Lack of the Antioxidant Glutathione Peroxidase-1 (GPx1) Exacerbates Retinopathy of Prematurity in Mice

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Purpose. Glutathione peroxidase-1 (GPx1) is highly expressed during normal retinal maturation; however, its role in retinopathy of prematurity (ROP) is not fully understood. We postulated that GPx1 plays an important role in protecting the premature retina from oxidative injury in a mouse model of ROP.

Methods. ROP was induced in wild-type (WT) and GPx1 knockout (KO) mice by exposing neonatal mice to 75% oxygen from postnatal days 7 to 11, followed by 1 week of room air. Structural effects of ROP were evaluated by retinal histology, and gene expression of retinal pro-angiogenic factors was measured by qRT-PCR.

Results. Retinas from ROP GPx1 KO mice had a significantly larger central avascular area compared to those from ROP WT mice (P < 0.001), indicative of a more severe vaso-obliterration. In ROP GPx1 KO mice, retinas also displayed increased preretinal neovascularization (P = 0.05) with a concurrent increase in the expression of vascular endothelial growth factor (P < 0.05) compared to values in ROP WT mice. Elevated oxidative stress was observed in ROP GPx1 KO retinas as evidenced by increased nitrotyrosine immunolabeling (P < 0.01) and superoxide (P < 0.05) in vessels compared to ROP WT retinas. In contrast to these findings of exacerbated retinal vascular injury in GPx1 KO mice, Müller cell gliosis and microglial density were similar in ROP GPx1 KO and ROP WT retinas.

Conclusions. GPx1, an important antioxidant enzyme of the premature retina, afforded protection against oxidative stress and oxidative injury in ROP. Lack of GPx1 was associated with increased oxidative stress, an increase in retinal avascular area, upregulation of retinal VEGF, and increased neovascularization in a mouse model of ROP. (Invest Ophthalmol Vis Sci. 2013;54:555–562) DOI:10.1167/iovs.12-10685
Materials and Methods

Full details of materials and methods have been published previously (see Supplementary Material, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10685/-/DCSupplemental).

Animals

GPx1 knockout (GPx1<sup>−/−</sup>) mice initially of mixed genetic background were created at the Centre for Functional Genomics and Human Disease, Monash University, Australia. These mice were then bred onto the C57Bl/J6 background (nine backcrosses). Wild-type (GPx1<sup>+/+</sup>) mice of the same background were generated via Mendelian segregation of heterozygous C57Bl/J6 GPx1 knockout (GPx1<sup>+/-</sup>) matings and maintained as a separate line. GPx1<sup>−/−</sup> mice are phenotypically normal, but are particularly susceptible to oxidative stress. Experimental procedures adhered to guidelines of the National Health and Medical Research Council (NHMRC) of Australia’s Code for the Care and Use of Animals for Scientific Purpose, conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee, Melbourne, Australia.

Retinopathy of Prematurity

ROP was induced in wild-type (WT) and GPx1 knockout (KO) mice by exposing neonatal mice to hyperoxia, which causes vaso-obliteration and death of endothelial cells, a model called oxygen-induced retinopathy. Briefly, 7-day-old (P<sup>7</sup>) WT and GPx1 KO pups, with their respective mothers, were placed in a sealed oxygen chamber containing 75% oxygen (O<sub>2</sub>) ± 5% O<sub>2</sub> and 2% carbon dioxide (CO<sub>2</sub>). Pups were exposed to 75% O<sub>2</sub> for 22 hours/d from P7 to P11, a hyperoxic period that suppresses normal retinal physiological angiogenesis (phase 1). Mothers and pups were removed from the chamber and placed in room air for 2 hours/d to avoid respiratory distress. On P12, mothers and pups were placed in room air until P18, a hypoxic period in which pathological angiogenesis occurs (phase 2). Additionally, pups of both genotypes were reared with their mothers in normal room air from birth until P18 and served as sham controls. At P18, pups were killed by intraperitoneal (IP) injection of pentobarbital (120 mg/kg body weight; Virbac, Peakhurst, NSW, Australia).

Retinal Whole Mounts for Angiogenesis

Retinal whole mounts were prepared and analyzed for angiogenesis as previously described.

Blood Vessel Profiles in Preretina

Blood vessel profiles were counted in the preretina as reported previously. A blood vessel profile consisted of an endothelial cell or a blood vessel with a lumen.

Immunohistochemistry for Microglial Marker (Ionized Calcium Binding Adapter Molecule 1) and Macroglial Marker (Glia Fibrillary Acidic Protein)

Immunohistochemistry for the microglial marker, Iba1, and the macroglial marker, GFAP, was performed as previously described.

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

Total retinal RNA extraction, preparation of DNA-free RNA, RNA reverse transcription, and gene expression of VEGF, GPx1, and HO-1 by qRT-PCR were as described previously. Probes and primers have been described elsewhere (see Supplementary Material and Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10685/-/DCSupplemental).

Oxidative Stress Measurements

Immunohistochemistry for nitrotyrosine, the marker for peroxynitrite-induced damage, was performed as previously described. Superoxide was assayed using dihydroethidium (DHE) according to a previously published protocol.

Statistical Analyses

The data are expressed as mean ± standard error of mean (SEM). Comparison between WT and KO groups in the avascular area (percentage) and preretinal blood vessel profiles were subjected to unpaired Student’s t-tests. All other data were analyzed by one-way ANOVA with Newman-Keuls post hoc test. All analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). A P value ≤ 0.05 was considered statistically significant.

Results

Angiogenesis and Preretinal Neovascularisation

In our mouse model of ROP, both WT and GPx1 KO mice displayed defects in vascular growth from the retinal periphery to the optic disc, rendering the central area of the retina avascular (Fig. 1). However, in ROP GPx1 KO mice, the avascular area was significantly larger than that of the ROP WT animals (Fig. 1E). As previously reported, blood vessel profiles, as an index for neovascularization, were increased in ROP animals compared with the sham group, with vessels protruding into the vitreous humor at the preretal layer (Figs. 1F–I). Preretinal blood vessels in the ROP GPx1 KO retina were increased compared to those in the ROP WT retina (P = 0.05, Fig. 1J).

Retinal Proangiogenetic and Proinflammatory Gene Expression

VEGF mRNA expression was significantly increased in both ROP WT and ROP GPx1 KO mice compared to their respective sham controls (Fig. 2A). This increase of VEGF gene expression in ROP was significantly more pronounced in the GPx1 KO retina compared to the WT retina. Other proinflammatory mediators of neovascularization, including intracellular adhesion molecule-1 (ICAM-1) and tumor necrosis factor-α (TNF-α), were increased in ROP, but were not significantly different between WT and GPx1 KO retinas (see Supplementary Material and Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10685/-/DCSupplemental).

Retinal Antioxidant Gene Expression

The expression of four selenium-dependent Gpx isoenzymes was evaluated to determine their relative contributions to Gpx function in ROP. As expected, GPx1 expression was significantly lower in the GPx1 KO compared to the WT retinas (Fig. 2B). The approximately 20% of residual GPx1 expression in the GPx1 KO retinas was most likely due to background and/or nonspecific primer amplification as previously described in the same animal model. There was a significant increase of GPx1 gene expression in the ROP WT retina compared to the sham WT retina, which was not observed in GPx1 KO retinas. In contrast, GPx2, GPx3, and GPx4 gene expression was not altered across all experimental groups (see Supplementary Table S2A–C, http://www.iovs.org/...
To examine if other antioxidants play a role in ROP, gene expression of heme oxygenase-1 (HO-1), catalase, and thioredoxin-1 (Trx-1) was also examined. While no difference in the expression of catalase and Trx-1 was observed across all experimental groups (see Supplementary Material and Supplementary Figs. S2D, S2E, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10685/-/DCSupplemental), HO-1 was significantly increased in both ROP WT and ROP GPx1 KO animals compared with their respective sham controls (Fig. 2C). However, expression of HO-1 was not different between ROP WT and ROP GPx1 KO groups.

**Retinal Oxidative Stress**

Superoxide levels were significantly increased in ROP GPx1 KO retinas compared to sham controls. (Fig. 3). Furthermore, this increase of superoxide in the ROP GPx1 KO retina was significantly higher than levels observed in ROP WT retinas (Fig. 3E), with higher intensity of DHE fluorescence observed predominantly in the ganglion cell layer (GCL). Interestingly, in the outer nuclear layer (ONL), which consists of the highly oxidative photoreceptors, superoxide levels were not different in ROP WT and KO mice (Fig. 3F), indicating that a lack of GPx1 has a more pronounced effect on the vasculature in ROP. High levels of superoxide can react with nitric oxide (NO) to form the oxidant peroxynitrite (ONOO⁻). Peroxynitrite-induced damage of proteins, measured as the formation of nitrotyrosine, serves as a marker of oxidative stress. In our ROP model, the presence of nitrotyrosine was detected mainly in the vasculature and was not observed in sham retinas (Fig. 4). The level of nitrotyrosine staining was significantly increased in ROP GPx1 KO retinas compared to ROP WT retinas (Fig. 4G).

**Retinal Glial Cells**

Expression of GFAP by Müller cells is a well-accepted indicator of gliosis.23 In our ROP model, GFAP expression increased in all retinal areas examined (peripheral, midperipheral, and central) in both WT and GPx1 KO retinas (Fig. 5). No significant difference was observed in Müller cell gliosis between ROP WT and ROP GPx1 KO retinas. In addition, the number of Iba1-immunoreactive microglia was significantly increased in both ROP groups compared to the sham control.
groups (Fig. 6). However, a deficiency in GPx1 did not significantly alter the level of Iba1 immunolabeling in the ROP GPx1 retinas compared to ROP WT retinas (Fig. 6E).

DISCUSSION

This study shows for the first time that the antioxidant enzyme GPx1 plays an important role in protecting the premature retina from oxidant-induced injury in ROP. Lack of GPx1 exacerbates key pathogenic features of ROP in mice, including vaso-obliteration and intravitreous neovascularization. In the first phase of ROP, lack of GPx1 and the associated oxidative stress results in enhanced vaso-obliteration under hyperoxic conditions, most likely as a result of vessel growth cessation. In turn, lack of GPx1 also exacerbates the proliferative phase of ROP (phase 2), resulting in a modest yet significant increase in preretinal neovascularization, most likely due to an increase in proangiogenic growth factors such as VEGF.

It is speculated that preterm retinas are particularly vulnerable to oxidant-induced damage because of their underdeveloped antioxidant defense systems coupled with their lipid-rich composition. Biological organisms utilize several endogenous antioxidant defenses to cope with oxidative stress. Superoxide radicals are neutralized to water via a two-step process involving superoxide dismutase (SOD) in a first step, and GPx or catalase in a second step. Increased
production of superoxide leads to the formation of peroxynitrite through its interaction with NO and a buildup of the intermediate H2O2. This leads to increased protein and lipid damage, respectively, the latter resulting in increased formation of lipid hydroperoxides.13 Since antioxidant defense systems mature only in the later phase of gestation within the preterm retina in preparation for transition into an oxygen-rich extrauterine environment, this leaves the premature retina vulnerable to oxidative injury.

Several studies have investigated the role of SOD in protecting the premature retina from ROP; however, conflicting results have been reported.26–28 In a recent study, Usui and colleagues showed that an increase in GPx or catalase, the peroxide-detoxifying enzymes, is more important than overexpression of SOD alone in attenuating oxidative stress-induced damage in retinal disease.29 However, since catalase and GPx both detoxify H2O2, the relative importances of these two enzymes cannot be distinguished in their study. In the current study, we found that the expression of catalase, at the mRNA level at least, was not altered in our model of ROP in WT retinas, while GPx1 was significantly upregulated. In addition, we showed that catalase is unaffected by the lack of GPx1, suggesting that catalase plays a lesser role in protecting the premature retina from oxidative damage.

On the other hand, a previous study reported a significant increase in the activity of GPx in the premature retina compared to the mature retina, implying that GPx functions as an important early defense mechanism in premature infants.15 GPx has previously been reported to play important roles in protecting the retina from phototoxicity24,30 and cataract formation.31 The GPx enzyme family consists of four selenium-dependent isoforms, GPx1, GPx2, GPx3, and GPx4. In this study, a significant increase in GPx1 gene expression was observed in ROP WT retina. However, the gene expression of GPx2, GPx3, and GPx4 was not changed in ROP WT retina compared to shams. Most importantly, the gene expression of
Lipid peroxidation of cell tissue, and the aforementioned molecules are known to cause may be of clinical relevance in ROP since retinal tissue has the protective role of GPx1 isoform plays a significant role in protection of the premature capillary endothelial cells, resulting in retinal microvascular peroxidation-induced injury. Furthermore, the role of VEGF in ROP has been well characterized. Indeed, when premature infants are exposed to supplementary oxygen, hyperoxia suppresses VEGF expression, resulting in the cessation of normal vessel growth, regression of existing vessels, and vaso-obliteration. In the second phase of ROP, hypoxia driven by the loss of vessels in phase 1 leads to induction of VEGF expression, resulting in neovascularization. Astrocytes are the normal source of VEGF that drives the formation of the inner layer of vasculature. Under intense hypoxia, retinal astrocytes degenerate, leading to compensation by other cell types such as neurons and Müller cells to secrete VEGF Aberrant vascular development and patterning ensues, which results in intravitreal neovascularization. In the ROP GPx1 KO retina, vaso-obliteration appeared to be more severely affected than preretinal neovascularization and increases in VEGF. These findings suggest that an increase in ROS due to a deficiency in GPx1 has a more potent effect on capillary degeneration than on new blood vessel growth. Nevertheless, the increase in DHE labeling in the GCL associated with preretinal neovascularization in ROP GPx1 KO retina indicates that ROS may have induced VEGF expression to promote preretinal neovascularization. VEGF is an endothelial-specific mitogen and chemotaxin that plays a key role in the normal development of the retinal vasculature.

Some data therefore suggest not only that GPx is the more important H2O2-detoxifying enzyme in the retina, but also that the GPx1 isoform plays a significant role in protection of the premature retina from oxidant-induced damage.

GPx1 neutralizes three major oxidants, namely H2O2, lipid peroxides, and peroxytniite. The protective role of GPx1 may be of clinical relevance in ROP since retinal tissue has the highest level of polyunsaturated fatty acids of any known tissue, and the aforementioned molecules are known to cause damaging peroxidation reactions. Lipid peroxidation of cell membranes results in loss of structural integrity and function, and retinal endothelial cells are particularly susceptible to peroxidation-induced injury. Furthermore, peroxytniire is known to mediate hyperoxia-induced apoptosis of retinal capillary endothelial cells, resulting in retinal microvascular degeneration and subsequent preretinal neovascularization. Indeed, the increased nitrotyrosine observed in the ROP GPx1 KO retinal tissue is indicative of increased peroxytniire. Together with the increased vaso-obliteration observed in the ROP GPx1 KO retina, this strongly suggests that GPx1 protects against apoptosis of retinal endothelial cells.

Another major antioxidant system that is capable of neutralizing H2O2 is the thioredoxin system in which thioredoxin 1 (Trx1) and Trx peroxidase (peroxiredoxin) scavenge H2O2 to produce water. However, our model of ROP had no effect on the gene expression of Trx1; and additionally, a lack of GPx1 did not affect the level of Trx1 gene expression. Heme oxygenase (HO) is an enzyme that catalyzes the degradation of heme to produce bilirubin, iron, and carbon monoxide. The inducible form of HO, HO-1, is increased in response to oxidative stress. In preterm infants, the expression of HO-1 is known to increase concurrently with a drop in total plasma bilirubin level and a reduction in total hydroperoxides, suggesting that HO-1 might play a role in reducing oxidative stress in premature infants. Furthermore, HO-1 is thought to play an important role in retinal glial cells to protect photoreceptors from oxidative damage. In this study, gene expression of HO-1 was increased in our model of ROP; however, the induction of HO-1 was not affected by the lack of GPx1. Therefore, although HO-1 may play a protective role in ROP, the increased oxidative damage and exacerbated retinal pathology observed in the GPx1 KO retina suggests that HO-1 was unable to prevent the overriding injury caused by a loss of GPx1 activity.

In the current study, expression of VEGF was significantly increased in the ROP GPx1 KO retina compared to the ROP WT retina, together with a concomitant increase in preretinal neovascularization. VEGF is an endothelial-specific mitogen and chemotaxin that plays a key role in the normal development of the retinal vasculature. Furthermore, the role of VEGF in ROP has been well characterized. Indeed, when premature infants are exposed to supplementary oxygen, hyperoxia suppresses VEGF expression, resulting in the cessation of normal vessel growth, regression of existing vessels, and vaso-obliteration. In the second phase of ROP, hypoxia driven by the loss of vessels in phase 1 leads to induction of VEGF expression, resulting in neovascularization. Astrocytes are the normal source of VEGF that drives the formation of the inner layer of vasculature. Under intense hypoxia, retinal astrocytes degenerate, leading to compensation by other cell types such as neurons and Müller cells to secrete VEGF. Aberrant vascular development and patterning ensues, which results in intravitreal neovascularization. In the ROP GPx1 KO retina, vaso-obliteration appeared to be more severely affected than preretinal neovascularization and increases in VEGF. These findings suggest that an increase in ROS due to a deficiency in GPx1 has a more potent effect on capillary degeneration than on new blood vessel growth. Nevertheless, the increase in DHE labeling in the GCL associated with preretinal neovascularization in ROP GPx1 KO retina indicates that ROS may have induced VEGF expression to promote preretinal neovascularization.

Glial cells, which include astrocytes and Müller cells, are thought to play important roles in ROP and their function is well characterized in mouse and rat models of ROP. Hypoxia is known to induce Müller cell gliosis, resulting in a loss of function. In ROP, gliotic Müller cells upregulate GFAP and are found predominantly in the avascular retina. Müller cell–derived VEGF is a major contributor to retinal neovascularization in ROP. Microglia increase in number and become activated in ROP, however, their role in ROP remains unclear. Microglia may contribute to ROP by releasing both neurotoxic and proangiogenic factors. In the current study, despite the increase in ROS and the oxidative damage observed in the ROP GPx1 KO retina, Müller cell gliosis and the number of microglia were unaffected by the lack of GPx1. This result is consistent with previous findings showing that retinal endothelial cells are especially vulnerable to ROS toxicity while other cell types, such as smooth muscle cells, astrocytes, pericytes, and neurons, are considerably less affected.
In summary, a deficiency in GPx1 was associated with increased oxidative stress, increased avascular area, and preretinal neovascularization in a mouse model of ROP. Our study has therefore shown the importance of the antioxidant GPx1 in the protection against ROP and provides evidence that a deficiency in this antioxidant enzyme leads to retinal vascular damage, most likely as a consequence of increased oxidative injury. Our data therefore strongly suggest that the reduced antioxidant capacity of preterm retinas is an important factor in the development of ROP. This idea would be further strengthened by gain-of-function studies to increase GPx1 activity, either via the overexpression of GPx1 or through the use of synthetic GPx1 mimetics in animal models of ROP. Furthermore, GPx1 mimetics might offer a novel antioxidant approach to limit retinal vascular damage associated with ROP. Thus far, treatment of preterm infants with nonselective antioxidants such as vitamin E has proved disappointing. Therefore, a deeper understanding of the antioxidants and the specific pathways that convey protection against ROP, as shown by this study, will lead to better treatment options to satisfy this unmet clinical need.

References


