Protection of Retinal Pigment Epithelial Cells from Oxidative Damage by Oltipraz, a Cancer Chemopreventive Agent

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OBJECTIVE. To determine whether oltipraz (4-methyl-5-pyrazinyl-1,2-dithiole-3-thione) protects against oxidative injury in cultured human retinal pigment epithelial (hRPE) cells.

METHODS. Primary cultured hRPE cells were incubated with various concentrations of oltipraz followed by treatment with the chemical oxidant tert-butylhydroperoxide (tBH). Cell viability was assessed by release of lactate dehydrogenase (LDH) and cleavage of WST-1. Intracellular and mitochondrial levels of glutathione (GSH) were measured by HPLC. Glutathione S-transferase (GST), NADPH-quinone reductase (NQR), and glutathione peroxidase (GPx) were measured by specific enzyme activity assays.

RESULTS. Treatment of hRPE cells with oltipraz inhibited tBH-induced cell death in a concentration-dependent manner with significant inhibition at 50 μM. Oltipraz (50 μM) increased GSH levels in hRPE cells by approximately 18% and in hRPE mitochondrial fractions by approximately 50% after 24 hours of exposure. Treatment with oltipraz increased GST and NQR activities by approximately 21% and 11%, respectively.

CONCLUSIONS. Oltipraz protects hRPE cells against tBH induced injury. The mechanism of protection is likely to include increased cellular and mitochondrial GSH levels and induction of detoxification enzymes, including GST and NQR. Dietary supplementation with oltipraz or other dithiolethiones may help protect the hRPE against oxidant induced injury. (Invest Ophthalmol Vis Sci. 2002;43:3550–3554)

Age-related macular degeneration (AMD) is a major cause of legal blindness in the United States.1,2 In AMD, pathologic changes in the retinal pigment epithelium (RPE) have been observed early in the disease process and, although a causal link has not been fully established, oxidative injury to RPE cells appears to contribute to the development of AMD.3–5 Recent results from the Age-Related Eye Disease Study (AREDS)6 have shown that antioxidants and zinc protected against progression of the disease.

Previous studies have shown that treatment of cultured human retinal pigment epithelial (hRPE) cells with reduced glutathione (GSH) or its amino acid precursors protects against cell death induced by oxidants.7,8 These results suggest that in addition to protection by dietary antioxidants, protection may be achieved by enhancing GSH-dependent systems and that at least two different mechanisms, including altering the supply of extracellular GSH or increasing the intracellular synthesis of GSH, may be involved.

It has been shown that plasma glutathione levels decrease with age,9 therefore dietary supplementation with GSH, its precursors, or the use of agents that induce GSH synthesis may be useful to maintain GSH during aging. These strategies, in addition to the use of dietary antioxidants, could be useful in protecting the RPE against oxidative injury and delay the onset or progression of AMD.

1,2-Dithiol-3-thiones are five-member cyclic sulfur-containing compounds with antioxidant, chemotherapeutic, and chemoprotective activities.10 Pharmacologic studies of oltipraz have shown that after oral administration, the drug is rapidly adsorbed, because it is detectable in the serum and urine after a short time and has a half-life of approximately 4 to 5 hours.11 Oltipraz, 4-methyl-5-pyrazinyl-1,2-dithiole-3-thione, is a compound that has been shown to increase the activity of enzymes involved in the synthesis of GSH, and certain phase II detoxification enzymes, including glutathione-S-transferase.12–14 In this study, oltipraz protected against oxidant-induced cell death in hRPE cells. This protection was associated with increased GSH levels in the cytosolic and mitochondrial compartments of the hRPE cells, as well as the induction of the detoxification enzymes glutathione-S-transferase and quinone reductase.

MATERIALS AND METHODS

Cell Culture Conditions

hRPE cell cultures were established from donor eyes obtained through the Georgia Eye Bank, as previously described.7 Methods for securing human tissues complied with the Emory University Human Investigations Review Board and the Declaration of Helsinki. Experiments were performed on hRPE cells cultured between the 4th and 10th passages in supplemented DMEM at 37°C in 95% air and 5% CO2. Oltipraz (Rhône-Poulenc Rôrer, Lyons, France) was dissolved in absolute ethanol (ETOH) vehicle and then added to the medium, resulting in a final ETOH concentration of 1.6%. Control cultures were treated with vehicle only. hRPE cells were treated with oltipraz for 24 hours, after which medium was removed and fresh medium added to the cultures. All experiments were performed in oltipraz-free medium (except for the experiment to determine whether oltipraz increases production of cellular reactive oxygen species (ROS)). Experiments were performed on standardized hRPE cultures (approximately 80% confluence). Data from replicate cultures were averaged before the calculation of the standard error of the mean.

Cytotoxicity Assays

Cell viability, in response to tBH treatment, was determined by monitoring release of lactate dehydrogenase (LDH) from hRPE cultures, as
previously described. LDH is a cytoplasmic enzyme released by dying cells. The activity of LDH in the culture medium after tBH treatment was measured spectrophotometrically, with pyruvate and reduced nicotinamide adenine dinucleotide (NADH) used as substrates. Total LDH activity was measured on Triton X-100 permeabilized hRPE cells (1% vol/vol). The ratio of the LDH released after tBH treatment compared with the LDH released after treatment with Triton X-100 was used to calculate the percentage of cell death. In some experiments, viability was assessed by reduction of the tetrazolium salt WST-1 (Roche Molecular Biochemicals, Indianapolis, IN). Mitochondrial dehydrogenases of intact and functional mitochondria reduce WST-1 to produce a colored formazan dye that can be determined spectrophotometrically. Cell viability was calculated as the activity after tBH treatment, expressed as percentage of control activity.

**GSH Analysis**

GSH concentrations were measured as previously described. Briefly, cells were lysed with 500 μL of perchloric acid solution (5% vol/vol) containing 0.2 M boric acid and 10 μM L-glutamyl glutamate (internal standard). Extracts were derivatized with iodoacetic acid and dansyl chloride and analyzed by high-performance liquid chromatography (HPLC) using fluorescence detection. The peaks were quantified by integration relative to the internal standard. GSH in enriched mitochondrial fractions was determined on samples that had been previously permeabilized with digitonin to release the cytosol. Post cytosolic samples were washed three times in ice-cold PBS and centrifuged. Cell pellets were processed in an identical manner to whole cells.

**Enzyme Assays**

Glutathione-S-transferase (GST) activity was measured spectrophotometrically at 340 nm, with 1-chloro-2,4-dinitrobenzene used as the substrate (CDNB; Sigma, St. Louis, MO). Activities obtained were normalized to protein content. Protein was determined by the Bradford method. Protein was determined by the Bradford manufacturer.

**Determination of ROS**

Production of ROS was estimated with the fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; Molecular Probes, Eugene, OR), which is a nonpolar compound that is converted into a nonfluorescent polar derivative (H2DCF) by cellular esterases. H2DCF is membrane impermeable and is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of intracellular ROS. hRPE cells were treated with oltipraz (50 μM), tBH (500 μM), or control vehicle (ethanol) for 3 hours. Cells were washed once in phosphate-buffered saline containing 10 mM glucose and incubated in H2DCFDA (50 μM) for 15 minutes. Fluorescence was measured with a confocal microscopy imaging system (Bio-Rad, Richmond, CA), using the manufacturer’s default settings for fluorescence.

**RESULTS**

**Cell Viability**

Initial experiments were performed with an oxidative stress model in which hRPE cells were treated with a toxic concentration of the oxidant tBH. Results showed that there was approximately 80% loss of viability after treatment with 300 μM tBH. Treatment with oltipraz (50 μM) 24 hours before the addition of tBH (300 μM) significantly protected against tBH-induced toxicity (Fig. 1A). LDH activity in the cell supernatants of tBH-treated hRPE cells that had been previously incubated with oltipraz for 24 hours was 16% ± 9% compared with 88% ± 6% in the supernatants from hRPE cells treated with tBH alone. Reduction of WST-1 to the colored formazan dye by hRPE cells treated with tBH (600 μM) was significantly inhibited with the reduction by control cells (the degree of this inhibition corresponded to an approximate viability of <20%). Treatment with oltipraz (24 hours) prevented loss of viability induced by tBH in a concentration-dependent manner with significant protection occurring at 50 μM (Fig 1B). These results show that oltipraz protected against tBH toxicity in hRPE.

**GSH Levels after Treatment with Oltipraz**

Because previous studies have shown that oltipraz induces an increase in GSH synthesis in other cell types, we measured GSH in hRPE after treatment with oltipraz. GSH levels were increased by 28% ± 12.5% after treatment with oltipraz (50 μM) for 24 hours (Fig. 2A), without causing a significant loss of cell viability (as measured by trypan blue, data not shown). Time course studies with 50 μM oltipraz showed that GSH was not significantly increased until 8 hours after treatment (Fig. 2B). Mitochondrial fractions from hRPE cells had significantly increased toxicity (Fig. 1A). LDH activity in the cell supernatants of tBH-treated hRPE cells that had been previously incubated with oltipraz for 24 hours was 16% ± 9% compared with 88% ± 6% in the supernatants from hRPE cells treated with tBH alone. Reduction of WST-1 to the colored formazan dye by hRPE cells treated with tBH (600 μM) was significantly inhibited with the reduction by control cells (the degree of this inhibition corresponded to an approximate viability of <20%). Treatment with oltipraz (24 hours) prevented loss of viability induced by tBH in a concentration-dependent manner with significant protection occurring at 50 μM (Fig 1B). These results show that oltipraz protected against tBH toxicity in hRPE.
higher GSH levels after treatment with oltipraz (50 µM) for 24 hours compared with control cultures with 3.6 ± 0.09 nmol/mg protein in oltipraz-treated cell samples compared with 2.3 ± 0.4 nmol/mg protein in control cell samples (P < 0.05; Fig. 2C).

GST, NQR, and GPx Activity after Treatment with Oltipraz

Oltipraz is known to increase the expression of detoxification enzymes. Measurement of GST and NQR activities 24 hours after treatment with oltipraz (50 µM) showed that the activities of both GST and NQR increased, although the magnitude of increase was relatively small. GST activity increased by 18% ± 5% compared with the control (Fig. 3). NQR activity increased by 11% ± 3% compared with the control (Fig 3). GPx activity was not increased by oltipraz, compared with the control (data not shown).

Production of ROS after Treatment with Oltipraz

To determine whether the increase in antioxidant response by oltipraz was due to the induction of oxidative stress, cells were treated with oltipraz and assayed for production of ROS by increase in fluorescence due to oxidation of H2DCF. Oltipraz-treated hRPE cells showed levels of DCF fluorescence similar to those of the ETOH-treated control cultures, indicating that oltipraz alone did not increase production of ROS in hRPE cells. As a positive control for increased production of ROS, cells were treated with 300 µM tBH (Fig. 4).

DISCUSSION

We have shown that the mechanism of cell death induced by tBH in hRPE cells involves induction of the apoptotic signaling cascade. In the present study, treatment with oltipraz protected hRPE cells against tBH-induced apoptosis, and that protection was associated with increased GSH levels in cytosolic and mitochondrial fractions of hRPE cells as well as the induction of the antioxidant systems GST and NQR. It is the parent compound that is active in the induction of phase II enzymes, because its metabolism by molecular rearrangement to yield a pyrrolopyrazine derivative is inactive in the induction of detoxification genes.

We have shown that dimethyl fumarate (DMF), a compound found in apples, also increases GSH concentrations in hRPE cells. However, in contrast to DMF with which an early decrease in GSH is followed by a large increase, treatment with oltipraz did not cause the early decline in GSH observed with DMF, although after 8 hours of treatment, a significant increase in GSH was observed. Because treatment of hRPE cells with oltipraz showed dramatic protection against tBH-mediated loss of viability, we thought that oltipraz might protect hRPE cells from this oxidative stress by increasing mitochondrial and cytosolic GSH. GSH was determined on mitochondrial fractions isolated from hRPE cells and results showed that oltipraz significantly increased mitochondrial GSH levels. Because mitochondrial GSH is a critical factor in protecting cells from oxidative stress, it is likely that oltipraz-mediated protection against tBH is attributable at least in part to increased mitochondrial GSH. It is known that phase II enzymes detoxify xenobiotics by a process of conjugation and excretion. However, these enzymes also protect against oxidative stress by increasing the generation of cellular antioxidants. Because Talalay et al. recently suggested that 1,2-dithiole-3-thiones induce the specific expression of GST and NQR, we
investigated whether oltipraz induces these enzymes. Our results showed that pretreatment with oltipraz caused a marginal increase in both GST and NQR, reflecting the possible involvement of these enzymes in the protection of hRPE against tBH.

The induction of these enzymes by oltipraz, combined with increased intracellular GSH, suggests that a multiplicity of factors may be involved in oxidant protection of hRPE cells by oltipraz.

Furthermore, because oltipraz contains two thiol groups, it may also protect against tBH-induced apoptosis by acting as a metal chelator and removing the potential for Fenton-mediated production of reactive hydroxyl radicals from the hydroperoxide. Indeed, it was recently shown that oxidative cell death in the presence of iron depends on the involvement of Fenton-type chemistry and peroxidative damage to lysosomal membranes, and could be prevented by lipoic acid and lipoamide which stabilize lysosomes, suggesting that the mechanism involves intralysosomal iron chelation that could prevent Fenton reactions.31

We also thought that, because it has been suggested that oltipraz induces the transcription of antioxidant-coding genes by a mechanism involving increased production of ROS,34 oltipraz may cause oxidative stress, and the cellular response to this could be an increase in antioxidant defenses.32,33 Oltipraz-mediated increase in GSH levels and elevated GST and NQR enzyme activities may be the result of oxidative stress induced by the compound. To investigate this we preloaded hRPE cells with the ROS-sensitive probe H2DCFHDA and treated cells with oltipraz. The treatment did not increase production of ROS in hRPE, suggesting that its ability to induce antioxidant protection of hRPE cells involves a mechanism other than ROS-dependent increase in antioxidant defenses. However, a possible reason for the failure to observe significant production of ROS after treatment with oltipraz could be altered experimental conditions.

Although the mechanism by which oltipraz induces antioxidant protection remains unknown, it may react with GSH, producing an electrophilic center which then binds with the nucleophilic center of a regulatory transcription factor. In this scenario, binding to Keap 1 could trigger release of Nrf2, allowing its translocation into nuclei and activation of transcription of phase II enzymes.34 Such a mechanism may explain the moderate decrease in GSH noted immediately after administration of oltipraz.

In summary, these data show that oltipraz protected against loss of hRPE cell viability due to oxidative stress by inducing a cellular antioxidant response. Because protection of the RPE from oxidative stress may be an important factor in the progression or development of AMD, these findings suggest that oltipraz or other dithiolethiones may be useful in preventing or delaying this disease.

References

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