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Oltipraz-induced phase 2 enzyme response conserved in cells lacking mitochondrial DNA ☆

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Abstract

Oltipraz, a member of a class of 1,2-dithiolethiones, is a potent phase 2 enzyme inducing agent used as a cancer chemopreventive. In this study, we investigated regulation of the phase 2 enzyme response and protection against endogenous oxidative stress in lymphoblastic leukemic parental CEM cells and cells lacking mitochondrial DNA (mtDNA) (ρ^0) by oltipraz. Glutathione (GSH) levels (total and mitochondrial) and glutathione *S*-transferase (GST) activity were significantly increased after pretreatment with oltipraz in both parental (ρ^+) and ρ^0 cells, and both cell lines were resistant to mitochondrial oxidation, loss of mitochondrial membrane potential, and cell death in response to the GSH depleting agent diethylmaleate. These results show that the phase 2 enzyme response, by enhancing GSH-dependent systems involved in xenobiotic metabolism, blocks endogenous oxidative stress and cell death, and that this response is intact in cells lacking mtDNA.
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Keywords: Oltipraz; Glutathione; ρ^0; mtDNA; Glutathione *S*-transferase; Mitochondrial permeability transition; Reactive oxygen species

1,2-Dithiol-3-thiones are five-membered cyclic sulfur-containing compounds with antioxidant, chemotherapeutic, and chemoprotective activities [1]. Oltipraz, 4-methyl-5-pyrazinyl-3H-1,2-dithiole-3-thione, is a compound that has been shown to increase the activity of enzymes involved in the synthesis of glutathione (GSH), and certain phase 2 detoxification enzymes, including glutathione-*S*-transferase (GST) [2–4]. While GSH or its precursor amino acids have been shown to protect cells against oxidant-induced cell death [5,6], GSH depletion or cysteine starvation, which leads to GSH depletion, has been shown to increase oxidative stress, induce the mitochondrial permeability transition (MPT), and cause cell death [7,8].

The factors involved in the regulation of mitochondrial integrity are complex and predominantly involve the Bcl-2 family of proteins which regulate mitochondrial outer membrane permeabilization [9,10] and GSH, which regulates the redox state of mitochondrial protein vicinal thiols involved in the mitochondrial permeability transition (MPT) independently of Bcl-2 [7,11,12]. The MPT is also regulated, in part, by the mitochondrial respiratory chain (MRC) since pharmacological inhibition of respiratory complex III (cytochrome bc_1) with stigmatellin and complex VI (adenine nucleotide translocator) with bongkrekic acid both of which block

☆ *Abbreviations:* GSH, glutathione; ρ^0, rho 0; MMP, mitochondrial membrane permeabilization; DEM, diethylmaleate; ANT, adenine nucleotide translocator; MPT, mitochondrial permeability transition; BgK, bongkrekic acid; Nrf2, nuclear transcription factor erythroid 2p45 (NF-E2)-related factor 2; Keap1, Kelch-like ECH-associated protein-1.

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oxidative phosphorylation (OX-PHOS) prevents or delays the MPT and cell death after GSH depletion [7,8,13].

In this study, we used a lymphoblastic leukemic CEM cell line deficient in mitochondrial DNA (mtDNA) and the phase 2 inducing agent oltipraz to determine: (1) whether enhancing GSH-dependent systems protected against endogenous oxidative stress caused by the GSH depleting agent diethylmaleate (DEM) and (2) whether this response was conserved in cells lacking mtDNA. We found that oltipraz pretreatment prevented reactive oxygen species (ROS) formation, MPT, and cell death after GSH depletion and that this effect was conserved in cells lacking mtDNA. These results suggest that oltipraz may be a useful pharmacological strategy to prevent oxidative stress-induced cell death caused by loss of GSH by enhancing GSH-dependent systems involved in xenobiotic metabolism.

Materials and methods

Chemicals and cell culture. All chemicals were of reagent grade and were obtained from Sigma chemical company. DEM was used at 5 mM for ROS assays, mitochondrial determinations, and cell viability studies. Tetra-methyl rhodamine methyl ester (TMRM) and dichlorofluorescein diacetate (DCFDA) were obtained from Molecular Probes (Eugene, Oregon). CEM parental (ρ^+) cells were cultured in RPMI, and 10% FBS and supplements as described previously [13]. Cells were passaged daily to maintain them in log-phase growth and kept at a nominal concentration of $2.5–5 \times 10^5$/ml. CEM ρ^0 cells were derived from CEM cells by culturing in the presence of 50 ng/ml of ethidium bromide for 4–6 weeks. These ρ^0 cells were then cultured in RPMI with 1 mM pyruvate and 50 μg/ml uridine, and regularly monitored for KCN-sensitive oxygen consumption. Oltipraz was obtained from LTK Laboratories (St. Paul, Minnesota) and cells were pretreated with 50 μM oltipraz for 16.5 h prior to experimentation. Cell viability was determined by trypan blue exclusion.

Biochemical methods. Oxygen consumption was determined polarographically using a Clarke-type oxygen electrode (Hansatech Instruments, Norfolk, England); GSH and GSSG levels and GST activity were measured using commercial kits (Cayman Chemicals, Ann Harbor, MI, USA); the GSH redox potential (E_h) was calculated using the *Nernst* equation using a cell volume as 7 μl per million cells [14] and E_0 at pH 7.0 was taken as −240 mV [15]. Mitochondrial isolation was performed according to [16] with modification. Protein concentration of each fraction was determined by the D*c* protein assay (Bio-Rad, Hercules, CA).

Measurement of oxidation (ROS) production and mitochondrial membrane potential ($\Delta\psi_m$). Flow cytometry determinations for cellular ROS formation and $\Delta\psi_m$ were performed as described previously [13]. Briefly, for ROS determination, cells were treated with DEM for 0, 10, 20, 30, 40, 50, and 60 min, loaded with 10 μM DCFDA for 15 min, washed with phosphate-buffered saline containing 10 mM glucose, and analyzed immediately by flow cytometry using the FITC channel (log mode). In each analysis, 10,000 events were recorded. For determination of $\Delta\psi_m$, cells were treated with DEM for 0, 30, 60, 90, 120, and 150 min, and loaded with 250 nM TMRM (TMRM is a cationic dye which accumulates within mitochondria in accordance with the $\Delta\psi_m$ *Nernst* potential) for 15 min and red fluorescence was determined by flow cytometry using the PE channel [13]. The protonophore carbonyl-CCCP (10 μM) was used to dissipate the chemiosmotic proton gradient ($\Delta\mu H+$) and served as a control for loss of $\Delta\psi_m$. In each analysis, 10,000 events were recorded.

Electron microscopic examination of CEM cells. CEM cells in the logarithmic proliferation phase were cultured with RPMI and DEM (5 mM) ± oltipraz for 120 min and were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at room temperature for 1 h. The cells were prepared for electron microscopic (EM) as described previously [13]. Briefly, cells were washed with 0.1 M cacodylate buffer and post-fixed with 1% osmium tetraoxide in 0.1 M cacodylate buffer. Finally, the cells were dehydrated with graded series of ethanol and embedded in LX112. Thin sections were prepared and stained with uranyl acetate. Specimens were examined on a JEOL 1000X electron microscope operating at 80 kV.

Statistical analysis. Statistical analyses were performed using one-way ANOVA and p values <0.05 were considered significant. Data are presented as means ± SEM.

Results

GSH levels are increased in CEM ρ^+ and ρ^0 cells after treatment with oltipraz

GSH and GSSG levels and GSH redox (E_h) potential were determined on aliquots of CEM ρ^+ and ρ^0 cells pretreated with oltipraz ± DEM. Oltipraz pretreatment increased GSH levels in ρ^+ cells from 60.8 ± 4.2 nmol/mg protein to 85.4 ± 5.9 nmol/mg protein ($P < 0.05$) and increased GSH levels in ρ^0 cell from 38.9 ± 2.2 nmol/mg protein to 64.7 ± 5.2 nmol/mg protein ($P < 0.05$) (Fig. 1A). This represents an approximate 40% increase in GSH levels in ρ^+ cells and an approximate 60% increase in GSH in ρ^0 cells. DEM treatment for 2 h significantly reduced GSH levels (>95%) in both ρ^+ (0.24 ± 0.07 nmol/mg protein) and ρ^0 (0.47 ± 0.07 nmol/mg protein) cell lines. Pretreatment of cells with oltipraz prevented GSH depletion after DEM treatment since GSH levels were 15.9 ± 1.2 nmol/mg protein in ρ^+ cells and 6.3 ± 1.0 nmol/mg protein in ρ^0 cells.

Oltipraz pretreatment increased mitochondrial GSH levels in ρ^+ cells from 6.2 ± 0.3 nmol/mg protein to 7.6 ± 0.5 nmol/mg protein ($P < 0.05$) and increased mitochondrial GSH levels in ρ^0 cells from 4.3 ± 0.5 nmol/mg protein to 5.1 ± 0.2 nmol/mg protein ($P < 0.05$) (Fig. 1B). This represents an approximate 20% increase in mitochondrial GSH levels in both ρ^+ and ρ^0 cells. DEM treatment for 2 h significantly reduced mitochondrial (>95%) in both ρ^+ (0.31 ± 0.04 nmol/mg protein) and ρ^0 (0.26 ± 0.02 nmol/mg protein) cell lines. Pretreatment of cells with oltipraz prevented mitochondrial GSH depletion after DEM treatment since mitochondrial GSH levels were 1.156 ± 0.5 nmol/mg protein in ρ^+ cells and 0.389 ± 0.1 nmol/mg protein in ρ^0 cells.

The GSH *Nernst* redox potential (E_h) increased ~120 mV in ρ^+ cells treated with DEM compared to control cells and ~30 mV in ρ^+ cells pretreated with oltipraz + DEM compared to control cells. $E_h = -264.3 \pm 13.6$ mV in control cells compared with $E_h = -138.5 \pm 5.1$ mV in DEM treated cells and $E_h = -287.6 \pm 12.1$ mV in ρ^+ control cells treated with oltipraz compared with -234.6 ± 11.1 mV in ρ^+ cells treated with oltipraz + DEM (Table 1).

The GSH *Nernst* redox potential (E_h) increased ~90 mV in ρ^0 cells treated with DEM compared to control cells and ~40 mV in cells treated with oltipraz + DEM compared to

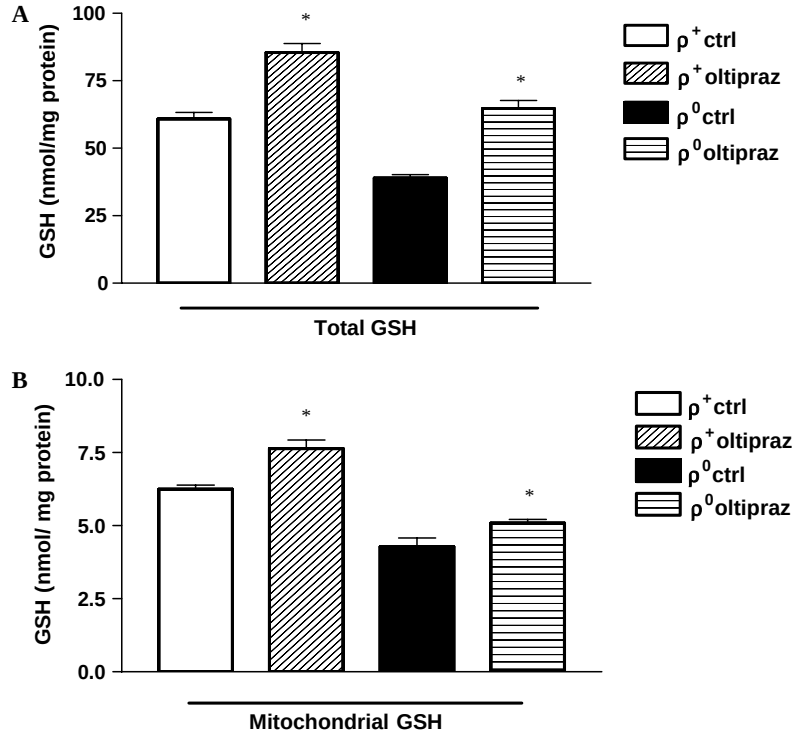

Fig. 1. (A) CEM parental ρ^+ and ρ^0 cells were incubated in RPMI ± 50 μM oltipraz for 16.5 h. GSH levels were determined on these samples as described under Materials and methods. The GSH concentration is plotted as nanomole per milligram protein. Data are expressed as means ± SEM ($n = 3$). (B) CEM parental ρ^+ and ρ^0 cells were incubated in RPMI ± 50 μM oltipraz for 16.5 h. Mitochondrial fractions were isolated as described under Materials and methods. GSH levels were determined on these samples as described under Materials and methods. The GSH concentration is plotted as nanomole per milligram protein. Data are expressed as means ± SEM ($n = 3$). (*$P < 0.05$).

Table 1
Aliquots of CEM parental ρ^+ and ρ^0 cells approximately 4×10^6/ml) preincubated in RPMI ± 50 μM oltipraz for 16.5 h and then treated with DEM or RPMI (control) for 0, or 120 min

Nernst GSH redox potential E_h (mV)	ρ^+	ρ^0
Control	−264.3 ± 13.6	−246.6 ± 12.7
DEM	−138.5 ± 5.1	−155.7 ± 10.1
Oltipraz	−287.6 ± 12.1	−272.4 ± 10.1
Olipraz + DEM	−234.6 ± 11.1	−202.8 ± 8.8

GSH and GSSG concentrations were determined by assays described under Materials and methods. The GSH redox potential (E_h) in millivolt was calculated using the Nernst equation as described under Materials and methods. Data are expressed as means ± SEM ($n = 3$).

control cells. $E_h = -246.6 \pm 12.7$ mV in control ρ^0 cells compared with $E_h = -155.7 \pm 10.1$ mV in DEM treated ρ^0 cells and $E_h = -272.4 \pm 10.1$ mV in ρ^0 control cells treated with oltipraz compared with -202.8 ± 8.8 mV in ρ^0 cells treated with oltipraz + DEM (Table 1).

GST levels are increased in CEM ρ^+ and ρ^0 cells after treatment with oltipraz

Oltipraz is known to increase the expression of phase 2 detoxification enzymes. GST activity increased from 9.4 ± 0.6 to 14.9 ± 0.3 nmol/min/mg protein in ρ^+ cells treated with oltipraz for 16.5 h ($P < 0.05$). This represents an approximate 60% increase in GST activity. GST activity increased from 13.5 ± 0.2 nmol/min/mg protein in ρ^0 cells to 18.6 ± 0.2 nmol/min/mg protein in CEM ρ^0 cells after treatment with oltipraz for 16.5 h (approximately 40%) ($P < 0.05$) (Fig. 2).

GSH depletion causes oxidation (ROS) production in CEM ρ^+ and ρ^0 cells which is blocked by pretreatment with oltipraz

Cellular oxidation and ROS production were determined in cells by monitoring the fluorescence of the ROS-sensitive dye DCF. Increased DCF fluorescence, a measure of intracellular oxidation and ROS production [7], was determined by a shift in DCF fluorescence to the right on the *x*-axis of the FACS histogram. CEM parental ρ^+ and ρ^0 cells were incubated in RPMI ± oltipraz. Fig. 3A shows representative DCF FACS histograms of CEM cells stained with DCFDA and analyzed using FITC channel as described in the methods section. Aliquots of ρ^+ and ρ^0 cells (2×10^6/ml) were treated with DEM for 0, 10, 20, 30, 40, 50, and 60 min, washed in PBS, and suspended in PBS containing 10 mM glucose. Cells were loaded with 10 μM DCFDA for 15 min and fluorescence was measured by flow cytometry using the FITC channel. Fig. 3A shows that DEM treatment caused the DCF fluorescence to shift right on the *x*-axis of the FACS histogram indicating increased ROS production. In each analysis,

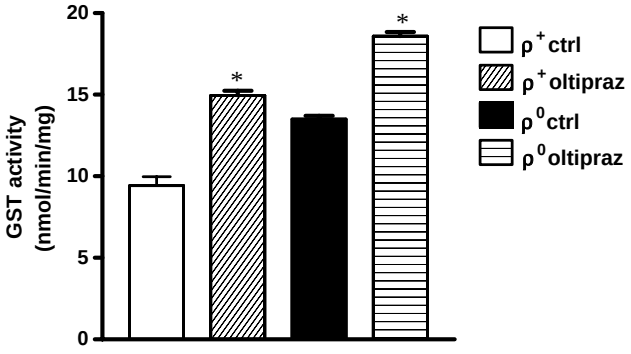

Fig. 2. CEM parental ρ^+ and ρ^0 cells were incubated in RPMI ± 50 μM oltipraz for 16.5 h and GST activity was determined as described under Materials and methods. GST activity in parental CEM ρ^+ cells was 9.4 ± 0.6 nmol/min/mg protein compared to 14.9 ± 0.3 nmol/min/mg protein after treatment with oltipraz. GST activity of CEM ρ^0 cells was 13.5 ± 0.2 nmol/min/mg protein compared to 18.6 ± 0.2 nmol/min/mg protein after treatment with oltipraz. (*$P < 0.05$).

10,000 events were recorded. Fig. 3B shows a bar graph of mean DCF fluorescence (a.u.) of CEM parental ρ^+ and ρ^0 cells before and after treatment with DEM ± oltipraz determined from mean fluorescence (a.u.) of FACS histograms as described above. In each analysis, 10,000 events were recorded. Data are expressed as means ± SEM ($n = 3$). Fig. 3C (top panel) shows a representative DCF FACS histogram of CEM cells stained with DCFDA and analyzed using FITC channel as described in the methods section before and after treatment with oltipraz. Figure shows that oltipraz increased the basal DCF fluorescence in both parental ρ^+ and ρ^0 cells. Fig. 3C (bottom panel) shows a bar graph of mean DCF fluorescence (a.u.) of CEM parental ρ^+ and ρ^0 cells. The basal DCF fluorescence increased from 80.3 ± 4.2 (a.u.) to 125.3 ± 14.8 (a.u.) in ρ^+ cells treated with oltipraz ($P < 0.05$). The basal DCF fluorescence increased from 138.0 ± 10.3 (a.u.) in ρ^0 cells to 205.8 ± 14.1 (a.u.) in CEM ρ^0 cells after treatment with oltipraz ($P < 0.05$).

GSH depletion causes ultrastructural changes in mitochondrial cristae of CEM cells and loss of $\Delta\psi_m$ that is blocked by pretreatment with oltipraz

Fig. 4A, caption "1" shows typical mitochondrial structure in untreated parental CEM cells. Inset arrowheads

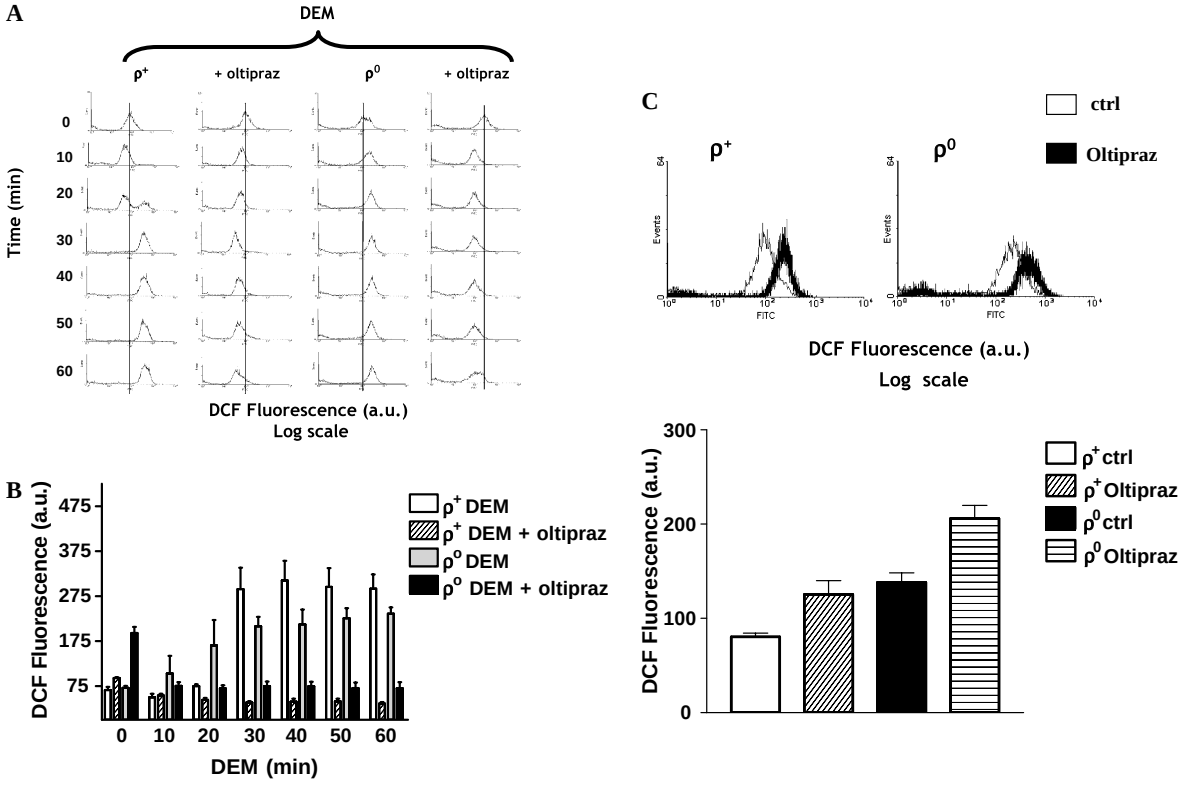

Fig. 3. (A) CEM parental ρ^+ and ρ^0 cells were incubated in RPMI ± 50 μM oltipraz for 16.5 h. Figure shows representative DCF FACS histograms of CEM cells stained with DCFDA and analyzed using FITC channel as described under Materials and methods. Aliquots of ρ^+ and ρ^0 cells (2×10^6/ml) were treated with DEM for 0, 10, 20, 30, 40, 50, and 60 min, washed in PBS, and suspended in PBS containing 10 mM glucose. Cells were loaded with 10 μM DCFDA for 15 min and fluorescence was measured by flow cytometry using the FITC channel. Representative example from three independent experiments. In each analysis, 10,000 events were recorded. (B) Bar graph showing mean DCF fluorescence (a.u.) of CEM parental ρ^+ and ρ^0 cells determined from mean fluorescence (a.u.) of FACS histograms as described above. In each analysis, 10,000 events were recorded. Data are expressed as means ± SEM ($n = 3$). (C) Top panel shows representative FACS histograms of CEM parental ρ^+ and ρ^0 cell of DCF fluorescence before and after treatment with 50 μM oltipraz for 16.5 h. Bottom panel shows bar graph of mean DCF fluorescence (a.u.) of CEM parental ρ^+ and ρ^0 cells before and after treatment with 50 μM oltipraz for 16.5 h. Data are expressed as means ± SEM ($n = 3$).

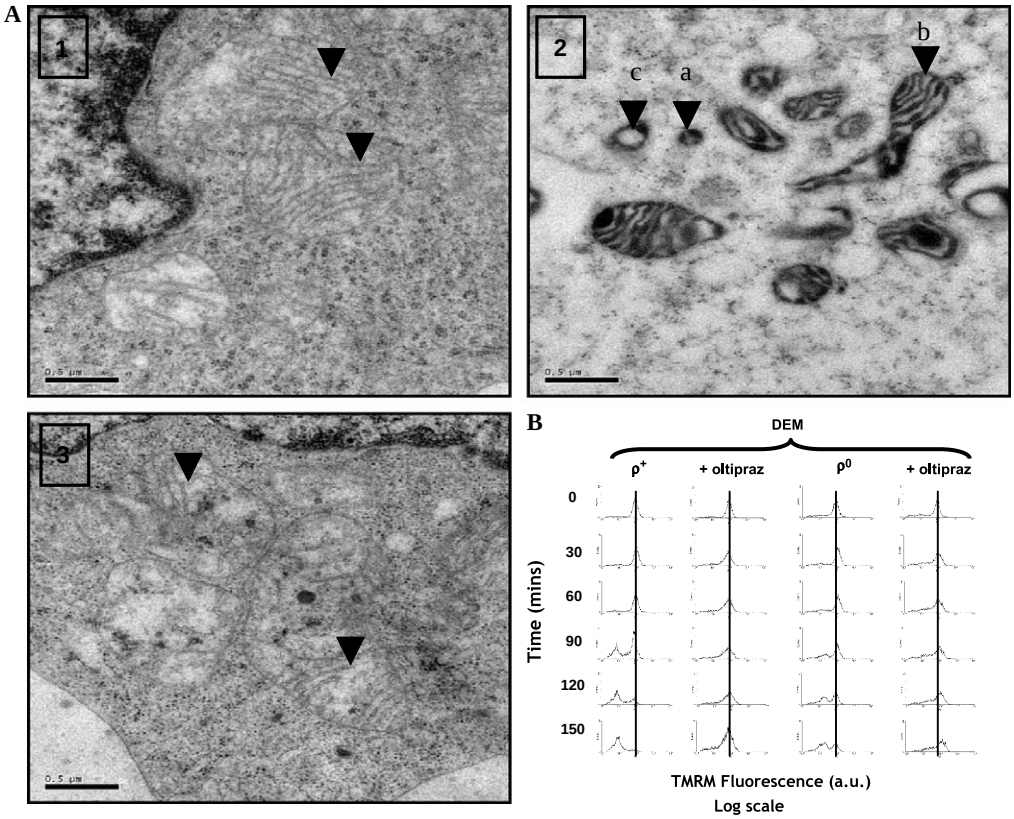

Fig. 4. (A) Representative electron micrographs (EM) of CEM cells incubated in RPMI ± 50 μM oltipraz for 16.5 h and then treated with DEM or RPMI (control) for 0, or 120 min. Fig. 2A, caption 1 shows mitochondria in control cells; inset arrowheads point to mitochondrial cristae. Caption 2 shows mitochondria of cells treated with DEM; inset arrowheads point to: (a) fragmented electron dense mitochondrion, (b) mitochondrion showing increased electron density of mitochondrial cristae, and (c) vacuolated mitochondrion. Caption 3 shows mitochondria of cells treated with DEM + oltipraz; inset arrowheads point to mitochondrial cristae. EM results suggest that oltipraz preserves mitochondrial cristae (the sites of electron transport and oxidative phosphorylation) after GSH depletion compared to DEM treatment alone in which the mitochondria ultrastructure is significantly altered. (B) Representative flow cytometric analysis of CEM cells stained with TMRM and analyzed using PE channel as described under Materials and methods. CEM parental ρ^+ and ρ^0 cells were incubated in RPMI ± 50 μM oltipraz for 16.5 h. Figure shows representative FACS histograms of CEM cells loaded with TMRM and analyzed using PE channel as described under Materials and methods. Aliquots of ρ^+ and ρ^0 cells (2×10^6/ml) were treated with DEM for 0, 30, 60, 90, 120, and 150 min, washed in PBS, and suspended in PBS containing 10 mM glucose. Cells were loaded with 250 nM TMRM for 15 min and red fluorescence was measured by flow cytometry using the PE setting. Representative example from at least three independent experiments. In each analysis, 10,000 events were recorded.

point to mitochondrial cristae showing typical crystal ultrastructure of CEM cell mitochondria. Caption "2" shows mitochondria of cells treated with DEM; inset arrowheads point to: (a) small fragmented electron dense mitochondrion, (b) mitochondrion showing increased electron density of remodeled mitochondrial cristae, and (c) typical vacuolated mitochondrion. Caption "3" shows mitochondria of cells pretreated with 50 μM oltipraz for 16.5 h and then treated with DEM for 120 min; inset arrowheads point to mitochondrial cristae. EM results indicate that oltipraz pretreatment preserved mitochondrial ultrastructure after GSH depletion. Fig. 4B shows the $\Delta\psi_m$ of ρ^+ and ρ^0 cells (determined by monitoring the fluorescence of cells loaded with the membrane potential dye TMRM). Decreased TMRM fluorescence, a measure of cells with decreased $\Delta\psi_m$, was determined by a shift in TMRM fluorescence to the left on the x-axis of the FACS histogram. Fig. 4B shows that cells with decreased TMRM fluorescence significantly increased in number approximately 90 min after treatment with DEM and this was blocked by pretreatment with oltipraz in both ρ^+ and ρ^0 cell lines.

Oltipraz blocks cell death in CEM ρ^+ and ρ^0 cells after GSH depletion

Since oltipraz blocked ROS production and preserved mitochondrial structure and $\Delta\psi_m$ of CEM cells, we determined cell viability after DEM treatment. Fig. 5 shows that oltipraz pretreatment significantly preserved cell viability in ρ^+ and ρ^0 cells after treatment with DEM.

Discussion

The phase 2 enzyme induction system is crucial for detoxification of carcinogens by conjugation with GSH which increases their solubility and allows their excretion

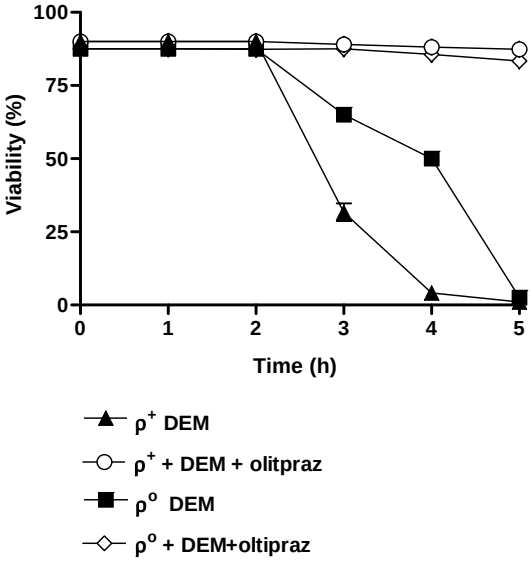

Fig. 5. CEM cells were incubated in RPMI ± 50 μM oltipraz for 16.5 h. Aliquots of cells were treated with either RPMI (control) or DEM for 10 h. Cell viability was determined at 1 h intervals during the time course of the experiment. Control omitted for clarity. Cell viability was determined by trypan blue analysis. Data are expressed as means ± SEM ($n = 3$).

[17–19]. It is unknown whether loss of mtDNA, as occurs in aging [20], has any effect on this important detoxification system; however, decline in the efficacy of this system would predispose cells to increased carcinogen overload and cell transformation. Our present results show that oltipraz induces the phase 2 enzyme response in both ρ^+ and ρ^0 cells with similar efficacy and this system protects cells against endogenous oxidative stress mediated by GSH depletion.

In agreement with previous reports we found that oltipraz significantly increased GSH levels and GST activity [5]; however, we also found that oltipraz caused a significant increase in GSH levels and in GST activity in ρ^0 cells, indicating that the pathway regulating the phase 2 response is conserved in cells lacking mtDNA. Interestingly, total GSH levels were lower in control ρ^0 cells compared to control ρ^+ cells and GSH *Nernst* redox potential showed that ρ^0 cells were more oxidized than ρ^+ cells which has previously been reported in ρ^0 osteosarcoma cells [21]. While, on the other hand, GST activity was significantly increased in the ρ^0 cell line which may be the result of the ethidium bromide used to create the cell line inducing a phase 2 enzyme response. Mitochondrial GSH is a critical factor in protecting cells from oxidative stress and oltipraz treatment significantly increased mitochondrial GSH, as previously reported [5], suggesting that protection against DEM-mediated toxicity may be due, in part, to the increased mitochondrial GSH fraction [22,23]. On the other hand, since the phase 2 response also protects against oxidative stress by increasing cellular antioxidants including manganese superoxide dismutase (MnSOD) and heme oxygenase-1 (HO-1) [24,25] suggests that a multiplicity of factors may be involved in the phase 2 response especially since cells lacking mtDNA are known to have increased levels of HO-1 and MnSOD [26].

Protection against DEM-mediated toxicity by pretreatment of cells with oltipraz appears to be the result of increased phase 2 detoxification potential (including GSH levels and GST activity) since after oltipraz treatment DEM did not completely deplete cellular GSH and the GSH *Nernst* redox potential of cells was significantly more reduced compared to DEM treatment alone (Table 1). Since it is known that GSH regulates the redox status of mitochondrial protein vicinal thiols involved in regulation of the MPT [7,11,12], it is likely that the preservation of cellular and mitochondrial GSH levels by oltipraz was responsible for blocking the activation of the MPT and cell death.

The major metabolite of oltipraz, a pyrrolopyrazine thione, has been shown to be a phase two enzyme inducer via the formation of a GSH stimulated redox cycle producing superoxide radical suggesting a possible mechanism by which oltipraz might increase in the transcription of phase 2 enzymes mediated by nuclear transcription factor erythroid 2p45 (NF-E2)-related factor 2 (Nrf2) [27]. The pyrrolopyrazine thione-dependent production of superoxide anion could cause redox-dependent dissociation of Nrf2 from Keap1, allowing it to translocate to the nucleus and activate its antioxidant response element (ARE), causing transcriptional regulation of target genes. In keeping with this idea, we found that oltipraz treatment increased basal DCF fluorescence in both cell lines (indicating increased oxidative stress) compared to untreated control cells (Fig. 3C). Our results, therefore, indicate that this redox mechanism of Nrf2 activation is independent of a functional respiratory chain and therefore of mitochondrial ROS production.

In summary, our data show that oltipraz protected cells against endogenous oxidative stress caused by GSH depletion, mitochondrial permeability transition, and cell death by inducing the phase 2 response and that this is conserved in cells lacking mtDNA indicating that the Nrf2-ARE core signaling pathway is intact in the absence of a functional MRC. Oltipraz, which is already in use in phase 2 clinical trials is rapidly adsorbed and is therefore ideally suited for oral use [28], may provide an alternative approach for the design and development of potential therapeutic antioxidants in age-related diseases where there is oxidative stress component, especially since progressive loss of mtDNA is characteristic feature of aging.

Acknowledgments

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