target for oxidative stress and increased oxidation of BH₄ to 7,8-BH₂ may cause eNOS uncoupling.³⁶⁻³⁸ Indeed, in the present study we observed that both decreased biosynthesis of BH₄ and increased oxidation of BH₄ caused impairment of endothelium-dependent relaxations to acetylcholine of aortas in GTPCH I-deficient mice. In contrast, endothelium-dependent relaxations to NO donor, DEA-NONOate, were unchanged in hph1 mice indicating that endothelial function is susceptible to decreased BH₄ availability.

While treatment with EPO increased enzymatic activity of GTPCH I and BH₄ levels in wild-type mice, it did not significantly influence these parameters in hph1 mice. Most notably, EPO treatment decreased 7,8-BH₂ levels and increased of BH₄ to 7,8-BH₂ ratio in aortas of hph1 mice. Likewise, endothelium-dependent relaxations to acetylcholine were significantly

improved in aortas of EPO treated hph1 mice despite low BH₄ concentrations, suggesting that decreasing 7,8-BH₂ levels may increase NO production in vascular endothelium. These findings are consistent with the concept that diminished intracellular BH₄ to 7,8-BH₂ ratio rather than absolute concentrations of BH₄ per se is the key determinant of endothelial dysfunction. Indeed BH₄ and 7,8-BH₂ bind eNOS with equal affinity and 7,8-BH₂ can efficiently replace eNOS-bound BH₄ resulting in eNOS uncoupling.³⁶⁻³⁸

It has been shown that increased shear stress caused by high number of circulating red blood cells is powerful stimulus for eNOS and NO production.^{16, 39, 40} However, we have shown that administration of EPO for three days does not affect the number of circulating red blood cells, but stimulates BH₄ biosynthesis and phosphorylation of eNOS suggesting that EPO possess direct vascular effects beyond erythropoiesis.^{16, 41} Moreover, the dose of EPO used in the present study was based on previous pharmacokinetic studies. The bioavailability of EPO is lowest in mice among tested animal species and therefore the dose of 1000 U/kg EPO is required to observe the vascular effects.²⁶⁻²⁸

Uncoupling of eNOS has been known to generate superoxide anion thereby playing critical roles in the initiation and progression of cardiovascular diseases.^{6, 7} In the present study we provided evidence that increased production of superoxide anions in hph-1 mice was abolished by NOS inhibitor L-NAME demonstrating that eNOS is the only source of increased superoxide generation during BH₄ deficiency. Indeed, we have shown that administration of BH₄ abolished the increase in superoxide anion production and normalized eNOS function in hph-1 mice.⁴² Treatment with EPO reduced levels of superoxide anion in the aorta of hph1 mice without normalizing BH₄ levels indicating that other mechanisms are responsible for prevention of superoxide anion and improvement of endothelial function.

Interestingly, we detected increased protein expressions of CuZnSOD and MnSOD in aortas of EPO-treated hph1 mice. It is conceivable that the decreased superoxide anion production and improvement of endothelium-dependent relaxations to acetylcholine by EPO could be explained by increased expression of CuZnSOD, because the latter is the major isoform of SOD in blood vessel wall.^{43, 44} In addition, we have previously shown that the ability of EPO to reduce vascular production of superoxide anion was abolished in CuZnSOD-deficient mice.²³ The exact molecular mechanisms responsible for increased CuZnSOD expression by EPO are unclear. However, several studies reported that EPO receptors are widely distributed in the cardiovascular system and that the vascular protective effects of EPO are dependent activation of EPO-receptor.^{14, 28, 45} Moreover, heterodimeric EPO-receptor/beta common receptor is required for mediation of the tissue-protective effects of EPO.⁴⁶ Irrespective of the exact receptor activated by EPO, downstream activations of phosphatidylinositol-3-kinase and/or nuclear factor-κB are most likely molecular mechanism underlying anti-inflammatory and anti-oxidative effects of EPO^{18, 47-50} including increased expression of CuZnSOD in response to EPO.^{24, 25, 51}

Increasing evidence indicates that vascular disease is also characterized by elevation of H₂O₂ because H₂O₂ is almost always formed under circumstances in which superoxide anion is produced.⁵² During chronic BH₄ deficiency, production of H₂O₂ by eNOS has been considered as an important cell signaling molecule and regulator of vascular tone.^{53, 54}

Indeed our study indicated that levels of H_2O_2 were increased in aortas of hph1 mice. Intriguingly, treatment with EPO reduced aortic H_2O_2 concentrations in hph1 mice. Similar observations were made in normal rat kidney cells showing that EPO receptors are involved in cytoprotection of EPO against H_2O_2 cytotoxicity.⁵⁵ The decrease in H_2O_2 levels after EPO treatment may be explained by the increased expression of H_2O_2 detoxifying enzymes catalase and GPx1. Indeed, EPO increased protein expression of catalase in both wild-type and hph1 mice while protein expression of GPx1 was unaffected. Inspection of mouse catalase protein sequence (National Center for Biotechnology Information accession # NP_033934) revealed that catalase possesses 5 consensus Yxx[L/I/V] motifs preferred by Janus kinase 2, the key kinase in the signal transduction pathways activated by EPO receptor.⁵⁶ From these results it is therefore conceivable that enhanced dismutation of superoxide anion to H_2O_2 and subsequent degradation of H_2O_2 to water and oxygen through increased protein expressions of CuZnSOD and catalase, respectively, contributes to protection of endothelial function from oxidative stress caused by eNOS uncoupling in hph1 mice.

We and others have shown that cGMP levels were increased by H_2O_2 generated in the vascular wall during chronic BH_4 deficiency.^{4, 19, 20, 57} Indeed, catalase was able to prevent the increased cGMP levels in hph1 mice suggesting that H_2O_2 can directly activate soluble guanylate cyclase. Similar observations have been reported in previous studies.^{58, 59} Interestingly, treatment with EPO reduced cGMP to levels comparable to those in wild-type mice indicating that prevention of oxidative stress caused by eNOS uncoupling is the most likely explanation for normalization of cGMP levels.

Conclusions

The present studies suggest that vascular reactive oxygen species such as superoxide anion and H_2O_2 contribute to decrease in NO bioactivity observed in endothelial dysfunction in GTPCH I-deficient hph1 mice. EPO exerts beneficial effects on endothelial dysfunction during BH_4 deficiency by increasing expressions of CuZnSOD, MnSOD, and catalase thereby protecting endothelial cells from excessive production of superoxide anion and hydrogen peroxide.

Acknowledgments

Source of Funding: This work was supported by the National Institutes of Health grants HL91867 and HL111062, by the Roche Foundation for Anemia Research, and by the Mayo Foundation.

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Figure 1.

Biopterin metabolism in hph1 mice and their wild-type littermates and those treated with EPO (+). **A)** Tetrahydrobiopterin (BH₄) levels, **B)** 7,8-dihydrobiopterin (7,8-BH₂) levels, and **C)** the ratio of BH₄ to 7,8-BH₂ were determined in aortas. All results are shown as mean ± SEM (n=10). * P<0.05 vs. wild-type mice; † P<0.05 vs. hph1 mice without EPO (-).

Figure 2.

Endothelium-dependent relaxations to acetylcholine (**A**) and endothelium-independent relaxations to NO-donor diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (DEA-NONOate, **B**) in isolated aortas of hph1 mice and their wild-type littermates and those treated with EPO. Please note that reduced relaxations to acetylcholine were significantly improved by EPO in hph1 mice ($P < 0.05$; ANOVA + Bonferroni's correction). * $P < 0.05$ vs. wild-type mice; † $P < 0.05$ vs. hph1 mice. All data are shown as mean $\pm$ SEM ($n=7$) and are expressed as percent relaxation of the sub-maximal contraction to phenylephrine.

Figure 3.

Effects of EPO on oxygen derived free radical in aortas of wild-type and hph1 mice. **A)** Superoxide anion levels were determined in aortas in the absence or presence of NOS inhibitor L-NAME (3×10^{-4} M). The data are mean $\pm$ SEM (n=9-12). **B)** H₂O₂ levels were analyzed in aortas using Amplex Red (n=6-8). All results were normalized against tissue protein levels. * P<0.05 vs. wild-type mice without EPO (-); † P<0.05 vs. hph1 mice without EPO (-); # P<0.05 vs. hph1 mice without L-NAME (two-way ANOVA).

Figure 4.

A) cGMP levels in aortas of hph1 mice and their wild-type littermates and those treated with EPO. **B)** Effect of catalase on cGMP levels in aortas of hph1 mice. All results were normalized against tissue protein levels and are shown as mean $\pm$ SEM (n=5-10). * $P < 0.05$ vs. wild-type mice without EPO; † $P < 0.05$ vs. hph1 mice without EPO; # $P < 0.05$ vs. hph1 mice without catalase.

Figure 5. Effects of EPO on eNOS protein expression in aortas of hph1 mice and their wild-type littermates. Bar graph indicates the results of the relative densitometry as compared to β -actin protein. All results are shown as mean $\pm$ SEM (n=9).

Figure 6.

Representative Western blot analysis of CuZnSOD (A), MnSOD (B), and EcSOD (C) protein expressions in wild-type littermates and hph1 mice aortas and those treated with EPO (+). Bar graphs indicate the results of the relative densitometry as compared to β-actin protein. Results are mean ± SEM (n=8-10). * P<0.05 vs. wild-type mice without EPO (-); † P<0.05 vs. hph1 mice without EPO (-).

Figure 7.

Representative Western blot analysis of catalase (**A**) and GPx1 (**B**) protein expressions in aortas of EPO-treated (+) wild-type littermates and hph1 mice. Bar graphs indicate the results of the relative densitometry as compared to β-actin protein. Results are mean ± SEM (n=8-9). * P<0.05 vs. wild-type mice without EPO (-); † P<0.05 vs. hph1 mice without EPO (-).

Table 1

Systolic blood pressure of wild-type and hph1 mice and those treated with EPO for 3 days.

Day	Wild-type	Wild-type + EPO	Hph1	Hph1 + EPO
0	112±3	112±2	115±2	111±2
3	112±2	115±2	114±2	114±2

EPO, erythropoietin. Data are expressed as mmHg and are means ± SEM of 6 mice.