VASCULAR BIOLOGY, ATHEROSCLEROSIS, AND ENDOTHELIUM BIOLOGY

Hyperoxia Depletes (6R)-5,6,7,8-Tetrahydrobiopterin Levels in the Neonatal Retina

Implications for Nitric Oxide Synthase Function in Retinopathy

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Retinopathy of prematurity is a sight-threatening complication of premature birth caused by nitro-oxidative insult to the developing retinal vasculature during therapeutic hyperoxia exposure and later ischemia-induced neovascularization on supplemental oxygen withdrawal. In the vasodegenerative phase, during hyperoxia, defective endothelial nitric oxide synthase (NOS) produces reactive oxygen and nitrogen free radicals rather than vasoprotective nitric oxide for unclear reasons. Crucially, normal NOS function depends on availability of the cofactor (6R)-5,6,7,8-tetrahydrobiopterin (BH4). Because BH4 synthesis is controlled enzymatically by GTP cyclohydrolase (GTPCH), we used GTPCH-depleted mice [hyperphenylalaninemia strain (hph1)] to investigate the impact of hyperoxia on BH4 bioavailability and retinal vascular pathology in the neonate. Hyperoxia decreased BH4 in retinas, lungs, and aortas in all experimental groups, resulting in a dose-dependent decrease in NOS activity and, in the wild-type group, elevated NOS-derived superoxide. Retinal dopamine levels were similarly diminished, consistent with the dependence of tyrosine hydroxylase on BH4. Despite greater depletion of BH4, the hph^{+/−} and hph1^{−/−} groups did not show exacerbated hyperoxia-induced vessel closure, but exhibited greater vascular protection and reduced progression to neovascular disease. This vasoprotective effect was independent of enhanced circulating vascular endothelial growth factor (VEGF), which was reduced by hyperoxia, but to local retinal ganglion cell layer-derived VEGF. In conclusion, a constitutively higher level of VEGF expression associated with retinal development protects GTPCH-deficient neonates from oxygen-induced vascular damage. (*Am J Pathol* 2015, 185: 1769–1782; <http://dx.doi.org/10.1016/j.ajpath.2015.02.021>)

Retinal neovascularization (NV) is a sight-threatening complication of retinopathy of prematurity (ROP), a disease that still represents the greatest cause of visual impairment in the young and that shows evidence of a resurgence because of the improvements in neonatal care and the increased survival of young babies.¹ The pathology of ROP occurs in two stages: normal vascular development of the inner retina occurs in the second or third trimester of pregnancy and is driven by physiological hypoxia in response to vascular endothelial growth factor (VEGF) production.^{2,3} During this developmental window, the

vessels that are formed are immature and highly susceptible to oxygen-induced cell damage.⁴ Thus, in preterm infants, the supplemental oxygen required to support their survival arrests normal vascular growth, resulting in an incompletely vascularized retina. During subsequent return to normal room air, this lack of vascularity results in tissue hypoxia, release of angiogenic growth factors, and retinal NV.

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In common with other retinal diseases, the new vessels do not correctly revascularize the ischemic inner retina but breach the internal limiting membrane and infiltrate the transparent vitreous, which, untreated, can lead to vision loss. Established treatment options involve destruction of the ischemic peripheral retina by either cryotherapy or laser photocoagulation, both of which are effective but reduce peripheral vision. Newer therapeutic options, such as VEGF inhibitors, have proved successful, but intraocular injections are invasive and carry the risk of endophthalmitis. Also, because VEGF inhibitors inhibit NV without correcting the underlying ischemia, they may require repeated injections.⁵ In addition, as an important cellular survival factor, the removal of VEGF could have significant impact on the developing neural retina.⁶ Thus, advances in the management of this disease would greatly improve the quality of life for a growing number of premature infants. Current treatments target the neovascular stage of disease; however, as a biphasic disease, an ideal therapeutic treatment strategy for ROP would be to target the initial vasodegenerative stage to preserve endothelial cell (EC) function and prevent progression to the sight-threatening proliferative phase of the disease.

The initiating insult in ROP is hyperoxia-induced EC regression and capillary closure, and the mechanisms leading to obliteration of the vessels are believed to be due to EC damage as a result of an increase in oxidative and nitrosative insult.^{7–10} Despite this knowledge, clinical studies using antioxidants have shown limited efficacy, demonstrating a need to clearly define the role of free radicals in the pathogenesis of ROP.

In the healthy vasculature, normal homeostasis is maintained by the vasodilator nitric oxide produced from endothelial nitric oxide synthase (eNOS), which has important anti-apoptotic and survival functions, resulting in vasoprotection.^{11–13} In disease, however, eNOS can become dysfunctional or uncoupled, resulting in the generation of superoxide and less nitric oxide, the combined products of which are the highly damaging free radical peroxynitrite.^{7,14–16} In parallel, there is an increase in other oxidation products of nitric oxide, such as nitrogen dioxide radical concomitant with enhanced NADPH oxidase activity, which together provide additional sources of oxidative free radicals.^{9,17} Overall, this causes a shift in the nitroso-redox balance, resulting in adverse consequences on vessel integrity. In the retinal microcirculation, during development, such an imbalance can be particularly detrimental, leading to a stunting of normal vascular development, vessel closure, and tissue ischemia. Accordingly, limiting reactive oxygen and nitrogen species production preserves EC integrity and reduces subsequent pathological NV.^{7,9,17,18} Previously, we and others have shown a pivotal role for eNOS-derived free radicals in the vasoobliterative phase of ROP.^{7,19} Currently, the mechanisms that lead to disturbed eNOS function and the nitroso-reduction oxidation imbalance are poorly defined. Further definition will aid in the development of therapeutic strategies for the treatment of this sight-threatening disease.

The function of eNOS is critically dependent on the cofactor, (6R)-5,6,7,8-tetrahydrobiopterin (BH4), which facilitates the production of nitric oxide from arginine. In the absence of

sufficient BH4, eNOS becomes dysfunctional. Indeed, there is now a significant amount of evidence to show that altered BH4 bioavailability plays a major contribution to cardiovascular disease.^{16,20–24}

BH4 is synthesized from GTP via a series of enzyme-dependent steps controlled by the first and rate-limiting enzyme, GTP cyclohydrolase (GTPCH), which catalyzes the formation of dihydroneopterin triphosphate from GTP. Accordingly, GTPCH plays a major role in controlling BH4 levels and, thus, NOS function.^{25,26}

Currently, there are no viable knockout models of GTPCH; however, the hyperphenylalaninemia strain (hph1), which has 90% reduction in GTPCH activity, is a well-described alternative that has been used to define the relationship between BH4 bioavailability and eNOS-mediated vascular function in disease.^{20–24,27–29} The hph1 mouse was originally generated by ethylnitrosourea mutagenesis for studies on hyperphenylalaninemia and was found to have a deficiency in BH4 production without effect on phenylalanine hydroxylase, similar to the human condition dopa-responsive dystonia.^{27,29,30} The significant reduction in GTPCH activity and BH4 levels is due to a marked decrease in the steady-state mRNA levels of GTPCH.²⁸ As a result of the deficiency, there is reduced NOS activity in the brain of hph1 animals, which can be improved by BH4 administration.^{31,32} Similarly, with regard to their vascular function, the animals are hypertensive.²⁰ In addition, they also show pulmonary hypertension due to reduced vascular-derived nitric oxide availability and an exaggerated vasoconstrictor response to hypoxia.^{22,23} Moreover, not only does the BH4 deficiency lead to a decrease in NOS activity, it also results in NOS dysfunction, with NOS becoming a significant source of superoxide production in aortas isolated from these animals.²⁰ It has also been suggested that the low levels of BH4 in the hph1 mice make them particularly susceptible to oxidation of this NOS cofactor, which can further exacerbate endothelial dysfunction. Together, these findings show that the hph1 strain is a well-characterized model to study the effects of BH4 deficiency on NOS function. Therefore, by using hph1 mice previously crossbred with wild-type (WT) C57BL6 to generate mice with differing levels of BH4, we were able to investigate the effect of varying levels of BH4 on retinal pathology by comparing WT animals with hph1 heterozygotes (hph1^{+/-}) and hph1 homozygotes (hph1^{-/-}).²²

Materials and Methods

hph1 Colony Breeding

The hph1 mouse strain on a C57BL6 background (a kind gift of Prof. Keith Channon, University of Oxford, Oxford, UK) were used throughout the present study.²² Routine genotyping was conducted as previously described, and breeding pairs of heterozygote animals were used to provide litters of experimental animals containing WT animals,

hph1^{+/-}, and hph1^{-/-}.²² This allowed the use of littermate control animals throughout, removing the confounding effects of experimental variations due to strain or genetic background. The term hph1 refers to previous publications in which only the original homozygotes were used.²⁷⁻²⁹ Animals were screened for the RD8 mutation in the rhodopsin gene and found to be negative. All animal studies were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Mouse Model of OIR

The protocol for oxygen-induced retinopathy (OIR) was that described by Smith et al.³³ This model mimics the two stages of pathology observed in ROP: exposure of mice to hyperoxia between postnatal day 7 (P7) and 12 (P12) retards normal retinal vascular development and causes vasoobliteration of existing retinal vessels, and return of the animals to room air at P12 renders the central retina ischemic and results in preretinal NV with maximal growth observed at P17.^{19,33} On P7, mouse pups and their nursing dams were placed in a chamber in which the oxygen level was maintained at 75% by a PROOX 110 oxygen regulator (Reming Bioinstruments, Redfield, NY). At P12, the mice were returned to room air and tissue was collected or placed with room air-maintained surrogate dams until P17; because nursing mice are adversely affected by hyperoxia, the use of room air surrogates ensures consistent nutrition between litters. Eyes were removed and processed by fixing in 4% paraformaldehyde (PFA) and frozen in optimal cutting temperature (OCT) medium for dividing into sections, or the retina was extracted and snap frozen for later analysis of BH4 levels, NOS activity, or enzyme-linked immunosorbent assay (ELISA). For BH4 and ELISA analysis, lungs, aorta, and serum were also removed and snap frozen immediately.

Assessment of Normal Retinal Development and Vascular Coverage in Retinal Flat Mount Preparations

Freshly isolated eyes were fixed in 4% PFA for 4 hours at room temperature. After washing with phosphate-buffered saline (PBS), the eyes were prepared for retinal flat mount in a Maltese cross conformation.¹⁹ Specimens were permeabilized for 4 hours at 37°C in permeabilization buffer (PBS with 0.5% Triton X-100, 1% normal goat serum, 0.1 mmol/L CaCl₂, and 0.05% NaN₃). Then, they were reacted with biotinylated isolectin B4 (Sigma-Aldrich, Gillingham, UK; 20 µg/mL) in permeabilization buffer overnight at 4°C and washed for 4 hours at 37°C before reaction with Alexa Fluor-488 streptavidin conjugate (Molecular Probes, Paisley, UK; dilution 1:500) in the dark for 3 hours at 37°C. After several further washes, the slides were mounted onto glass coverslips with Vectashield medium (Vector Laboratories, Peterborough, UK). Low-power images of the whole specimen were acquired using a Nikon Eclipse E400

fluorescent microscope fitted with a Nikon DS-i1 camera (Nikon, Kingston Upon Thames, UK).

Vascular coverage at P7 and P12 was determined using Volocity software version 6.3 (PerkinElmer, Waltham, MA). Isolectin-stained vessels were detected in an automated manner, and the total vessel coverage area was measured. For OIR-treated animals, the vascular, avascular, and neovascular areas were demarcated and measured in each quadrant of each retina in square micrometers using the NIS Elements Basic Research software version 3.0. Avascular areas were those that displayed no vessel-positive lectin staining, and neovascular areas were the neovascular tufts extending out of the retina and were more brightly fluorescent than normal vascular areas. We have previously shown that these hyperfluorescent areas correspond to NV tufts extending into the vitreal compartment of the eye.¹⁹ The quantified areas were expressed as a percentage of the total retinal area.

Normal astrocyte coverage was identified using the glial cell marker, glial fibrillary acidic protein, and morphological characterization. Lectin-stained retinal flat mounts were immunostained with anti-glial fibrillary acidic protein antibody (Dako, Cambridgeshire, UK) overnight at 4°C, followed by goat anti-rabbit Alexa Fluor-568 (Molecular Probes), and imaged by confocal microscopy using a collection of z-series images from the nerve fiber layer through the ganglion cell layer to ensure capture of the full astrocyte thickness. For histological examination, eyes from P7 mice were fixed overnight at 4°C in 10% neutral-buffered formalin solution. The specimens were then dehydrated through graded alcohol (50%, 70%, 90%, 95%, and 100%), then cleared in xylene, and immersed in molten wax using an automated processing system. After this, eyes were embedded in fresh wax in moulds. The paraffin blocks were divided into sections (6 µm thick) using a microtome (Reichert-Jung 1130/Biocut; Leica Biosystems, Nussloch, Germany) and floated on a warmed water bath to flatten the sections before placing onto a slide. For hematoxylin and eosin staining, the slides were placed in xylene for 10 minutes to clear the wax, then hydrated through graded alcohol to distilled water before being placed in Harris' hematoxylin for 15 minutes. Slides were then differentiated in acid alcohol, then blued in ammonia water before being placed in eosin for 5 minutes. After washing in distilled water, the specimens were dehydrated and cleared, then a coverslip was mounted with DPX mountant (Sigma-Aldrich). The number of nuclei in the ganglion cell layer was quantified at 200-µm intervals at four points at each side from the center of retinal sections processed for hematoxylin and eosin staining. The number of hematoxylin-stained nuclei in the ganglion cell layer, excluding any peripheral elongated cell nuclei that are likely to be EC nuclei, was determined from 16 sections per eye and from three eyes per group.

Determination of BH4 and BH4/BH2 Ratios

Tissue BH4 and dihydropterin (BH2) levels were measured by high-performance liquid chromatography with fluorescence

detection after differential iodine oxidation in acidic or alkaline conditions.³⁴ Briefly, tissue was homogenized in cold extraction buffer [50 mmol/L Tris-HCl (pH 7.4), 1 mmol/L dithiothreitol, and 1 mmol/L EDTA] and centrifuged at $16,060 \times g$ for 15 minutes at 4°C. The samples were then subjected to differential oxidation. To determine total biopterin (BH4, BH2, and oxidized biopterin), half of the sample was subjected to acid oxidation by the addition of a 1:1 mixture of 1.5 mol/L HClO₄ and 2 mol/L H₃PO₄ to the samples to yield a final concentration of 7.5 and 10 mmol/L, respectively, before adding 1% iodine in 2% KI solution. To determine BH2 and oxidized biopterin, the other half of the sample was subjected to alkali oxidation by the addition of 10 mmol/L NaOH before receiving the 1% iodine/2% KI solution. After incubation at room temperature for 1 hour in the dark, alkaline oxidation samples were acidified by the addition of 167 mmol/L H₃PO₄. Iodine was reduced in both groups by the addition of 5 mmol/L freshly prepared ascorbic acid before centrifugation at $16,060 \times g$ for 5 minutes. The supernatant was then ready for injection into a 250-mm-long, 4.6-mm-inner diameter ODS column (5- μ m particle size; Phenomenex, Cheshire, UK) with a methanol-water (8:92, v/v) mobile phase containing 15 mmol/L potassium phosphate buffer (pH 6.4) at a flow rate of 0.5 mL/minute. Fluorescence detection (350-nm excitation and 450-nm emission) was performed using a fluorescence detector (model RF20AXS; Shimadzu, Buckinghamshire, UK). Stock solutions of BH4 (Schrick's Laboratories, Jona, Switzerland) were prepared in oxygen-free water and stored at -80°C. For each experiment, serial dilutions of BH4 stock were performed using freshly prepared extraction buffer to yield standards of 10, 5, 2.5, 1.25, and 0 ng/mL BH4. BH4 concentrations, expressed as nanograms per milligram of protein, were calculated by subtracting BH2 plus oxidized biopterin from total biopterins. BH4 levels and BH4/BH2 ratios were determined by high-performance liquid chromatography from a variety of tissues, including retina, lung, and aorta of animals after 5 days of hyperoxia (Supplemental Figure S1) or short-term exposure (6 hours) for vascular perfusion analysis (lung).

NOS Activity Assay

NOS activity was determined by the conversion of radio-labeled arginine to citrulline using a NOS Activity Assay Kit (Cayman, Ann Arbor, MI) with modifications as described previously.³⁵ NOS activity assays can be confounded by the presence of arginase in the sample, which competes with NOS for arginine (Supplemental Figure S2).^{35,36} Therefore, to ensure measurements of NOS activity only, pooled mouse retinas were homogenized in buffer containing 5 μ mol/L arginase inhibitor, (S)-(2-boronoethyl)-L-cysteine-HCl (BEC; Calbiochem/EMD Chemicals, Gibbstown, NJ), and added to the reaction mixture containing 0.05 μ Ci [¹⁴C] arginine (Amersham Biosciences, Piscataway, NJ) and incubated for 1 hour at 37°C. After incubation, ice-cold HEPES buffer containing EDTA (pH 5.5) was added and

unreacted arginine and ornithine removed by filtration through a spin column before determination of [¹⁴C]-L-citrulline levels. For each sample, background was determined by incubation in the presence of L-NNA (L-N^G-Nitroarginine), further confirming NOS activity, and subtracted from the total counts (Supplemental Figure S3). To provide a true reflection of tissue BH4 levels, assays were conducted in the absence of the additional BH4 normally used in such assays. The BH4 dependency of the NOS activity assay was further validated by demonstrating that the reduced NOS activity in the hph1 groups was reversed by BH4 supplementation (Supplemental Figure S4). For NOS activity, five to six neonatal retinas from three independent OIR experiments were pooled and assayed in triplicate.

Western Blot Analysis

Retinas were dissected from freshly enucleated eyes, snap frozen individually in liquid nitrogen, and stored at -80°C until use. Pooled retinas ($n = 4$ to 5) were homogenized in ice-cold extraction buffer [1% Igepal (Sigma-Aldrich), 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 100 mmol/L Tris-HCl (pH 7.4)] with protease inhibitors (Complete Mini EDTA free protease inhibitor cocktail; Roche Applied Science, Penzberg, Germany) on ice for approximately 3 minutes and then rotated for 1 hour at 4°C. Equivalent amounts of protein sample were separated on a 9% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and immunoblotted with anti-eNOS, neuronal NOS (nNOS; BD Biosciences, San Jose, CA), or anti-nitrotyrosine (Cayman) antibodies or, to verify equivalency of loading, anti- β -actin (Sigma). The membrane-bound protein was detected by enhanced chemiluminescent detection, and band densitometry was performed.

Preparation of Retinal Tissue Sections for Quantification of Reactive Oxygen and Nitrogen Species and VEGF Production

Freshly isolated eyes embedded in OCT compound (Tissue Tek; Fisher Scientific, Waltham, MA), frozen using liquid nitrogen-cooled isopentane, and stored at -80°C were used. Before dividing into sections, OCT blocks were removed from -80°C storage and allowed to equilibrate in the cryostat to -20°C, and the eyes were divided into sections (10 μ m thick) using a Leica Biosystems cryostat maintained at -20°C and collected on Superfrost plus slides (Fisher Scientific, Loughborough, UK).

DHE as an *in Situ* Fluorescent Indicator of ROS Production

Dihydroethidium (DHE) was used to quantify reactive oxygen species (ROS) production, as described previously, and the specificity of the reaction and enzymatic source of ROS production was confirmed by inhibition of the

Figure 1 Quantification of (6R)-5,6,7,8-tetrahydrobiopterin (BH4) levels verifies reduced production in the retinas of 5- to 6-month-old adult hyperphenylalaninemia strain (hph1)^{+/-} and hph1^{-/-} mice. Biopterin levels were measured by high-performance liquid chromatography. There is an incremental reduction in BH4 levels in retinal (A) and lung (B) samples from hph1^{+/-} and hph1^{-/-} animals compared with wild type (WT). Dihydropterin (BH2) levels are not significantly different between groups; therefore, the BH4/BH2 ratios are decreased in a similar manner as the BH4 levels. **P* < 0.05, ***P* < 0.01. *n* = 3 individual pools containing two retinas each (A); *n* = 4 independent lung samples (B).

DHE fluorescence with the inhibitors polyethylene glycol-superoxide dismutase (SOD) and *N*_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME).³⁵ Briefly, a ring was drawn around fresh frozen retinal sections using a Dako pen (Dako) and allowed to dry. Sections were incubated with inhibitor compounds for 30 minutes at room temperature: 1 mmol/L L-NAME (Sigma) or 400 U/mL SOD (Sigma). DHE stock solutions (10 mmol/L) were diluted to 10 μmol/L with the same inhibitors and incubated with tissue sections for 1 hour at 37°C in a dark, humidified chamber. Slides were washed before viewing using a confocal microscope with similar laser settings maintained throughout collection of all images. Retinal section area was demarcated and fluorescence intensity quantified by automated image analysis using ImageJ software version 1.47 (NIH, Bethesda, MD). For each group, a mean fluorescence value was determined from eight images per eye, and three eyes were assayed per experimental group from three independent OIR experiments.

Dopamine ELISA

Retinal samples were homogenized in lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 1% Igepal CA-630] containing 10 mmol/L ascorbic acid and 0.1 mmol/L EDTA. Lysates were then centrifuged for 5 minutes at 1000 × *g*, and supernatants were assayed for dopamine using an ELISA kit (Labor Diagnostika Nord, Nordhorn, Germany) and following the manufacturer's instructions. Briefly, samples along with standards were added to the extraction plate for 1 hour at room

temperature before derivatization to an acylated form. Standards, controls, and samples, similarly processed, were then added to precoated dopamine microtiter strips, and dopamine antiserum was added. After overnight incubation at 4°C and several washes, the amount of bound dopamine antiserum was detected using horseradish peroxidase-conjugated antibody and the substrate 3,3',5,5'-tetramethylbenzidine and read on a microplate reader (BMG Labtech, Ortenberg, Germany) at 450 nm, with correction at 650 nm. Sample dopamine concentrations were determined by reference to the standards provided.

VEGF ELISA

Pooled retinas or lung samples from P7, P12 room air control (RAC), or OIR-treated mice were lysed by two freeze-thaw cycles and homogenized in PBS containing protease inhibitors. The homogenates were then centrifuged for 5 minutes, and VEGF in the supernatant was determined by ELISA (R&D Systems, Abingdon, UK), following the manufacturer's instructions. Serum samples were diluted fivefold using calibrator diluent supplied with the kit before assay.

VEGF Immunocytochemistry

OCT-embedded, frozen retinal sections were fixed for 30 minutes in 4% PFA, then rinsed with PBS before being permeabilized with 0.2% Triton X-100 in PBS. Sections were then blocked using 1% bovine serum albumin and 2% goat serum in PBS for 1 hour. Primary antibody, anti-VEGF (Santa Cruz Biotechnology, Dallas, TX), was then applied diluted 1:50 in 2% goat serum overnight at 4°C. Sections were washed three times with PBS containing 0.1% Triton X-100, then incubated with secondary antibody, Alexa Fluor goat anti-rabbit 568 (Life Technologies, Paisley, UK; A11036) diluted 1:500 for 1 hour. After three further washes, sections were incubated with DAPI for 20 minutes for nuclear staining and rinsed with PBS before being mounted with Vectashield mounting medium (H-1000).

ConA Staining for Analysis of Perfusion

At P7, pups were placed into hyperoxia, as described above, for 6 hours before perfusion for 5 minutes with 50 μg/mL rhodamine red-conjugated concanavalin A (ConA) lectin (Dako). After removal, eyes were fixed in 4% PFA solution for 4 hours, and retinas were dissected for flat mounting, labeled with isolectin, and detected with Alexa Fluor-488-conjugated streptavidin, to visualize all retinal vessels. Total vascular coverage was calculated from isolectin (green) stained vessels using NIS Elements software automated to detect by staining intensity. The amount of vascular perfusion was estimated from the amount of dual rhodamine labeling (red) and isolectin (green) positive vessels, and this value was subtracted from the total vascular coverage measurement to yield the area of non-perfusion [isolectin (green) positive and ConA (red) negative stained vessels].

Figure 2 Normal vascular development in hyperphenylalaninemia strain (*hph1*) mice from postnatal day 7 (P7) retinas. Assessment of normal vascular development and retinal phenotype shows no significant morphological deviation in *hph1* mice compared to wild type (WT) at P7. Retinal vasculature was visualized with lectin from P7 *hph1* mice and shows normal vascular development. **A:** Representative retinal quadrant images from WT, *hph1*^{+/−}, and *hph1*^{−/−} groups. **B:** High-power images showing retinal vascular branching. **C:** Glial fibrillary acidic protein (GFAP)—positive astrocyte coverage is also normal in developing *hph1* retinas. Lectin, green; GFAP, red. **D:** Retinal layer specification in hematoxylin and eosin—stained sections from paraffin-embedded eyes of P7 mice demonstrates no genotype-specific differences. *N* = 5 or more retinas per group (**C**). Scale bars: 100 μ m (**B** and **C**); 25 μ m (**D**). GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer.

Statistical Analysis

Statistical analysis was performed by one-way analysis of variance, followed by Bonferroni's multiple-comparison post

hoc test. All data are presented as means $\pm$ SEM from three to six individual OIR experiments, unless otherwise indicated.

Results

Retinas of *hph1*^{+/−} and *hph1*^{−/−} Animals Display Incremental Decreases in BH4 Levels Compared to WT Controls

In retinal samples from 5- to 6-month-old adult animals, as expected, there was an incremental decrease in BH4 levels from WT, through *hph1*^{+/−} to *hph1*^{−/−} retinas (Figure 1). Levels of BH2, the major oxidation product of BH4, were similar in all groups, yielding a similar BH4/BH2 ratio profile to that observed for the differences in BH4 for all groups. Taken together, this confirmed a diminished ability to produce BH4 in the *hph1* groups.

Inner Retinal Vascular Development in GTPCH-Deficient *hph1* Mice

ROP primarily affects the inner retinal vasculature with maximal impact of hyperoxia-induced damage observed when exposure is initiated at P7. Therefore, before subjecting the animals to OIR, it was important to investigate superficial vascular development at P7 (Figure 2). Analysis of the spreading of the vascular plexus in flat-mounted retina showed that the *hph1* genotype did not have any significant effect on normal retinal vascular development in any of the genotypes (Figure 2, A and B). Vascular coverage at P7 and P12 did not vary significantly between genotypes at P7 or P12. Astrocyte coverage was also normal and displayed the typical stellate morphology and close apposition with the retinal vessels (Figure 2C). Retinal cross-section and subsequent hematoxylin and eosin staining confirmed similar degrees of retinal layer compartmentalization in all genotypes (Figure 2D).

Hyperoxia Depletes Tissue BH4 Levels in Neonatal Animals at P12

When comparing across genotypes, BH4 levels in all tissue tested were significantly lower in *hph1*^{+/−} and *hph1*^{−/−} animals compared to littermate controls (Figure 3). In the neonatal retinas, the levels of BH4 were much lower than in adult retinal samples, and this is likely to be due to the developmental increase in liver GTPCH levels previously observed in these animals.²⁸ In these studies, a developmental change in GTPCH and, thus, BH4 levels has been described in the *hph1* animals, with a marked increase in hepatic GTPCH activity and BH4 levels observed after weaning (3 to 4 weeks), but still reduced compared to controls.²⁸ This may account for the marked differences we observed in the adult retina compared to the neonatal retinal levels. Compared to age-matched RACs, all groups showed a significant depletion of BH4 levels after hyperoxia, which

Figure 3 Comparison of (6R)-5,6,7,8-tetrahydrobiopterin (BH4) and BH4/dihydrobiopterin (BH2) level measurements in tissues from hyperphenylalaninemia strain (hph1) animals at postnatal day 12 (P12) after exposure to oxygen-induced retinopathy (OIR). Biopterin levels were measured by high-performance liquid chromatography from hph1 P12 OIR and room air control (RAC) tissue samples. **A:** There is a significant reduction in BH4 level seen in retinal samples from hph1^{-/-} and hph1^{+/-} animals compared with wild type (WT). BH4 level in OIR-treated animals is also significantly reduced compared to RAC for all groups. The BH4/BH2 ratios show a similar pattern reflecting unchanged BH2 levels. **B and C:** There is a significant reduction in BH4 level seen in aorta (**B**) and lung (**C**) samples from hph1^{-/-} and hph1^{+/-} animals compared to WT. BH4 level in OIR-treated animals is also significantly reduced compared to RAC in both WT and hph1 animals. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. $n = 2$ individual experiments, each containing five pooled retinas (**A**); $n = 3$ aortas (**B**); $n = 3$ lung samples (**C**).

was decreased in accord with GTPCH expression levels in WT, hph1^{+/-}, and hph1^{-/-} in all tissues tested. For example, in lung samples from WT mice, BH4 level changed from 3.97 ± 0.37 in RAC animals to 1.43 ± 0.19 after OIR, in hph1^{+/-} pups from 2.67 ± 0.26 animals to 1.39 ± 0.32 , and in hph1^{-/-} pups from 0.4 ± 0.03 animals to 0.27 ± 0.06 . Similarly, in retinal samples, WT pups' BH4 levels decreased from 2.6 ± 0.01 in RAC animals to 1.68 ± 0.15 after OIR, in hph1^{+/-} pups from 1.35 ± 0.08 to 1.03 ± 0.03 , and in hph1^{-/-} pups from 1.11 ± 0.06 to 0.61 ± 0.003 . There was a small, but not significant, change in BH2 levels after hyperoxia; therefore, the overall ratio of BH4/BH2 decreased, consistent with the incremental decrease in BH4 levels between genotypes.

Hyperoxia-Induced Vaso-Obliteration Is Reduced in hph1 Mice

Next, we investigated the impact of hyperoxia-induced BH4 depletion on retinal vascular integrity by examining the extent of vascular closure at P12 after 5 days of hyperoxia. In WT mice, exposure to hyperoxia typically produces a significant avascular area in the central retina, representing the combined impacts of vaso-oblivation and retarded development (Figure 4A). In the hph1 animals, the avascular area was markedly reduced compared to the WT control and reflected the stepwise decreases in BH4 in hph1^{+/-} and hph1^{-/-} groups (Figure 4C). RAC animals showed typically complete vascular coverage at P12 (Figure 4B).

NOS Activity Is Dependent on BH4 Availability in P12 Retinas

NOS activity was measured by the conversion of radio-labeled arginine to citrulline using a modified NOS activity assay (Supplemental Figure S2 and Figure 5). Nitric oxide is synthesized from NOS by the enzymatic oxidation of the N-terminal guanidino group of L-arginine. This amino acid is also a substrate for the enzyme arginase. Previously, we and others have shown that during tissue preparation, arginase in the sample can reduce the amount of radiolabeled arginine, leading to problems interpreting the measurement of NOS conversion. This occurs such that in the absence of the arginase inhibitor, BEC, radiolabeled arginine is converted to citrulline plus urea and is, thus, a measure of NOS plus arginase enzymatic activity. Indeed, in the absence of BEC, we observed no measureable difference in the conversion rates between any of the genotype groups (Supplemental Figure S2). However, when the inhibitor is added, the expected difference in NOS activity between groups is observed. Arginase is known to be up-regulated during OIR; therefore, to ensure we were determining NOS activity, all experiments were performed in the presence of arginase inhibition.³⁷ In parallel with the reduced endogenous BH4 levels in the hph1 groups, there were corresponding decreases in NOS activity when compared to WT littermate controls: NOS activity was 171.4 ± 9.3 in WT and reduced to 128.1 ± 0.5 in hph1^{+/-} and 97.0 ± 0.2 in hph1^{-/-} groups (Figure 5A). These values were further reduced after hyperoxia treatment: in WT samples, NOS activity was 28.1 ± 4.3 compared to 15.7 ± 6.6 in hph1^{+/-} and 11.2 ± 2.2 in hph1^{-/-} (Figure 5B). Comparison of eNOS and nNOS protein levels (inducible NOS was not detectable) in the same samples showed no change in NOS protein level in any of the genotypes (Figure 5) and confirmed that the hyperoxia-induced effect on NOS (eNOS plus nNOS) was independent of changes in NOS protein levels. Nitrotyrosine (NT) levels, in contrast, showed evidence of a reduction in the hph1^{+/-} and hph1^{-/-} groups, which was particularly pronounced in the hyperoxia-exposed groups.

Figure 4 Hyperphenylalaninemia strain (hph1) retinas are protected from hyperoxia-induced vascular damage: reduced vaso-obliteration at postnatal day 12 (P12) after oxygen-induced retinopathy (OIR) in hph1 mice. Extent of vascular closure at P12 was examined in flat-mounted retina stained with lectin. **A:** Vascular areas are outlined in images from flat-mounted retina of wild-type (WT) and hph1 mice. **B:** Room air control (RAC) animals show normal vascular coverage at P12. **C:** There is significantly less vaso-obliteration in the hph1^{-/-} mice compared to both WT and hph1^{+/-} animals. **P* < 0.05, ***P* < 0.01. *n* = 3 independent litters (C). Scale bar = 1 mm.

Reactive Oxygen and Nitrogen Species Production Is Altered in P12 Retinas after Hyperoxia

Because BH4 insufficiency can result in increased oxidative stress, the production of reactive oxygen and nitrogen species was measured in P12 retinas after hyperoxia using fresh frozen retinal sections of the two genotypes showing the most extreme differences in all parameters: the WT and hph1^{-/-} groups. *In situ* free radical production was assayed with the oxidative fluorescent dye, DHE, in the presence and absence of the inhibitors polyethylene glycol–SOD and L-NAME. In the WT group, there was a significant inhibition in DHE fluorescence after SOD and L-NAME treatment, confirming that the free radical was superoxide and that dysfunctional NOS represented its source (Figure 6A). Retinal DHE fluorescence was significantly reduced in the hph1^{-/-} retinas compared to WT controls (726.7 ± 141 in WT compared with 423.3 ± 35 in hph1^{-/-} animals). In contrast to WT mice, L-NAME had no significant effect on DHE fluorescence in hph1^{-/-} retinas (L-NAME incubation reduced WT levels to 447 ± 206 and hph1^{-/-} levels to 452 ± 77), whereas polyethylene glycol–SOD reduced DHE fluorescence in WT

and hph1^{-/-} retinas by a similar proportion, 338 ± 51 and 271 ± 18.4 , respectively. Taken together, these results indicate a global diminution in retinal NOS activity regarding its enzymatic capacity to produce nitric oxide or superoxide in the hph1^{-/-} group. In agreement with this, NT, the stable end product of tyrosine nitration and indicative of peroxynitrite production, was similarly reduced in the hph1^{-/-} group compared to the WT group (Figure 6B): 24.02 ± 3.8 and 51.7 ± 14 , respectively.

Retinal Dopamine Levels in P12 hph1 Retinas before and after Hyperoxia Exposure

In addition to being a cofactor for the NOS isoenzymes, BH4 is also a cofactor for the aromatic hydroxylases: phenylalanine, tyrosine, and tryptophan hydroxylase. Tyrosine hydroxylase is the rate-limiting enzyme in the synthesis of dopamine, the most abundant and important catecholamine in the retina, which plays a significant role in coupling light detection to a neurochemical output.^{38,39} Therefore, we measured retinal dopamine levels by ELISA (Figure 7) in P12 RAC and P12 hyperoxia-treated animals. Correlating with the incremental decrease in BH4 levels across genotypes, there was a similar stepwise decrease in dopamine levels in the RAC P12 retinas: 3.24 ± 0.02 in WT compared to 2.98 ± 0.03 in hph1^{+/-} and 2.51 ± 0.03 in hph1^{-/-}. Again, in parallel with the greatly reduced BH4 bioavailability after hyperoxia, there was also a further decrease in retinal dopamine levels after hyperoxia in all

Figure 5 Nitric oxide synthase (NOS) activity is significantly lower in postnatal day 12 (P12) hyperphenylalaninemia strain (hph1) retinas and further reduced after hyperoxia despite comparable levels of NOS protein levels. **A:** P12 room air control (RAC) retinal protein samples show no significant difference in endothelial NOS (eNOS), neuronal NOS (nNOS) protein, or nitrotyrosine (NT) between wild-type (WT) and hph1 groups. In contrast, NOS activity is significantly reduced across genotypes, consistent with the observed decrease in (6R)-5,6,7,8-tetrahydrobiopterin (BH4) levels. **B:** Similarly, in the P12 oxygen-induced retinopathy-treated samples, there is a further decrease in NOS activity reflecting the additional hyperoxia-induced depletion of BH4. NOS activity was measured from freshly isolated retinas by conversion of radiolabeled arginine to citrulline. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. *n* = 5 pooled retinas assayed in triplicate (A and B).

Figure 6 Reduced superoxide production and nitrotyrosine (NT) immunofluorescence in hyperphenylalaninemia strain (hph1^{-/-}) postnatal day 12 (P12) oxygen-induced retinopathy (OIR) retinal sections. **A:** Investigation of *in situ* superoxide generation in hph1 retinal sections at P12 using the oxidative fluorescent dye dihydroethidium (DHE), with addition of inhibitors *N*_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) and polyethylene glycol (PEG)–superoxide dismutase (SOD) in sequential sections. Significantly reduced superoxide is detected in hph1^{-/-} retinal sections compared to wild type (WT). The nitric oxide synthase dependence of this superoxide generation in the WT is indicated by the significant inhibition by L-NAME. **B:** NT fluorescence intensity was measured in retinal sections from WT and hph1^{-/-} mice after OIR at P12. Quantification of NT immunoreactivity shows significantly greater NT in WT retinal sections than hph1^{-/-} sections. **P* < 0.05. *n* = 3 eyes. Scale bar = 100 μm. AU, arbitrary unit.

three groups: 2.18 ± 0.01 in WT compared to 1.98 ± 0.01 in hph1^{+/-} and 2.03 ± 0.04 in hph1^{-/-}.

VEGF Levels Are Elevated in hph1 Retinas and Remain Elevated after Exposure to Hyperoxia

Next, we determined if the increased vasoprotection in the hph1 groups was due to increased levels of the important EC survival factor, VEGF.^{2,3} Compared to WT mice, retinal VEGF protein levels were significantly higher in the hph1 groups at P12 without hyperoxia exposure, 79.35 ± 5.1 in WT compared to 110 ± 7.6 in hph1^{+/-} and 132.8 ± 0.48 in hph1^{-/-} (Figure 8A). After 5 days of hyperoxia, levels remain elevated: 69.6 ± 0.72 in WT compared to 81.5 ± 1.1 in hph1^{+/-} and 88.1 ± 2.1 in hph1^{-/-}; these values paralleled the enhanced vascular preservation observed at P12 in these groups after hyperoxia exposure. VEGF immunocytochemistry in retinal sections demonstrated predominant localization to the retinal ganglion cell layer (Figure 8, B and C) and quantification confirmed the ELISA results: 185 ± 16.6 in WT P12 OIR-treated animals compared to 268 ± 7.9 in hph1^{+/-} and 306 ± 2.6 in hph1^{-/-}.

Retinal-Specific VEGF Levels Are Elevated in the Inner Nuclear Layer at P7 in hph1^{-/-} Animals and Correlate with Reduced Vascular Closure after 6 Hours of Hyperoxia

Vascularization of the inner retina is driven by VEGF gradients produced by retinal ganglion cells in response to the increasing neural activity of a developing phototransduction cascade.^{40,41} Thus, having observed elevated VEGF levels at

P12, next we examined if this was also elevated at the earlier time point of P7, before the animals are exposed to hyperoxia. In agreement with the P12 observation, VEGF immunoreactivity in the retinal ganglion layer was also elevated at P7 (Figure 9A) in the hph1^{-/-} group compared to control: 128 ± 10.8 in WT compared to 185 ± 21 in hph1^{+/-} and 252 ± 22 in hph1^{-/-}. The greater VEGF levels in the hph1^{-/-} and hph1^{+/-} groups correlated with a higher density of cell nuclei in the ganglion cell layer (Figure 9B) at P7, which was consistently elevated throughout the entire retina.

To determine the vasoprotective impact of the elevated VEGF, animals were exposed to 6 hours of hyperoxia and the extent of vessel closure was determined by ConA perfusion (Figure 9D). The higher VEGF levels in the hph1^{-/-} group correlated with significantly fewer areas of vascular non-perfusion than in WT animals: 59.0 ± 5.5 in hph1^{-/-}, 100.0 ± 11.6 in hph1^{+/-}, and 110.6 ± 8.5 in WT. At the same time point, VEGF was quantified in serum, lung, and retinal samples by ELISA, revealing a tissue-specific increase in retinal VEGF levels, which was not evident in the serum or lung samples. Retinal levels were 68.5 ± 0.55 in WT compared to 81.96 ± 2.5 in hph1^{+/-} and 121.4 ± 5.6 in hph1^{-/-}; serum levels were 13.4 ± 1.2 in WT compared to 8.1 ± 1.7 in hph1^{+/-} and 6.3 ± 0.85 in hph1^{-/-}; and lung sample levels were 119.8 ± 2.6 in WT compared to 112.2 ± 4.5 in hph1^{+/-} and 97.5 ± 1.5 in hph1^{-/-} (Figure 9C). Quantification of BH4 levels in parallel lung samples confirmed a similar incremental decrease in BH4 tissue levels across genotypes to that described for the P12 groups (data not included). Thus, taken together, these results suggest a developmental increase in inner retinal VEGF levels that facilitates vessel protection through hyperoxic exposure.

Figure 7 Retinal dopamine levels are significantly reduced in hyperphenylalaninemia strain ($hph1^{-/-}$ and $hph1^{+/-}$ mice compared to wild-type (WT) controls and decreased in all groups after hyperoxia. Dopamine was measured in retinal samples from $hph1$ mice at postnatal day 12 in room air controls (RACs) and oxygen-induced retinopathy (OIR)-treated mice by enzyme-linked immunosorbent assay. There is a significant decrease in dopamine level in $hph1^{+/-}$ and $hph1^{-/-}$ retinas compared to both WT and in $hph1^{-/-}$ compared to $hph1^{+/-}$ group. Pooled samples of four retinas were assayed in duplicate. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Neovascular Response in the Proliferative Stage of ROP Is Reduced in Animals with the $hph1$ Genotype

The pathology of ROP occurs in two phases: with hyperoxia-induced vascular closure, resulting in retinal ischemia and sight-threatening NV. The proliferative stage of ROP is the most damaging for sight; therefore, it was important to show the relationship between early vascular preservation during hyperoxia and progression to the vasoproliferative stage of disease. In the murine OIR model, the neovascular response is maximal 5 days after return to room air at P17 and is measurable as the area of neovascular tuft formation. Thus, at P17, 5 days after return to room air, the neovascular regions were demarcated and quantified in retinal flat mounts (Figure 10, A and B). We found significantly less formation of neovascular tufts in $hph1^{+/-}$ and $hph1^{-/-}$ mice ($3.9\% \pm 0.7\%$) compared to WT ($7.8\% \pm 1.4\%$) controls, corresponding to the decreased

levels of vaso-oblivation in these groups at P12. Retinal VEGF protein levels measured by ELISA were not significantly different across genotypes: 61 ± 15.4 in WT compared to 67 ± 9.2 in $hph1^{+/-}$ and 68 ± 2.3 in $hph1^{-/-}$ (Figure 10C). Together, these results demonstrate a reduced hypoxic drive in the $hph1$ groups that would have arisen from enhanced vasculature preservation at P12.

Discussion

eNOS-derived nitric oxide plays an important role in maintaining vascular homeostasis; thus, any alterations in the bioavailability of its essential cofactor, BH4, have serious consequences for EC function and vascular integrity. Importantly, in the absence of sufficient BH4, eNOS becomes dysfunctional, producing superoxide in place of nitric oxide, diminishing nitric oxide function, and elevating the levels of ROS and reactive nitrogen species.^{14,15} During development of the retinal microcirculation, such a nitroso-reduction oxidation imbalance can be particularly detrimental, leading to damage to the endothelium, vascular closure, and subsequent tissue ischemia. GTPCH is the major rate-limiting enzyme controlling the production of BH4; thus, alterations to the activity of this protein, as occurs in $hph1$ mutant mice, have a significant impact on the availability of BH4, and GTPCH-deficient $hph1$ mice have played an important role in demonstrating the importance of BH4 to eNOS-mediated vascular function.^{20,22,23} Thus, our aim was to investigate the effect of hyperoxia on the bioavailability of BH4 in the neonate and examine the consequences on NOS function in the murine model of OIR using the well-characterized $hph1$ strain.

Initially, we quantified the levels of BH4 in retinal tissue of adult animals and confirmed incremental decreases in BH4 levels in tissue from $hph1^{+/-}$ and $hph1^{-/-}$ compared to their WT counterparts. Next, we investigated the effect of BH4 deficiency on vascular development in $hph1$ animals. Normal retinal vascular development occurs postnatally in

Figure 8 Vascular survival at postnatal day 12 (P12) is associated with an incremental increase in vascular endothelial growth factor (VEGF) levels in $hph1^{+/-}$ and $hph1^{-/-}$ retinas compared with wild type (WT). **A:** VEGF levels were measured in retinal samples at P12 in room air control animals (RACs) and after oxygen-induced retinopathy (OIR) by enzyme-linked immunosorbent assay. **B:** VEGF immunolabeling is localized predominantly to the ganglion cell layer (GCL). **C:** Quantification of VEGF in GCL shows a significant increase in VEGF in this region in $hph1$ retinal sections compared to WT. * $P < 0.05$, *** $P < 0.001$. $n = 2$ individual experiments, each containing three pooled retinas (**A**); $n = 3$ eyes from three independent OIR experiments (**C**). AU, arbitrary unit; IPL, inner plexiform layer.

Figure 9 Enhanced ganglion cell layer (GCL) vascular endothelial growth factor (VEGF) in hyperphenylalaninemia strain (hph1^{+/-}) and hph1^{-/-} animals at postnatal day 7 (P7) correlates with reduced vascular closure in response to short-term hyperoxia (6 hours). **A:** VEGF immunohistochemistry in P7 retinal sections shows increased VEGF localization to the GCL in hph1^{-/-} animals compared to wild type (WT). **B:** Cell density in the GCL in hph1^{+/-} and hph1^{-/-} animals is significantly increased in both central and peripheral regions compared to WT. **C:** VEGF levels were measured at P7 after 6 hours of hyperoxia, in serum, lung, and retinal samples by enzyme-linked immunosorbent assay. **D:** Green biotinylated isolectin B4-stained vessels lacking corresponding red concanavalin A staining indicate nonperfusion, and are reduced in hph1^{-/-} animals. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. *n* = 3 eyes (**A**); *n* = 3 eyes per group (**B**); *n* = 3 (**C**, serum and lung); *n* = 2 pools of two retinas assayed in duplicate (**C**, retinal samples); *n* = 3 individual litters (**D**). AU, arbitrary unit; IPL, inner plexiform layer.

mice, with migration of the superficial vascular plexus complete at P7. This is also the stage of development when the mice are most susceptible to oxygen-induced damage; therefore, it was important to ensure that there were no major differences in vascular development between WT and hph1 mice. In this regard, we found that the superficial vascular plexus developed normally in the hph1 mouse, with no difference in the peripheral extent of the retinal vasculature between the hph1 animals and WT.

Having established that there were no adverse consequences of the hph1 phenotype on vascular development, we then sought to analyze the effect of hyperoxia on BH4 levels. The greatest impact of hyperoxia on BH4 levels was measured in lung tissue, a phenomenon that was possibly related to the highly vascularized nature of the tissue or its role as the primary interface with the hyperoxic atmosphere. BH4 was decreased in each of the three genotypes studied, with the maximal reduction in the WT mice. Interestingly, when BH4 was depleted by hyperoxia, there was no corresponding significant increase in BH2 levels, suggesting BH4 was oxidized to another product or that production was inhibited.¹⁶ Thus, the BH4/BH2 ratios mirrored the incremental decrease in BH4 across the three groups. An important advantage of our study was that we used mice previously

backcrossed onto a C57 background—vascular development is well characterized in this strain, as is their responsiveness to the OIR model.^{19,33} Moreover, whenever possible, all experiments were performed with littermate WT controls, further reducing the likelihood of variations in genetic background confounding our results.

Next, we investigated the effect of BH4 deficiency on vascular integrity in OIR. BH4 deficiency can result in two possible outcomes: eNOS may become dysfunctional and increase superoxide production, resulting in increased oxidative stress; alternatively, total NOS activity could be attenuated, resulting in lower nitric oxide and superoxide levels, with insufficient concentrations of these radicals to combine and produce peroxynitrite.

Previously, we have shown that eNOS-derived reactive oxygen or nitrogen species production exacerbates vascular closure in response to hyperoxia, which we hypothesized was due to limited BH4 bioavailability in hyperoxia.¹⁹ Indeed, consistent with this notion, in the WT group of the present study, there was a direct correlation between the extent of BH4 depletion and the following changes: defective NOS activity after hyperoxia, increased ROS and NT levels, and enhanced vaso-oblivation. Taken together, these alterations indicate insufficient BH4 for normal NOS

Figure 10 Ischemia-induced neovascularization (NV) formation is significantly reduced in hyperphenylalaninemia strain (hph1) retinas in the neovascular phase of oxygen-induced retinopathy (OIR). Retinas from postnatal day 17 (P17) mice were flat mounted and lectin stained to investigate vascular growth after OIR. **A:** Representative images of retinal flat mounts from wild-type (WT) and hph1 mice showing neovascular areas (arrows). **B:** The extent of NV is reduced in hph1 mice compared with WT. **C:** Retinal vascular endothelial growth factor (VEGF) was measured by enzyme-linked immunosorbent assay from P17 OIR retinal samples. There is no significant difference between WT and hph1 mice. $**P < 0.01$. $n = 4$ individual litters (**B**); $n = 2$ pooled retinal samples of three retinas each (**C**). Scale bar = 1 mm.

function and provide further evidence for hyperoxia-depleted BH4 bioavailability as a causative factor in the vasodegenerative phase of ROP.

Surprisingly, when BH4 levels were limited even further, as was the case in the hph1 groups, we obtained results opposite to those found in other studies with these animals and seemingly consistent with the second of the two scenarios proposed above: a reduction in global NOS activity and correspondingly less vaso-obliteration in the hph1 groups compared to WT. Moreover, in accordance with a decrease in vessel closure in this group, there was also a decrease in the severity of NV growth. These outcomes were considered suggestive of less peroxynitrite-induced damage to the vasculature. This, however, is in contrast to findings observed in other studies in which there is usually a worsening of pathology in the hph1 groups (eg, their pulmonary hypertension is associated with greater vascular-derived oxidative stress).^{22,23,42} One of the studies showed that the pulmonary hypertension in hph1s was present from birth and that neonates gained weight more slowly when compared to control animals on a CBA genetic background.⁴² In our hands, however, there were no differences in body weights between any of the genotypes. Moreover, because low birth weight and poor nutrition are significant risk factors for ROP, which is associated with exacerbated NV, the findings that body weights were equivalent and that there was a lessening of disease severity, rather than a worsening, suggest that the nutritional status was similar between groups.⁴³ Thus, taken together, our findings suggest the possibility of an alternative mechanism that aided vascular preservation.

VEGF is an important EC survival factor, which is known to be protective in the hyperoxic retina.^{2,3} Accordingly, because VEGF production is driven by hypoxia, hyperoxia exposure inhibits production of this important growth factor, resulting in vascular regression.³ Conversely, enhancing VEGF levels during the vasodegenerative phase is protective. Moreover, in addition to reducing VEGF levels, hyperoxia-induced peroxynitrite production from dysfunctional NOS reduces the pro-survival effects of VEGF, resulting in further EC damage.^{44,45} Thus, next, we sought to investigate if there was a correlation between increased VEGF levels and vasoprotection. This was indeed the case, with significantly elevated levels observed in the hph1 groups, which, although reduced by hyperoxia, remained elevated compared to WT controls. Notably, VEGF expression was also higher at the earlier time point of P7 and specifically in retinal, but not lung or serum, samples, indicating a local source at the level of the retinal ganglion layer. In addition, the finding that increased VEGF levels in the hph1 retinas correlated with diminished hyperoxia-induced vascular closure in the short-term (6 hours) and vaso-obliteration after prolonged hyperoxia suggests that peroxynitrite does not have a detrimental effect on VEGF function in this group. Thus, collectively, our results show that the hph1 animals have constitutively high expression of retinal ganglion cell layer–derived VEGF that preserves the inner retinal vasculature during hyperoxia. Further evidence of this vasoprotective effect was demonstrated by diminished inner retinal hypoxia and VEGF production at P17, which resulted in lower NV.

There is increasing evidence to show that during development, neural activity drives vascular growth. For example, in the retina, development of the inner retinal circulation is coupled to maturation of the phototransduction cascade driven by VEGF gradients produced in response to the metabolic demand of retinal ganglion cells.^{40,41} Because ROP manifests primarily as a vascular pathology, initially we had focused our studies on investigating the consequences of BH4 deficiency on endothelial-derived eNOS function. However, in addition to being a cofactor for eNOS, BH4 is also a cofactor for neuronal NOS and tyrosine hydroxylase, whose products, nitric oxide and dopamine, play an important role in facilitating phototransduction.^{39,46,47} Herein, we have shown that the activity of these enzymes and their corresponding products is reduced in hph1 retinas and affected further by hyperoxia, indicating their dependency on BH4 bioavailability and susceptibility to oxygen-induced damage.

Mechanistically, these neurotransmitters may modulate vascular function directly or indirectly. For example, dopamine has been shown to reduce VEGF–VEGF receptor 2 signaling in ECs; therefore, the lower levels in the hph1 groups may make the hph1 animals resistant to this anti-angiogenic effect.⁴⁸ In addition, dopamine and nNOS-derived nitric oxide may indirectly affect vascular growth, through their modulation of neural processing in a similar manner to that described for animals that have been dark

reared during development or are deficient in melanopsin (*Opn4^{-/-}*) and, thus, unresponsive to light. In these situations the higher energy and oxygen requirements of the dark adapted retina were shown to drive vascular growth.⁴⁹ Interestingly, these models phenocopy our findings with the *hph1* strain: an increased retinal neuron population in the inner retina and greatly elevated VEGF production. The transmittance of light in the retina, as a chemical signal, is achieved by dopamine, whose production in amacrine interneurons is light inducible; nNOS is also produced in amacrine and retinal ganglion cells and is also involved in modulation of this cascade.^{39,46,47} Thus, it is conceivable to hypothesize that the diminished nitric oxide and dopamine we observed in the *hph1* groups leads to a reduction in light perception mimicking the dark-reared or melanopsin-deleted state, which presents as elevated retinal ganglion cell (RGC)-derived VEGF. Taken together, our results show that a constitutively higher level of VEGF expression, associated with retinal development, protects GTPCH-deficient neonates from oxygen-induced vascular damage.

In summary, we have shown that hyperoxia depletes BH4 levels, resulting in reduced NOS function but increased eNOS-derived ROS levels and elevated ROS/reactive nitrogen species levels in hyperoxia during the vasodegenerative phase. Surprisingly, when BH4 levels were limited even further, as was the case in the *hph1^{+/-}* and *hph1^{-/-}* groups, there was an increase in vessel survival, rather than a worsening. This enhanced vascular preservation correlated with a developmental increase in neuronal cell number and elevated RGC layer-derived VEGF production, which is vasoprotective during hyperoxia. Thus, taken together, our results indicate the involvement of a BH4-dependent mechanism that controls neurovascular communication to couple vascular supply to neural demand in the maturing retina.

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Supplemental Data

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