Selective Degeneration of Central Photoreceptors after Hyperbaric Oxygen in Normal and Metallothionein-Knockout Mice

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PURPOSE. Metallothioneins (MTs) in the brain and retina are believed to bind metals and reduce free radicals, thereby protecting neurons from oxidative damage. This study was undertaken to investigate whether retinal photoreceptor (PR) cells lacking MTs are more susceptible to hyperbaric oxygen (HBO)-induced cell death in vivo.

METHODS. Wild-type (WT) and MT-knockout (MT-KO) mice lacking metallothionein (MT)-1 and MT-2 were exposed to three atmospheres of 100% oxygen for 3 hours, 3 times per week for 1, 3, or 5 weeks. The control animals were not exposed. Histologic analysis of PR viability was performed by counting rows of nuclei in the outer nuclear layer (ONL). Ultrastructure studies verified PR damage.

RESULTS. HBO exposure produced a major loss of PR cells in the central retinas of WT and MT-KO mice, with no effect on the peripheral retina even at the longest (5 weeks) exposures. The degree of PR damage and cell death increased with duration of HBO exposure. One week of HBO exposure was insufficient to cause PR death, but tissue damage was observed in the inner and outer segments. At 3 weeks, the rows of PR nuclei in the central retina were significantly reduced by 38% in WT and 28% in MT-KO animals. At 5 weeks, PR loss was identical in WT (34%) and MT-KO (34%) animals and was comparable to that in WT at 3 weeks.

CONCLUSIONS. The data suggest that MT-1 and -2 alone are not sufficient for protecting PRs against HBO-induced cell death. The selective degeneration of central PRs may provide clues to mechanisms of oxidative damage in retinal disease. (Invest Ophthalmol Vis Sci. 2008;49:3207–3215) DOI:10.1167/iovs.07-1039

Oxidative stress is rapidly gaining attention as an integral component in many neurodegenerative disorders of the central nervous system (CNS). However, the factors linking oxidative stress to neuronal cell death remain unclear. One component of neuronal antioxidant defense is the family of metallothioneins (MTs), intracellular metal-binding proteins that act as free-radical scavengers to mitigate cell destruction triggered by normal oxidative processes. By binding cations (e.g., iron), MTs reduce the risk for the Fenton reaction, which generates highly reactive hydroxyl radicals (powerful oxidants). The multiple isoforms of MTs (i.e., MT-1, -2, -3, and -4), are expressed in most cells and across many species. MTs play a protective role in the retina. MT-1, -2, and -3 are all found in PRs, retinal pigment epithelium, and retinal neurons, and MT-1 and -2 are induced by oxidative stress. MT protein in the retinal pigment epithelium (RPE) of patients with age-related macular degeneration (ARMD) declines with age, with proportionately greater loss in the macula than in the peripheral retina. Retinal MT mRNA expression was lower in a monkey model of ARMD than in healthy animals. Delayed progression of ARMD by antioxidant therapy emphasizes the possible role of redox imbalance in PR loss.

The goal of the present study was to gain a better understanding of the potential link between oxidative stress, MTs, and PR degeneration. We used a hyperbaric-oxygen (HBO)-exposure model to induce oxidative stress in the mouse eye. It has long been known that hyperoxia can induce PR cell death. Recent studies in rodents have confirmed these findings. The experiments described in the present study tested the hypothesis that MT expression in the retina modulates PR death in hyperoxia. To assess the role of MT directly, we used advantage of MT-knockout (KO) mice in which targeted gene disruptions prevents the expression of MT-1 and -2. Using a high-resolution imaging system, we compared HBO-induced PR loss in WT versus MT-KO mouse retinas to determine whether MT-deficient PRs were more susceptible to oxidative damage.

METHODS

Knockout and Control Animals

Adult male MT-KO mice, 129S7/SvEvBrd-Mt1mt2 (stock 002211), and wild type (WT) mice, 129S1/SvImJ (stock 002448), were obtained from the Jackson Laboratory (Bar Harbor, ME). The mice were approximately 200 days old at the start of the study. WT and MT-KO mice were either treated with HBO or maintained in room air (untreated controls), resulting in four study groups: (1) WT mice + HBO treatment (total of nine animals; i.e., n = 3 for each of three different time points), (2) MT-KO mice + HBO (total of nine animals; i.e., three groups of n = 3 animals each), (3) WT mice, untreated (n = 3), and (4) MT-KO mice, untreated (n = 3). All animals used were maintained and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, under provisions of an approved protocol from the Institutional Animal Care and Use Committee (IACUC).
HBO Treatment

The mice were exposed to 3 atmospheres absolute pressure (29.4 psig [pounds per square inch gauge], equivalent to 66 feet of seawater) of 100% oxygen for 3 hours in the morning, three times per week on alternate days, for 1, 5, or 5 weeks. The procedure for treating experimental animals with HBO has been described.20 In brief, mice were kept in the dark inside the chamber during treatment to eliminate any possibility of light-induced damage to the retina. After the appropriate exposure duration, the animals were euthanatized with an overdose of cylinder delivered CO₂. The untreated WT and MT-KO animals were kept in the dark inside the chamber during treatment to eliminate any possibility of light-induced damage to the retina. After the appropriate exposure duration, the animals were euthanatized with an overdose of cylinder delivered CO₂. The untreated WT and MT-KO animals were kept on a normal light–dark cycle and euthanatized after 5 weeks.

Tissue Preparation: Light (LM) and Electron Microscopy (EM)

The eyes were enucleated and fixed in 2.5% glutaraldehyde and 2% paraformaldehyde, bisected along the vertical meridian, embedded, and sectioned, according to LaVail et al.29,30 One half of the eyeball was processed for LM; the other half for EM. Each 2-μm-thick LM section contained superior retina, optic nerve, and inferior retina, spanning from one ora serrata to the other (Fig. 1). For EM, the inferior retinal quadrant from each eye was embedded as described previously,31 and thin sections were collected on 300-mesh grids and stained with uranyl acetate and lead citrate. Thin sections were viewed on a transmission EM (Morgani 268; FEI Co., Hillsboro, OR) with attached digital camera (ORCA-HR; Hamamatsu, Hamamatsu City, Japan), and the areas of interest were photographed.

Analysis of PR Cell Loss

HBO-induced PR cell loss was quantified in LM sections by counting PR cells. A series of digitized images was acquired (Fig. 1; Q-imaging Retiga 1300 camera [Quantitative Imaging Corporation, Burnaby, British Columbia, Canada], and Metamorph Imaging System [Universal Imaging Corporation, Downingtown, PA]). Contiguous regions (divided into bins) were measured along the vertical meridian from the optic nerve outward along the inferior and superior hemispheres. Each bin was numbered, with bin 1 being closest to the optic nerve head (ONH). Bins of the inferior hemisphere were labeled i1, i2, i3, . . . i12; those of the superior hemisphere were labeled s1, s2, s3, . . . s12. With an oil objective (1.4 NA, 63×; Planapo), and a 1.25× optivar lens, each image represented a single 200-μm-wide bin. A 50-μm-wide field in the center of each photographic field was analyzed. The total number of PR nuclei and rows of PR nuclei were counted in each bin. On average, there were 12 bins per hemisphere, yielding 24 sets of measurements for the entire retinal section. Data from three consecutive bins were pooled and averaged. In our numbering scheme, bins 1 to 6 represented the central retina (i.e., closest to the optic nerve) and bins 7 to the ora serrata (bin 12), the peripheral retina. To compare the total number of PR nuclei per sampling area, we pooled the corresponding eccentricities (i.e., distance from the optic nerve along the vertical meridian) for the inferior and superior retina of each section, since the central to peripheral gradient of cell loss was similar in both hemispheres.

Statistical Analysis

For each mouse treatment group, one to two retinal sections per animal were analyzed, yielding a total of four sections from three animals for any given time point and treatment protocol. For each eccentricity, data from multiple animals (n = 3) were pooled for each bin (inferior and superior values combined), descriptive statistics were calculated, and a two-way t-test was performed across groups. A test was considered significant if its probability was P < 0.05. In the case of multiple comparisons, a Bonferroni correction was applied32 that adjusts significance levels for the number (n) of comparisons (i.e., P = 0.05/n). To illustrate the relevance of changes obtained with treatment and across animals, effect sizes were calculated. Effect size is a measure of clinical or meaningful variation and is calculated by dividing the mean sample difference by the SD for each control group.33 Effect sizes of 0.20 are considered small, 0.50 moderate, and 0.80 large.
**Results**

Within the treatment period studied, the early pathologic effect of HBO exposure on the retina was evident only in the PR cells. In LM sections, the inner nuclear layer, inner plexiform layer, and ganglion cell layer showed no signs of disease or cell loss, even up to the maximum 5 weeks’ exposure. Therefore, we restricted our statistical analysis to PR damage by counting nuclei and rows of PR in the outer nuclear layer (ONL; data for rows of PR nuclei in WT and MT-KO mice are presented in Table 1).

**Effect of HBO Treatment on PR Viability**

**Wild-Type and MT-KO Retinas.** There were several striking similarities between WT and MT-KO retinas in their response to HBO treatment. In both the normal and KO mice, HBO treatment for 3 and 5 weeks caused a dramatic loss of PRs from the central retinas, measured as a thinning of the ONL (see Table 1). The loss of PRs was regionally selective, with large decreases in ONL thickness occurring exclusively in the central retinas of WT and MT-KO animals (Table 1, Fig. 2, bins 1–6), but not in the periphery (Fig. 2, bins 7–12). To compare the WT and MT-KO mice, we took values in Table 1 and pooled them across eccentricities 1 to 3 and 4 to 6 to obtain an average for the central retina. Specifically, in central retinas (bins 1–6) of WT animals, HBO exposure caused a significant reduction in the number of rows of PR at 3 weeks (38%, P < 0.0001) and 5 weeks (34%, P < 0.002), compared with untreated control retinas. The effect sizes were large, corresponding to 0.90 and 0.81, respectively. Similarly, in the central retinas (bins 1–6) of MT-KO mice, the number of rows of PR nuclei was 28% lower (P < 0.0001) at 3 weeks and 34% lower after 5 weeks of HBO exposure than in retinas not exposed to HBO. The effect sizes for these differences were also large, corresponding to 1.01 and 1.24, respectively. In contrast, no PR cell loss was detected in the peripheral retinas of either WT or MT-KO mice, even after 5 weeks of HBO exposure (Table 1; Fig. 2, bins 7–12). Shorter HBO exposures of 1 week did not produce any measurable PR loss, even in central retinas of either normal or KO mice, compared to unexposed (0 weeks) control retinas (Fig. 2, bins 1–6).

It should be emphasized that in WT and MT-KO retinas, the effects of HBO were most striking in bins 1 to 6 (central retina), as clearly illustrated in Figure 2. Therefore, we performed most of our statistical comparisons in that part of the retina. In contrast, since the effects of HBO on the periphery (bins 7–12) showed some fluctuation, but overall appeared similar to 0-week control retinas, we did not perform a detailed analysis of that region.

Our finding that the PRs in MT-KO retinas were damaged by HBO in a pattern and time-course that closely resembled that of the WT retinas led us to make a direct comparison of WT and MT-KO retinas at each time point. There were no differences (t-test, P > 0.05) in the total number of rows of PR nuclei in the central retinal regions of unexposed WT and MT-KO mice (Fig. 3, 0 weeks). Similarly, no differences were detected in the number of PR rows of WT and MT-KO retinas after 1 week or 5 weeks of HBO (Fig. 3). On the other hand, with intermediate exposure (3 weeks) the MT-KO retinas lost significantly fewer rows of PR than did WT retinas (P < 0.002). This was the only time that a difference was detected between the WT and MT-KO animals. Thus, the average decrease in rows of PR nuclei in the central retina (bins 1–6) was only 28% in the MT-KO retinas, compared with a decrease of 38% in WT retinas. The number of PR rows in the peripheral retina (bins 7–12) was identical in both WT and MT-KO retinas at 3 weeks of HBO.

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**O2-Induced Degeneration of Central Photoreceptors**

**Table 1. Rows of Photoreceptor Nuclei in the Wild-Type (WT) and Metallothionein Knockout (KO) in Control Mice (0-week HBO) and Animals Exposed to HBO**

<table>
<thead>
<tr>
<th>Eccentricity</th>
<th>0 wk HBO</th>
<th>1 wk HBO</th>
<th>3 wk HBO</th>
<th>5 wk HBO</th>
<th>0 wk HBO</th>
<th>1 wk HBO</th>
<th>3 wk HBO</th>
<th>5 wk HBO</th>
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<td>Mean ± SD</td>
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<td>3.6 ± 0.8</td>
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<td>6.6 ± 1.0</td>
<td>6.4 ± 0.8</td>
<td>4.3 ± 0.9</td>
<td>4.0 ± 0.8</td>
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<td>% Loss vs. 0-wk control</td>
<td>1.5%</td>
<td>45.1%</td>
<td>43.4%</td>
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<td>34.2%</td>
<td>38.2%</td>
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<td>Significance (P)</td>
<td>6.61E-01</td>
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<td>Mean ± SD</td>
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<td>4.7 ± 1.6</td>
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<td>% Loss vs. 0-wk control</td>
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<td>32.3%</td>
<td>25.9%</td>
<td>0.9%</td>
<td>21.5%</td>
<td>28.3%</td>
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<td>Significance (P)</td>
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<td>Mean ± SD</td>
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<td>6.7 ± 0.8</td>
<td>5.9 ± 1.0</td>
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<td>6.3 ± 1.1</td>
<td>6.1 ± 0.9</td>
<td>5.7 ± 1.3</td>
<td>5.3 ± 1.9</td>
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<td>8.7%</td>
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<td>Significance (P)</td>
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<tr>
<td>% Loss vs. 0-wk control</td>
<td>-1.5%</td>
<td>-4.5%</td>
<td>-15.0%</td>
<td>7.7%</td>
<td>-3.4%</td>
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<td>Significance (P)</td>
<td>8.57E-01</td>
<td>8.43E-01</td>
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<td>8.72E-01</td>
<td>9.93E-01</td>
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</table>

Values are given for each retinal eccentricity (the distance from the optic nerve). An eccentricity of 1 is closest to the optic nerve and an eccentricity of 12 is closest to the ora serrata. Data for eccentricities 1–3, 4–6, 7–9, and 10–12 were pooled, and the mean ± SD, percentage loss in comparison with the control, and two-way ANOVA (t-test, P < 0.012) were computed. Probabilities are given in scientific notation (bold percentages indicate significant loss).
HBO-Induced Abnormalities in WT and MT-KO PRs: LM Analysis. In the absence of HBO treatment (0 weeks), the retinas of MT-KO mice were morphologically similar to those of WT control retinas. After 1 week of HBO, although no PR cell death was detected, there were major signs of early PR degeneration in the inner (IS) and outer (OS) segments (Fig. 4B) of both WT and MT-KO retinas. At the LM level, the IS were swollen, and a darkening of both IS and OS was apparent (Fig. 4B). In addition, OS were fragmented (Fig. 4B) and frequently shortened compared with the control retinas (Figs. 4A, 4C). These early pathologic changes were observed only in the central (and not peripheral) retina and were seen before any loss of PR nuclei (Figs. 4A, 4B).

The swelling of IS and darkening of the IS and OS was more pronounced in PRs surviving longer exposures (Fig. 4D, 5 weeks). In addition, after HBO exposure for 3 and 5 weeks, the structural damage in the IS and OS was accompanied by significant PR cell loss and corresponding thinning of the ONL (Figs. 5A, 5B, 6A, 6B, 6D). The localization of the HBO-induced PR damage to the central retina was striking, as no cell loss or structural changes were observed in the peripheral retinas of WT (Figs. 5C, 5D) or MT-KO mice (not shown).

Some differences between WT and MT-KO retinas were noted at the LM level after 3 weeks of HBO exposure. Whereas WT retinas usually incurred most of their damage by 3 weeks of HBO and thus showed cell loss similar to retinas exposed for 5 weeks, damage to PRs of MT-KO mice at 3 weeks typically showed various degrees of severity (Figs. 6C, 6D); sometimes appearing similar to 0- or 1-week HBO retinas with little or no damage or cell loss (Fig. 6A; see also Fig. 4B), and sometimes appearing more like a 5-week exposed retina, with extensive PR damage and thinning of the ONL (Fig. 6B). (There were no apparent changes in the morphology of the inner retina or RPE in the WT or MT-KO retina as a result of HBO exposure).
appeared fragmented and disorganized, and in some cases it was difficult to distinguish between the OS and IS layers (Figs. 7C, 7F). As a result of deterioration of the OS and IS, the PR nuclei of the ONL were located progressively closer to the RPE (compare Figs. 7B to 7C and 7E to 7F). Thus, our EM analysis confirmed the LM observations.

**FIGURE 4.** HBO-induced structural abnormalities in the PR IS and OS of WT and MT-KO mice preceded the loss of PR nuclei. (A, B) MT-KO retina, central region. (A) Control, no HBO. OS and IS were long and well aligned, with no signs of IS swelling, and the ONL was of normal thickness. (B) 1-week HBO. The number of rows of PR nuclei in the ONL was similar to that in the untreated retina (A), however, signs of IS swelling and OS shortening were already observed. (C, D) WT retina, central region, showing a higher magnification of the OS and IS layers at 0 and 5 weeks of HBO. (C) At 0 weeks of HBO, OS and IS were long and well aligned. (D) At 5 weeks of HBO, OS and IS became shortened, fragmented, swollen, and darkened. In addition, significant loss of PR nuclei had occurred by this stage (also see Figs. 5B, 6B). All findings were the same for both WT and MT-KO retinas. Scale bars, 25 μm.

**FIGURE 5.** Regional variation in HBO-induced PR damage. Central retinas (A, B) and peripheral retinas (C, D) of WT mice; similar findings were obtained in MT-KO mice (see Figs. 6A, 6B). (A) Central retina of WT mouse that was not exposed to HBO. The ONL, which contains the PR nuclei, was approximately 6 to 10 rows thick, and the PR IS and OS appeared healthy and vertically aligned. (B) Central retina of WT mouse exposed to 5 weeks of HBO. The ONL thickness has been reduced to only three to five rows of nuclei, indicating a significant loss of PRs. In (B), thinning of PR IS and OS layers, as well as swelling and disorganization of the remaining IS and OS also accompanied PR cell loss (compare to control retina in A). (C) Peripheral retina of WT mouse, not exposed to HBO. The ONL contained six to nine rows of PR nuclei, the IS appeared intact, and the OS were well organized. (D) Peripheral retina of WT mouse, after HBO exposure. Even after 5 weeks of HBO, the peripheral retina appeared virtually identical with the control (C), showing normal PR nuclei, IS, and OS. RPE, retinal pigment epithelium; OPL, outer plexiform layer; INL, inner nuclear layer. Scale bar, 25 μm.
DISCUSSION

The most important finding of this study was the extensive damage to the central retinas of both WT and MT-KO mice with HBO exposure. The marked thinning of the ONL due to loss of PRs decreased with retinal eccentricity and was absent (i.e., no PR loss) in the peripheral retina. The trend with eccentricity was similar in the superior and inferior retina. During the 5-week period of HBO exposure that we analyzed, the PRs were the only retinal cells with observable structural damage early on, due to the imposed oxidative stress; the inner retina and RPE did not show any signs of oxidative damage.

FIGURE 6. Variability of cell damage in MT-KO central retinas at 3 weeks of HBO. (A) Untreated MT-KO retina, 0 weeks of HBO. Similar to untreated WT retina, PRs appeared healthy, with approximately 10 rows of nuclei in the ONL and intact, well-aligned IS and OS. (B) MT-KO retina, 5 weeks of HBO. As in the WT, HBO has caused a remarkable thinning of the ONL to only three to five rows of nuclei, reflecting a dramatic loss of PRs. (C, D) Various degrees of damage were observed at 3 weeks, ranging from minimal damage without any cell loss (C), similar to untreated (A) or 1-week treated (Fig. 4B) retinas, to extensive damage with considerable cell loss (D), comparable to 5-week exposed retinas (B). Scale bar, 25 μm.

FIGURE 7. Ultrastructural analysis of PR IS and OS of WT and MT-KO mice after different HBO exposures. (A–C) WT retina at 0, 3, and 5 weeks of HBO. (D–F) MT-KO retina at 0, 3, and 5 weeks of HBO. Overall, the MT-KO retinas looked similar to the wild-type retinas at each successive stage of HBO exposure. Well-aligned PR OS at 0 weeks of HBO (A, D) became shortened (B, E) and fragmented (C, F). (Note in D that despite the slightly cross-sectional cut through the OS, the thickness of the OS layer is well illustrated.) IS became swollen (C, E, F), and there was a progressive thinning of the OS and IS layers, as evidenced by the repositioning of the ONL, more proximate to the RPE, with longer HBO exposures (compare A and D, in which the ONL was not visible in the micrograph, to C and F, in which the ONL was relatively closer to the RPE). Evidence of degenerating PRs is represented by darkened, pyknotic tissue (B, C, E: ✽, double arrows). At 3 weeks of HBO, the transition from normal morphology to pathologic was evident at various stages. The micrograph in (B) highlights the shortening of OS and IS, whereas (E) shows more prominent swelling of the IS. Scale bars, 5 μm.
damage, even with the longest (5 weeks) HBO exposure. Nevertheless, we cannot rule out the possibility that secondary changes involving neuronal and glial plasticity in the inner retina may occur as a consequence of PR cell loss, as seen in other forms of PR degeneration.\textsuperscript{34} Such late-stage changes, which may have become evident only at time points beyond 5 weeks, could include glial cell activation, proliferation, hypertrophy and migration; vascular changes; relocation of surviving inner retinal neurons; or reactive sprouting of neurites.

In contrast to other retinal cell types, the selective vulnerability of PRs to HBO that we have observed has also been reported in rat retinas exposed to hyperoxia,\textsuperscript{22} and may be related to the tightly packed mitochondria in the inner segments of the PRs, which are among the most metabolically active tissues of the body.\textsuperscript{35} Hyperoxia rapidly interferes with key mitochondrial enzymes in a variety of cellular systems, thereby inhibiting oxygen consumption and causing cell death.\textsuperscript{36--38} In fact, the first signs of HBO-induced cell damage that we observed in the mouse retina occurred in the PR inner segments. One proposed mechanism of O₂ toxicity involves the accumulation of reactive oxygen species, particularly superoxide anion, in the mitochondria.\textsuperscript{36,37} The specificity of the O₂ damage to the PRs may also be due to a lack of the antioxidant, reduced glutathione, in these retinal neurons (Winkler BS et al. IOVS 2005;46: ARVO E-Abstract 1676). Further, initial damage in the inner segment may be related to the low levels of glutathione in mitochondria.\textsuperscript{39}

Our results are consistent with other reports of toxic effects of HBO on the retina in a variety of species.\textsuperscript{40--46} When HBO treatment of dogs at 3 atmospheres was extended beyond 3 hours, retinal pathology became evident.\textsuperscript{40} Similarly, we observed morphologic changes in PRs of mice treated with HBO for 1 week (total of 9 hours of HBO exposure). These histopathologic changes began in the central retina, involved the PR IS and OS, and preceded any loss of PR cells. The early signs of degeneration may explain why patients reported impaired central vision with decreased visual acuity for form and color beginning just after 3 hours of HBO treatment\textsuperscript{40} and why the visual deficits were reversible on return of the patients to normal air.\textsuperscript{40} In other reports, HBO treatment caused cataracts,\textsuperscript{47,48} especially in aging patients,\textsuperscript{49} and has been shown to be detrimental to lens clarity in a guinea pig model.\textsuperscript{19,20}

**Similar HBO Sensitivities in Normal and MT-KO Retinas**

This study investigated whether PR cells deficient in MTs are more susceptible to HBO-induced cell death in vivo than are WT control cells, since metallothioneins protect cells against oxidative stress; MT-1\textsuperscript{10,12} and -2\textsuperscript{10--12} are normally present in mouse and human PRs; and MT-1 and -2 expression is upregulated in mouse PRs in response to an oxidative insult.\textsuperscript{10} In fact, our results did not support this hypothesis. Rather, experiments showed an unexpected similarity in the response of WT and MT-KO retinas to HBO, except for one intermediate exposure time (3 weeks). We, therefore, conclude that MT-1 and -2 do not contribute significantly to the protection of PRs against HBO-induced oxidative stress.

**Possible Protective Effect of MT-KO**

At 3 weeks of HBO exposure, a surprising difference was found between WT and MT-KO retinas: The amount of PR cell loss in MT-KO retinas was significantly lower than that in normal retinas. This early and transient protective effect, which initially contributed to an overall slower rate of HBO-induced PR death in the KO, was no longer evident by 5 weeks of HBO exposure. Perhaps MT-3, which is present in the PRs,\textsuperscript{10} but is not usually induced by oxidative stress, is sufficient to protect the MT-KO PRs against oxidative damage imposed by HBO. The possibility that other MT isoforms\textsuperscript{3,6} were upregulated in the MT-1 MT-2 KO retinas also cannot be eliminated. Another possible explanation for this difference is that there is an upregulation of other (nonmetallothionein) antioxidants in the MT-KO retina, which partially compensate for the loss of MT and provide alternative pathways for protecting PRs against a brief oxidative insult. Moreover, since levels of MT in a normal retina decrease with age,\textsuperscript{44} the WT retinas could, in fact, be more susceptible to oxidative damage than the MT-KO retinas, in which compensatory mechanisms may have been long activated. However, after a more prolonged oxidative insult, such as 5 weeks of HBO exposure, existing antioxidant systems may no longer provide sufficient protection, resulting in similar PR degeneration in normal and MT-KO mouse retinas.

**Regional Variation in HBO Sensitivity-Selective Degeneration of Central Retinal PRs**

Oxidative stress in the retina, induced by light damage or high oxygen, may lead to preferential death of central PRs. We observed a central-to-peripheral gradient of PR degeneration in the mouse retina with HBO exposure. The selective HBO-induced loss of PR cells in the central retina was consistent with the still unexplained greater vulnerability of central PRs to light-induced damage.\textsuperscript{50} Our results were also in agreement with those of Yamada et al.,\textsuperscript{24} who reported a similar central-to-peripheral degeneration pattern in a study of 6-week-old mouse retinas exposed to normobaric hyperoxia (i.e., 75% oxygen at normal atmospheric pressure). However, these investigators did not present high-resolution images of the retinas, nor did they systematically measure ONL thickness or the number of PR nuclei. Similarly, in a neonatal rat model of retinopathy of prematurity (ROP), oxygen exposure for 11 days caused thinning and disorganization of central rod OS,\textsuperscript{51} and in the young adult rat retina, PR cell death induced by hyperoxia likewise exhibited regional variation, with more profiles of dying cells present in the center of the retina compared with the edge of the retina.\textsuperscript{52} The results in those studies suggest that it is the high oxygen level (and oxidative stress) rather than the high HBO pressure that causes extensive PR damage and death. However, what accounts for the elevated oxidative stress in the central retina? One report led to the idea that the vessels closest to the ONH provide a greater amount of oxygenated blood than that supplied to the peripheral retina.\textsuperscript{5,2} This study proposed that selective loss of blood vessels closest to the ONH in the mouse model of ROP was a consequence of maximum O₂ concentration there.\textsuperscript{52} Thus, the deleterious effects of high oxygen would be most concentrated near the ONH where vascular perfusion is greatest, and would taper off with increasing eccentricity as observed in our studies and in those by Yamada et al.\textsuperscript{24} and Wellard et al.\textsuperscript{27}

The greater vulnerability of central retinal PRs to hyperoxia may conversely be attributable to the greater resistance of PRs in the periphery or edge of the retina to acute hypoxic stress. There is evidence that chronic oxidative stress, which leads to edge-specific degeneration of the retina, also stimulates the expression of protective growth factors at the edge of the retina, thereby imparting increased resistance of the peripheral retina to acute hyperoxia.\textsuperscript{53}

**HBO Therapy: Clinical Use and Considerations**

The implications of the present findings are significant. HBO therapy is increasingly used clinically to treat a wide variety of ailments and diseases, including those of the eye and the retina. For example, HBO therapy is commonly used to treat decompression sickness (the bends) in deep-sea divers and is used to promote wound healing and in the treatment of carbon mon-
oxide poisoning, diabetic neuropathies, and serious bacterial infections, especially in patients with type II diabetes. In addition, HBO treatment has been successful in the management of various ocular disorders involving retinal hypoxia, such as cystoid macular edema as a complication of retinal vein occlusion or postoperative cataract surgery, and recurrent branch retinal artery occlusion in Susac syndrome. HBO therapy has been documented to improve electroretinogram (ERG) responses in a small number of patients with retinitis pigmentosa. In many of these cases, HBO therapy has resulted in significant improvements in visual acuity after other treatment methods failed.

The apparent discrepancy with the damaging effects of HBO on PRs observed in the present study may be reconciled by considering the level of oxygenation of the PRs at the time of HBO administration. It is not surprising that cells lacking sufficient oxygen would benefit from HBO treatment. For instance, in a study involving retinitis pigmentosa, the beneficial effects of HBO were attributed to the increased supply of available oxygen to the otherwise hypoxic and therefore, degenerating, PRs. In contrast, healthy PRs with an adequate supply of oxygen may be thrown into a state of oxidative stress with prolonged HBO exposure; this overload of oxygen could then lead to PR death.

In conclusion, we have demonstrated that PR degeneration is a direct consequence of HBO treatment in normal mice and that MT-1 and -2 do not play a significant role in protecting PRs from HBO-induced oxidative stress. Moreover, the HBO-induced PR cell death was regionally specific, affecting only the central retina. This striking finding invites further exploration into the mechanisms of neurodegeneration, perhaps leading to the elucidation of molecular variations among PRs in different parts of the retina, which influence their susceptibility to oxidative stress. Our HBO mouse model provides a useful system in which to pursue these questions.

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References

