SOD2 Protects against Oxidation-Induced Apoptosis in Mouse Retinal Pigment Epithelium: Implications for Age-Related Macular Degeneration

Emiko Kasahara,1 Li-Ren Lin,1 Ye-Shib Ho,2 and Venkat N. Reddy1

PURPOSE. Oxidative stress from reactive oxygen species (ROS) has been implicated in many diseases, including age-related macular degeneration (AMD), in which the retinal pigment epithelium (RPE) is considered a primary target. Because manganese superoxide dismutase (SOD2), localized in mitochondria, is known to be a key enzyme that protects the cells against oxidative stress, this study was undertaken to examine oxidation-induced apoptosis in cultured RPE cells with various levels of SOD2.

METHODS. Primary cultures of RPE cells were established from wild-type (WT), heterozygous Sod2-knockout mouse (HET) and hemizygous Sod2 mice with overexpression of the enzyme (HEMI). Purity of the RPE cell cultures was verified by immunostaining with antibody to RPE65 and quantified by flow cytometry. Oxidative stress was induced in RPE cells by exposing them to H2O2 (0–500 μM) for 1 hour and reculturing them in normal medium for various times (0–24 hours). Apoptosis in the RPE was examined by TUNEL staining and quantified by cell-death–detection ELISA. Mitochondrial transmembrane potential (MTP) was measured by a cationic dye, and cytochrome c leakage from mitochondria was analyzed by Western blot analysis.

RESULTS. More than 95% of the cells in each culture were RPE65 positive, and the relative SOD2 levels in HET, WT, and HEMI cells were 0.6, 1.0, and 3.3, respectively. H2O2-induced apoptotic cell death was both dose and time dependent, and apoptosis in these cells was related to the cellular SOD2 level. Disruption of MTP and release of cytochrome c were observed to occur before apoptotic cell death, and they correlated with cellular SOD2.

CONCLUSIONS. The results demonstrate a critical role of SOD2 in protection against oxidative challenge. Cells from HET mice showed greater apoptotic cell death, whereas in those from HEMI mice, cell death induced by oxidative injury was suppressed. (Invest Ophthalmol Vis Sci. 2005;46:3426–3434) DOI:10.1167/iovs.05-0344

From the 1Kellogg Eye Center, Department of Ophthalmology and Visual Sciences, University of Michigan, Ann Arbor, Michigan; and the 2Institute of Environmental Health Sciences, Wayne State University, Detroit, Michigan. Supported by National Eye Institute Grants EY00484 (VNR), Vision Core Grant EY07003, and National and Heart, Lung, and Blood Institute Grant HL56421 (Y-SH). Submitted for publication March 17, 2005; accepted April 25, 2005.

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Corresponding author: Venkat N. Reddy, Kellogg Eye Center, Department of Ophthalmology and Visual Sciences, University of Michigan, 1000 Wall Street, Ann Arbor, MI 48105; venreddy@umich.edu.
bryonic or neonatal lethality, and thus, SOD2 has been consid-
ered a critical enzyme for aerobic life. However, the Sod2-
knockout mice survive for only a short time, and it has not
been possible to study the ocular manifestations resulting from
the total absence of the SOD2 protein. Heterozygous Sod2-
knockout mice (HET) appeared relatively normal, and no phe-
notype has been reported, although the activities of this en-
zyme in the mitochondria were reduced in all the tissues
studied. SOD2 activity in HET is roughly 50% of wild-type (WT)
mice, but activity of other antioxidant enzymes such as glu-
thione peroxidase and copper/zinc superoxide dismutase
(SOD1) is not affected. 18 Although the partial reduction of this
enzyme induces mitochondrial oxidative damage and altered
mitochondrial function, there is no significant oxidative dam-
age in cytosolic protein or nuclear DNA. 19 Thus, the heterozy-
gous knockout mouse that shows mitochondrial oxidative
damage could serve as a useful model for studying the func-
tional role of this enzyme in apoptotic cell death. We have also
studied the role of this enzyme in Sod2 transgenic mice (HEMI)
in which the enzyme was overexpressed. Specific activity of
SOD2 in the lungs of these animals increased by 170% com-
pared to WT control animals and provides antioxidant defense
in the lung. 19, 20 We have reported that SOD2 overexpression
in human lens epithelial cell culture suppresses apoptotic cell
deat induced by photochemical oxidative stress. 21 These
studies demonstrated increased cytochrome c leakage from
the mitochondria and greater apoptosis in SOD2-deficient cells
when compared with the cells overexpressing the enzyme.

In this study, we investigated the protective role of SOD2
against H2O2-induced oxidative stress in RPE cells containing
various levels of the enzyme. The primary cultures were de-
ferred from WT and mutant mice, partial knockout mice (HET),
and transgenic mice that overexpress the enzyme (HEMI).

Materials and Methods

Animals

Sod2 heterozygous knockout mice were obtained from the Jackson
Laboratory (Bar Harbor, ME; stock no. 002973, strain B6.129S7-
Sod2tm1Leb /H9262 and were fed standard laboratory chow and water ad
libitum. To minimize genetic variations, HET and WT mice were
initially derived from breeding two heterozygous knockout mice. The
Sod2 HEMI mice were first generated by microinjecting the human
SOD2 expression vector into fertilized mouse eggs isolated from mat-
ing of (C57BL/6 X C3H; hereafter called B6C3 F1) male and female
mice, as described previously. 19 Experimental animals were generated
by breeding of hemizygous Sod2 transgenic mice with WT B6C3 F1
mice. PCR was used for genotyping the offspring, as described previ-
ously. 21 All animals used were maintained and treated in accordance
with ARVO Statement for the Use of Animals in Ophthalmic and Vision
Research.

Cell Culture

Mouse RPE cells were isolated from eyes obtained from 1-year-old HET,
WT, and HEMI mice. In brief, the mice were killed by CO2 asphyxia,
eyes were enucleated, and the anterior segment, lens, and neuroretina
were removed. Eyecups were treated for 30 minutes with papain (5
U/mL) to isolate the RPE from Bruch’s membrane. After incubation,
the RPE was removed by trituration with a plastic pipette and cultured in
Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen Gibco, Grand
Island, NY) containing 15% fetal bovine serum (FBS; Invitrogen Gibco)
and 50 μg/mL of gentamicin in culture plates (Falcon; BD Biosciences,
Bedford, MA). Mouse eyecups became soft and flat when treated with
papain, because both the inside and outside of the eyecups were in
contact with the enzyme solution. Preliminary studies showed that the
harvested RPE cells were often contaminated with fibroblasts from the
scera. To avoid fibroblast contamination of the RPE, the early stage of
the initial culture was carefully monitored, and all possible fibroblast
growth (which appear earlier than RPE cell growth) was ablated with a
red-hot platinum loop that burned out all the fibroblast colonies, and
cell culturing continued. When RPE cells became confluent they were
subcultured by splitting in a 1:3 ratio. After three passages, the cells
were frozen in liquid N2 and later used for the various experiments.

Purity of the cells was verified by immunostaining with antibody to
RPE65, a specific marker for RPE, and the SOD2 level was determined
by Western blot analysis, as described below. These cells were cul-
tured in DMEM with 15% FBS at 37°C in a 5% CO2 environment. After
the cells were confluent, they were trypsinized and recultured to
adjust the number of cells in each group, as desired.

RPE65 Immunohistochemistry and Flow Cytometry

The purity of the cell culture was established by an immunocyto-
chemical method, with an antibody against RPE65, and quantified by flow
cytometry. The primary cell cultures (2 x 10⁵/well) from WT, HET,
and HEMI mice were cultured on eight-well chambered slides (Nunc,
Roskilde, Denmark) for 24 hours and then stained with RPE65 antibody
and analyzed by fluorescence microscopy. Briefly, the cultured cells
were fixed with 4% methanol-free formaldehyde for 30 minutes at
room temperature and washed with PBS. They were then blocked with
a blocking solution (20% sheep serum and 0.5% Triton X-100 in PBS)
for 30 minutes at room temperature and incubated overnight at 4°C
with primary antibody to RPE65 (kindly provided by Debra Thompson,
Kellogg Eye Center) diluted in wash solution (2% sheep serum and
0.2% Triton X-100 in PBS). The cells were then rinsed again with wash
solution and incubated for 1 hour at room temperature with tetra-
ethylrhodamine isomer R (TRITC)-conjugated secondary antibody (Mo-
lecular Probe, Eugene, OR), which was diluted in wash solution (1: 250).

For quantification, the stained cells were analyzed by flow cytom-
etry, as follows. Cells (5 x 10⁷) were trypsinized and collected by
centrifugation at 1000g for 5 minutes and then washed and incubated with
4% methanol-
free formaldehyde for 30 minutes at room temperature. They were
then incubated in PBS containing 0.25% Triton X-100 for 5 minutes at
4°C to permeabilize the cell membrane. After the cells were washed
with PBS, they were incubated with primary antibody to RPE65 or
without the antibody, as a negative control, in wash solution for 1 hour
at room temperature. The cells were washed two times and then
incubated for 30 minutes at room temperature with TRITC-conjugated
secondary antibody diluted in wash solution (1:100). The cells were
analyzed by flow cytometry (FACS; BD Biosciences, San Jose, CA), with
FITC signal detector (FL2).

Analysis of SOD2 Protein Expression

SOD2 levels in these cells were measured by Western blot analysis.
Trypsinized cultures were washed with PBS and centrifuged at 1000g
for 5 minutes. Collected cells were dissolved in 0.5% Triton buffer
containing protease inhibitor (Roche Diagnostics, Mannheim, Ger-
many) and incubated for 10 minutes on ice. After incubation, each
sample was centrifuged at 10,000g for 10 minutes at 4°C, to remove
the debris. Supernatants were assayed for protein and all samples
adjusted to the same protein concentration and mixed with lithium
dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA) and 2-mercap-
toethanol, according to Invitrogen protocols. The samples were sub-
jected to SDS/PAGE followed by Western blot analysis using a protein
expression assay (Western Breeze; Invitrogen, Carlsbad, CA). Electro-
phoresed proteins were transferred from gel onto nitrocellulose mem-
brane (Trans-Blot transfer medium; Bio-Rad, Hercules, CA) and incu-
bated with 0.1 μg/mL primary antibody against SOD2 (Anti MnSOD;
product no. SOD-111; Stressgen, Victoria, British Columbia, Canada)
onight at 4°C and then incubated for 30 minutes at room temper-
avor with alkaline-phosphatase–conjugated secondary antibody. The

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staining reaction was quantified by densitometry with a computerized image analysis program (NIH Image, ver. 1.63; available by ftp at zippy.nimh.nih.gov or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

H$_2$O$_2$-Induced Oxidative Stress

Primary cultures were incubated in serum-free DMEM containing different levels of H$_2$O$_2$ (0–500 μM) for 1 hour, and then the media were replaced with DMEM with 15% FBS. After 0, 4, 8, 16, and 24 hours of reculturing, these cells were used for experiments involving apoptosis and mitochondrial transmembrane potential.

Analysis of Apoptosis

The cells were cultured on eight-well chambered slides (2 × 10^5/well) for 24 hours and then exposed to H$_2$O$_2$, as described earlier. They were fixed with 4% paraformaldehyde and subjected to terminal deoxynucleotidyltransferase dUTP nick-ended labeling (TUNEL) staining using a cell-death–detection kit (In Situ Cell Death Detection Fluorescein kit; Roche Applied Science, Penzberg, Germany). For quantification of apoptosis under different experimental conditions, apoptotic cell death was determined by a cell-death–detection ELISA kit (Roche Applied Science), which is based on a quantitative sandwich-immunoenzyme immunoassay using antibody binding to DNA and histones H1, H2A, H2B, H3, and H4 and photometric measurement at 405 nm with a microplate reader (Spectra Max 190; Molecular Devices, Sunnyvale, CA).

Measurement of Mitochondrial Transmembrane Potential

After exposure to H$_2$O$_2$ as previously described, the cells on the slide were examined for change in mitochondrial transmembrane potential (MTP), by using a cationic dye (MitoCapture; BioVision, Mountain View, CA), and detected with fluorescence microscopy. The cultured cells on the chamber slide after exposure to H$_2$O$_2$ were incubated with prewarmed cationic dye solution (MitoCapture; BioVision) for 15 minutes at 37°C and then immediately observed under a fluorescence microscope. The assay is based on the aggregation of the dye and its fluorescence in the mitochondria. In the undamaged mitochondria, the aggregated dye appears as red fluorescence, whereas in the apoptotic cell with altered mitochondrial transmembrane potential, the dye remains as monomers in the cytoplasm with diffuse green fluorescence.

Measurement of Cytochrome c Leakage from Mitochondria

After 8 hours of reculturing after exposure to 300 μM of H$_2$O$_2$ for 1 hour, the RPE cells (5 × 10^5) were collected by centrifugation at 600g for 5 minutes at 4°C and washed with ice-cold PBS. The cells were assayed with the cytochrome c-releasing apoptosis kit (BioVision). Briefly, they were homogenized with the cytosol extraction buffer provided in the kit and then centrifuged at 700g for 10 minutes at 4°C to remove the debris. The supernatant was then centrifuged at 10,000g for 30 minutes at 4°C. The pellet was used as the mitochondrial fraction, and the supernatant was collected as the cytosolic fraction. These fractions were analyzed for cytochrome c by Western blot as described earlier, with the cytochrome c antibody provided in the kit.

RESULTS

Purity of RPE Cell Cultures

In this study, first it was important to establish the purity of the RPE cultures. Preliminary studies indicated considerable contamination with fibroblast cells, probably derived from the sclera. However, ablation of the fibroblast colonies, which appeared first in the initial cultures, with a hot platinum wire was highly successful. The purity of the RPE cells was verified by immunostaining with antibody to RPE65 and quantified by flow cytometry. RPE65-positive stain was observed in nearly all the cells (Fig. 1A). Quantification with flow cytometry (Fig. 1B) showed that nearly 100% of the cells in each of the cell cultures were RPE65 positive. Incubation without RPE65 antibody, as negative control, showed less than 5% of the cells were stained. Thus, we concluded that the RPE cell cultures derived from WT and the two mutant mice are not contaminated with other cell types.

Levels of SOD2 Protein in RPE Cells

Expression of SOD2 in the RPE cells from the three groups of mice (WT, HET, and HEMI) was detected by Western blot analysis with antibody against SOD2. A 25-kDa band was observed in each of the samples (Fig. 2A). Density scanning of these immunoblots showed that the relative expression of SOD2 in RPE cells from HET was approximately 60% of the endogenous level in the control cells (WT), whereas the expression in RPE cells from HEMI was approximately 3.4 times that of the control cells (Fig. 2B).

Effect of Oxidative Stress on Apoptosis in RPE Cell Culture

The normal phagocytosis of outer segments of photoreceptor by RPE is known to accumulate H$_2$O$_2$, which is detoxified by antioxidant enzymes. We therefore assessed the protective effect of SOD2 against oxidation-induced apoptosis after exposure to H$_2$O$_2$. To detect apoptosis, we analyzed by fluorescence microscopy the occurrence of DNA fragmentation in the cells (Fig. 3). The number of TUNEL-positive cells increased with H$_2$O$_2$ treatment in a dose- and time-dependent manner (data not shown). Figure 3 shows that when RPE cells were exposed to a 300-μM concentration of H$_2$O$_2$ for 1 hour and then recultured for 16 hours, apoptotic cell death increased compared with that in untreated control cells (without H$_2$O$_2$). The number of TUNEL-positive RPE cells from HET mice was much greater than that in cells derived from WT or HEMI mice. Furthermore, in cells derived from HEMI mice, in which the SOD2 level was nearly 3.4 times that of WT, apoptosis was suppressed. It is notable that the least number of TUNEL-positive cells was present in the cells derived from the HEMI mice. To obtain further confirmation of peroxide-induced apoptosis, additional experiments were performed for quantitative assessment. RPE cells from the three groups of mice were exposed to different doses of H$_2$O$_2$ for 1 hour and recultured for various times, and apoptotic cell death was measured by cell-death–detection ELISA kit (Figs. 4, 5). H$_2$O$_2$-induced apoptosis was both dose and time dependent. Figure 4 shows cell death as a function of the concentration of H$_2$O$_2$, ranging from 0 to 500 μM. The cell death in SOD2-deficient cells (HET) was significantly greater than in those from WT and HEMI mice at all concentrations of H$_2$O$_2$ higher than 200 μM. The time dependency of H$_2$O$_2$-induced apoptosis was also related to the cellular level of SOD2 (Fig. 5). The cells overexpressing SOD2 (HEMI) had a greater protective effect against apoptosis induced by H$_2$O$_2$ than did those from WT or HET mice.

Effect of Oxidative Stress on MTP

Altered MTP is known to be an early event in the apoptosis signaling process. We therefore investigated the relationship between mitochondrial damage and apoptosis when cells were treated with H$_2$O$_2$. The cells were exposed to 300 μM of H$_2$O$_2$ for 1 hour and then recultured for 4, 8, and 16 hours. Disruptions of the MTP in these cells increased in a time-dependent manner, and the extent of the mitochondrial damage was related to the level of SOD2. Fig. 6 shows mitochondrial membrane damage (green fluorescence) in cells derived from WT and HET mice first noted after 4 hours of incubation.
At this time point, no apoptotic cell death was observed (Fig. 5). Loss of membrane potential after 4 and 8 hours was much greater in HET than WT mice, whereas the MTP in cells derived from HEMI mice was still preserved until 8 hours of incubation. Thus, deficiency of SOD2 results in greater disruption of the MTP, whereas overexpression of the enzyme protects against mitochondrial membrane damage. Furthermore, altered MTP was observed before apoptosis was detected.

**Effect of Oxidative Stress on Cytochrome c Leakage**

The leakage of cytochrome c from mitochondria into the cytoplasm is known to activate caspases and initiate apoptosis.22,25 We therefore examined leakage of cytochrome c from the three groups of the RPE cell cultures derived from WT, HET, and HEMI after oxidative insult. The cells were exposed to 300 μM H2O2 for 1 hour and then incubated for 8 hours, and the cytochrome c in the cytoplasm was determined, as described in the Methods section. As seen in Figure 7, there was a marked increase in cytochrome c leakage in H2O2-treated RPE cells compared with that in the untreated control cells (shown only for WT). Moreover, there was a greater cytochrome c leakage in H2O2-treated cells derived from HET mice compared with those of WT and HEMI mice. The relative amounts of cytochrome c in the cytosolic fractions of H2O2-treated RPE cells from HET, WT, and HEMI mice were 1.4, 1.0, and 0.6 respectively (cytochrome c leakage in non-H2O2-treated cells was similar in the three groups; data not shown). Thus, the leakage of cytochrome c was directly related to the SOD2 levels in the RPE cell cultures.

**DISCUSSION**

This study demonstrates the critical role of the mitochondrial enzyme SOD2 in protecting RPE cells in culture against H2O2-induced apoptotic cell death. The protective effect of SOD2 against oxidation-induced apoptosis was directly related to the cellular level of the enzyme. The cells derived from heterozy-
gous mice (partial knockout, +/−), with SOD2 levels of 60% compared with WT controls, showed a greater number of
TUNEL-positive cells. In contrast, the cells derived from transgenic (hemizygous mice) that overexpressed, having an SOD2
level nearly 3.4 times that of WT mice, were resistant to the
cytotoxic effect of H2O2. Apoptosis in these cells was sup-
pressed or prevented when exposed to similar concentrations
of H2O2. The studies also show that RPE cells exposed to H2O2
exacerbation of cerebral infarction caused by overgenerated
mitochondrial oxidative stress by arterial ischemia and reperfusion injury, there is
mitochondrial damage and cytochrome c leakage, in response to oxidative stress.30 It has also been reported
that mitochondrial DNA deletions accumulated in the aging
retina may contribute to the changes in macular function
observed in aging and age-related maculopathy.29 As mitochon-
dria are organelles that are vulnerable to oxidative stress, in-
creased intracellular oxidative challenge in the aging process
could be a cause of mitochondrial damage. Although multiple
processes are involved in the pathogenesis of various retinal-
degeneration diseases, alteration of mitochondrial function
appears to play an important role in triggering apoptosis in RPE
cells.

For a model, we used cells with different levels of SOD2
protein, which was varied by gene manipulation of the mice,
because this protein is well known to be an important enzyme
in maintaining mitochondrial function against oxidative
stress.30 In an earlier study on human lens epithelial cells, in
which SOD2 was up- and downregulated with plasmids con-
taining sense and antisense vectors for cDNA for SOD2, it was
demonstrated that the cells with higher enzyme levels were
more resistant to the cytotoxic effect of H2O2, O2−, and UVB
radiation as evidenced by decreased apoptosis.21,31 Further-
more, SOD2-deficient cells revealed dramatic mitochondrial
damage, increased cytochrome c leakage, and activation of
caspase 3 and enhanced apoptotic cell death.21

Although the functional role of SOD2 in protecting against
oxidant-induced damage has been studied in animals or cells
derived from knockout animals, this approach was not possible in the current investigation because the homozygous
(Sod2−/−) mice null for the enzyme survive only for a short
postnatal period (3 days in our hands). In previous reports16,17
Sod2-knockout mice survived for 5 to 18 days. In one study, it
was reported that treatment of these mice with SOD2 mimetic
compound briefly extended the lifespan of these animals, which exhibited progressive thinning of the retina with early
effects on photoreceptors.32 Postnatal reduction of SOD2 ex-
pression in mouse eyes using ribozyme targeted against SOD2
mRNA to reduce expression of SOD2 was found to increase
ROS generation from mitochondria leading to thinning of reti-
nal layer and loss of axons and myelin in the optic nerve and
ganglion cells.33 The aforementioned studies and those de-
scribed in this communication clearly indicate the importance
of SOD2 in the retina in protection against mitochondrial
oxidative stress.

Because of our inability to breed Sod2-knockout mice, we
studied the role of this enzyme in apoptotic cell death in RPE
cells that were derived from mutant mice with different levels
of SOD2. It was thought that this model, with heterozygous
mice with roughly half the enzyme of the WT, might be
analogous to decreased antioxidant status during the aging
process and might give some insight into the ocular manifes-
tation of SOD2 deficiency. There is little information regarding
the ocular disease in partial Sod2-knockout mice because of
the absence of a phenotype in this model. Although the ani-
imals grow and reproduce normally, when subjected to oxidative
stress by arterial ischemia and reperfusion injury, there is
exacerbation of cerebral infarction caused by overgenerated
O2− in the mitochondrial compartment.54,55 There is another report that chronic mitochondrial oxidative stress in the Sod2
heterozygous mouse results in the age-related decline of mito-
ochondrial function and induction of premature apoptosis in the
liver.56 Similar mitochondrial decline was also observed in the
mice with normal SOD2 levels, but it occurred later in life.56
Thus, it is possible that similar changes may occur in the ocular
processes are involved in the pathogenesis of various retinal-
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![Figure 2](image-url)

**Figure 2.** Differential expression of SOD2 in RPE cells. (A) Extracts containing 10 μg protein from each of the cell cultures were subjected to SDS-PAGE followed by Western-blot analysis with antibody to SOD2. (B) The enzyme level was quantified by integrated density scanning of the immunoblot membrane. The relative SOD2 levels in WT, HET, and HEMI mice were 1.0, 0.6, and 3.4, respectively. Data are expressed as the mean ± SEM (n = 4). *P < 0.05 compared with those of WT.
tissues in Sod2 heterozygous mouse during the aging process, leading to pathologic changes. In the present investigation, RPE cells derived from heterozygous mice had a normal growth rate in culture (data not shown), and there were no differences in mitochondrial transmembrane potential or apoptosis, unless the cells were subjected to oxidative stress by H$_2$O$_2$ exposure. Thus, under normal physiological conditions in vivo, the RPE cells with a partial deficiency of SOD2 (60% of that in WT) may be able to withstand the oxidative stress generated by ROS under normal metabolic conditions of the retina.

The RPE is known to play an important role in phagocytosis of the photoreceptor outer segment with the generation of H$_2$O$_2$. A decrease in SOD2 (and other antioxidants) may compromise the ability of the cells to remove or detoxify the continuous oxidative challenge from H$_2$O$_2$ and other ROS. The retina is particularly susceptible to oxidative insult, because of the high concentration of polyunsaturated fatty acid, such as docosahexaenoic acid (DHA) in the membrane of photoreceptor/pigment epithelium complex. DHA has been considered a potential target of lipid peroxidation induced by ROS in retina. Yet another source of oxidative challenge to the retina

**FIGURE 3.** The effect of oxidative stress on apoptosis in RPE cells derived from mice with different levels of SOD2. Cell cultures were exposed to 300 μM H$_2$O$_2$ for 1 hour and recultured for 16 hours in a normal medium, and apoptosis was determined by TUNEL staining. H$_2$O$_2$-treated cells from HET mice showed more TUNEL-positive cells than did those from the WT or HEMI mice, whereas apoptotic cell death was suppressed in the cells from the HEMI mice.

**FIGURE 4.** Dose-dependent effect of H$_2$O$_2$ exposure on apoptosis in RPE with different levels of SOD2. An equal number of RPE cells derived from WT (○), HET (△), and HEMI (■) mice were exposed to various concentrations of H$_2$O$_2$ for 1 hour and recultured in normal medium for 16 hours, and apoptosis was determined by a cell-death-detection ELISA kit. Cells from HET mice showed greater apoptosis than did those from WT or HEMI mice at all levels of H$_2$O$_2$ exposure. A higher level of SOD2 (HEMI) protected the RPE cells from apoptosis. Data are expressed as the mean ± SEM (n = 4 to 7). *P < 0.05 compared with those of WT.

**FIGURE 5.** Time course of the effect of H$_2$O$_2$ exposure on apoptosis in RPE with different levels of SOD2. An equal number of RPE cells derived from WT (○), HET (△), and HEMI (■) mice were exposed to 300 μM H$_2$O$_2$ for 1 hour and cultured in normal medium for the different periods indicated. Apoptosis was determined by a cell-death-detection ELISA kit. Apoptotic cell death increased progressively as a function of time. Also, greater apoptosis was noted in HET cells than in WT and HEMI cells. Apoptotic cell death in HEMI mice was well suppressed until 16 hours of incubation. Data are expressed as the mean ± SEM (n = 4 to 7). *P < 0.05 compared with those of WT.
is the continuous generation of $\text{O}_2^-$ radicals, since nearly 4% of oxygen consumed by the electron transport chain in mitochondria is thought to be converted to superoxide anions, which must be removed by SOD2 through the dismutation of $\text{O}_2^-$ to $\text{H}_2\text{O}_2$. The retina is known to be one of the highest oxygen-consuming tissues of the human body because it requires a large amount of metabolic energy for visual processes. Because the RPE is located between the choroidal plexus and neural retina, which forms the outer blood-retinal barrier, it may be exposed to relatively high oxygen concentration in the retina and may be targeted by a large amount of ROS under physiological conditions. In the retina, deficiency of SOD2 enzyme in the mitochondria could increase generation of ROS and lead to perturbation of mitochondrial metabolism and the redox status in the cells. $\text{O}_2^-$ is a major source of ROS produced by mitochondria, and these ROS possibly induce decline of mitochondrial function and increase levels of oxidative products, which positively correlate to aging. To the extent that similar changes may occur in the human retina during aging, we hypothesize that an accumulation of oxidative stress from ROS during the aging process may be one of the factors that contributes to AMD, a leading cause of legal blindness in the developed world. We therefore suggest that partial knockout mice and mice with overexpression of SOD2 protein could serve as a useful model for studying mitochondria-based AMD.

In conclusion, we have shown that SOD2 plays a critical role in apoptosis in RPE cells in vitro and may have a similar

![Figure 6](image-url)

**Figure 6.** Effect of $\text{H}_2\text{O}_2$ on mitochondrial transmembrane potential in RPE cells with various levels of SOD2. (A) An equal number of cells from each of the cultures were exposed to 300 $\mu$M of $\text{H}_2\text{O}_2$ for 1 hour and then recultured for 4, 8, and 16 hours in normal medium. The cells were then treated with a cationic dye and observed under a fluorescence microscope. The decreased fluorescence (orange) began to appear at 4 hours in the WT and HET cells, indicative of the dispersion of MTP. This was more marked in cells from the HET mice than in those from the WT and HEMI mice. The images are representative of one experiment after 4, 8, and 16 hours of reculture in three separate experiments. (B) Higher magnification of the stained cells with exposure to 300 $\mu$M of $\text{H}_2\text{O}_2$ for 1 hour and reculture for 4 hours.
function in vivo. We hypothesize that a decrease of this enzyme below some critical level during the aging process may be a significant contributory factor in AMD.

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References


