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Depleted folate pool and dysfunctional mitochondria associated with defective mitochondrial folate proteins sensitize Chinese ovary cell mutants to *tert*-butylhydroperoxide-induced oxidative stress and apoptosis☆

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Abstract

The functional role of mitochondrial (mt) folate-associated proteins in mammalian cells is not clearly understood. We investigated the respiratory function and apoptosis phenotype of Chinese hamster ovary (CHO) mutant cells with defective mt serine hydroxymethyltransferase (SHMT) activities (glyA) or with defective mt folate transporter (glyB) in the absence/presence of oxidant challenge. The mechanisms underlying their aberrant phenotypes were explored. Compared with CHOK1 wild-type cells, both mutants carried dysfunctional mitochondria with reduced respiratory complex IV activity and depolarized mt membrane potential ($P<.05$). Elevated superoxide levels and accumulated mtDNA large deletions were observed in glyB in association with a depleted compartmental folate pool ($P<.05$). *tert*-Butylhydroperoxide (tBH) treatment at 50 μM for 72 h significantly depleted mt and cytosolic folate levels, impaired antioxidant defenses, and aggravated mt oxidative dysfunction in both mutants ($P<.05$), more severely in glyB. Only tBH-treated glyB cells displayed an elevated ratio of mt Bax/Bcl-2, activation of procaspases 9 and 3, and apoptosis promotion. The apoptotic phenotype of tBH-treated glyB could be partially corrected by folate supplementation (10–1000 μM), which enriched compartmental folate levels, restored antioxidant defenses, eliminated mt oxidative injuries, and normalized mt membrane function. Our data identify previously unrecognized roles of mt folate-associated proteins in the protection of mitochondria against oxidative insults. Defective mt folate transporter sensitized glyB cells to elevated oxidative stress and tBH-induced apoptosis, partly mediated by depleted compartmental folate and mt dysfunction. Defective mt SHMT sensitized glyA to respiratory dysfunction and tBH-induced oxidative injury without apoptosis promotion.

Keywords: Folate metabolism; Apoptosis; Oxidative stress; Respiratory dysfunction; Chinese hamster ovary cell mutants

1. Introduction

Biochemical defects in mitochondrial (mt) folate-associated proteins may affect mt folate metabolism and cellular growth [1]. A Chinese hamster ovary (CHO) mutant cell line, glyB, carries a point mutation in mt folate transporter, leading to ineffective transport of folate into mitochondria [2–4]. CHO mutant cell line glyA is deficient in activities of mt serine hydroxymethyltransferase (SHMT) with functional cytosolic isozyme [5,6]. SHMT catalyzes the reversible interconversion of serine and tetrahydrofolate (THF) to glycine and 5,10-CH_2-THF; mt SHMT is the main source of one-carbon units for both mt and cytoplasmic folate metabolism and nucleoside biosynthesis [7]. The biochemical defects of both mutants result in glycine auxotrophy [1–7]. With a folate-dependent supply of glycine, the mutants can survive, raising the question of the role of mt folate-associated proteins in cellular function.

Mitochondria are central to the life of mammalian cells, but mt respiratory phosphorylation is a major intracellular source of reactive oxygen species (ROS) [8,9]. Elevated oxidative stress that exceeds the capacity of detoxification and repair pathways may damage mtDNA, proteins and phospholipids, disrupting respiratory function and further aggravating the endogenous ROS-generated vicious cycle [10]. Mitochondria with dysfunctions induced by toxic chemicals and oxidants act as killer organelles, the central executors of apoptosis or programmed cell death [11]. Damaged mitochondria localize the proapoptotic proteins Bax and Bak to interact with the antiapoptotic protein Bcl-2 in the regulation of oxidative stress-inducible collapse of the inner mt membrane potential [12–14]. This mt permeability transition releases cytochrome *c* from mitochondria, which may subsequently activate the apoptotic factor Procaspase 9 followed by the apoptosis executor Caspase 3 to promote apoptosis [15]. Deregulation of mt respiratory function was associated with dissipation of mt membrane potential and apoptosis [16]. Antioxidants could suppress expression of death signaling to inhibit apoptosis of oxidant-treated cells [17].

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Folate has been proposed as an antioxidant vitamin that plays a crucial role in the maintenance of mt function. Folate deprivation leads to mt degeneration and an increase in depolarized mitochondria in neurons [18,19]. Folate deficiency induces mt oxidative decay [20] and promotes apoptotic injury [21,22]. Folate supplementation may protect against the accumulation of the mtDNA 4834 bp large deletion (mtDNA^{4834} deletion) associated with elevated oxidative stress [23] in the livers of chemotherapeutic drug-treated rats [24] and of aging rats [25]. In light of the antioxidant role of folate to protect against mt oxidative decay, and the influence of defective mt folate-associated enzymes on one-carbon metabolism [1–7], we hypothesize that the defects in mt folate-associated proteins in glyA and glyB affect compartmental folate levels and mt function, possibly sensitizing these mutants to oxidant-induced oxidative injury and apoptosis. We characterized the oxidative stress, compartmental folate status, respiratory function and apoptosis phenotype in CHOK1 wild-type cells and glyA and glyB mutants before and after oxidant challenge. We compared the altered phenotypes of mutants and wild-type cells and discuss the mechanistic links between defective mt folate metabolism, mt dysfunction and regulation of apoptosis.

2. Materials and methods

2.1. Materials

tert-Butylhydroperoxide (tBH), folate (pteroylmonoglutamic acid), hypoxanthine, thymidine, rhodamine 123, propidium iodide (PI), vitamin C, vitamin E, *N*-acetyl-L-cysteine (NAC), 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and hydroethidine (HE) were purchased from Sigma Chemical (St. Louis, MO, USA). Minimal essential medium (MEM) alpha without ribosides, ribotides, deoxyribosides, deoxyribotides, glycine, serine and folate was specially formulated by JRH (Lenex, KS, USA). Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT, USA). Antibiotics and trypsin were purchased from Gibco Laboratories (Grand Island, NY, USA). Rabbit antihuman Bcl-2, Bax, Bak, procaspases 3 and 9 and cytochrome *c* antibodies came from BD Biosciences (San Jose, CA, USA) and R&D Systems (Minneapolis, MN, USA). DNA markers came from Pharmacia Biotech (Piscataway, NJ, USA).

2.2. Cell culture

CHOK1, a Chinese hamster ovary cell line, was purchased from the Cell Bank of the National Health Research Institutes in Taipei, Taiwan (CCRC 60027). The glyA and glyB cell mutants, which were derived from CHOK1 cells, were generous gifts from Professor Barry Shane (University of California at Berkeley). Cells were cultured in complete MEM Alpha supplemented with 10% FBS and antibiotics (2000 U/L penicillin and 20 mg/L streptomycin). The complete MEM Alpha contained 10 g powdered basal medium and 2.2 g $NaHCO_3$ per liter of deionized water, supplemented with penicillin (20 U/ml), streptomycin (20 mg/ml), 600 μM glycine, 36 μM hypoxanthine, 36 μM thymidine and 2 μM folate. Cells were maintained in a humidified 5% CO_2 atmosphere at 37°C. Cellular viability was determined by trypan blue exclusion.

2.3. Oxidant and antioxidant preparation

For all experiments, a tBH stock solution was prepared at 10 mmol/L by dissolving 6.44 μl 70% tBH in 5 ml phosphate-buffered saline solution. A stock solution of folate was prepared at 10 mmol/L by dissolving 44 mg folate in 10 mL $NaHCO_3$ (10 g/L) solution. Stock solutions of 5 mmol/L vitamin C and 200 mmol/L NAC were prepared in the culture medium. A stock solution of vitamin E was prepared at 30 mmol/L in ethanol; the ethanol concentration in the culture medium has no effect on cell viability. All antioxidant stocks were freshly prepared before experiments.

2.4. Experimental treatment

The susceptibility of mutants to oxidative stress–induced cytotoxicity was previously tested [26]. Treatment with 50 μM tBH for 72 h reduced viability of glyA and glyB mutants to 80% and 36%, respectively, but was sublethal to CHOK1 wild-type cells. We thus used tBH at this concentration as the oxidant stimulus. CHO cells were preincubated in control medium with various levels of folate or antioxidants for 72 h and then treated with tBH to induce apoptosis. Cultures without tBH treatment were used as additional controls. After the treatments were terminated, cells were harvested for assay.

2.5. Analysis of antioxidant defense systems

Compartmental folate levels were assayed by using cryoprotected *Lactobacillus casei* in 96-well microtiter plates [27]. The levels of reduced glutathione (GSH) [28], Cu–Zn superoxide dismutase (SOD) [29], glutathione peroxidase (GPx) [30] and catalase [31] were determined as previously described.

2.6. Analysis of mtDNA^{4834} deletion

Total DNA was extracted for the analysis of mtDNA^{4834} deletion. The specific primers for the detection of the mtDNA^{4834} deletion and the undeleted mtDNA region (mt D-loop) are described elsewhere in detail [32]. The thermal profile for amplification of the region flanking mtDNA^{4834} deletion was a 10-min denaturation period at 95°C; 40 cycles of 95°C for 15 s, 60°C for 60 s and 72°C for 60 s, followed by 10 min at 72 °C. All reactions were carried out in a GeneAmp DNA thermal cycler (Perkin Elmer, Wellesley, MA, USA). The cycle at which a significant increase in normalized fluorescence was first detected was designated as the threshold cycle number (Ct). The ratio of mtDNA^{4834} deletion to mt D-loop DNA was calculated as $2^{-\Delta Ct}$, where delta Ct=mt Ct_{del}−mt $Ct_{D\text{-}loop}$.

2.7. Analysis of respiratory complex activities

We assayed the activities of oxidative phosphorylation enzyme complexes using the methods as described previously [33]. Briefly, NADH-cytochrome *c* oxidoreductase (NCCR) was measured by the complex I+III coupled assay with NADH-dependent reduction of cytochrome *c* at $A_{550}-A_{540}$ (ε_{550}=19.0 mM^{-1} cm^{-1}). Succinate–cytochrome *c* oxidoreductase (SCCR) was assayed via the reduction of cytochrome *c* by Complex III coupled to succinate oxidation through complex II at 550 minus 540 nm. Cytochrome *c* oxidase (CcOX) activity (Complex IV) was measured by following the oxidation of reduced cytochrome *c* at 550 nm at an extinction coefficient of ε_{550}=27.7 mM^{-1} cm^{-1}. Cytochrome *c* was reduced by L-ascorbate, purified through a Sephadex G-25 column (Pharmacia Biotech, Piscataway, NJ, USA) and checked for full reduction by absorbance at 550 and 565 nm.

2.8. Quantification of depolarized cells with mitochondrial membrane potential dissipation and intracellular ROS production

Mt membrane potential was quantified by rhodamine 123 staining and flow cytometric analysis as described [20]. Intracellular ROS were assayed by using the fluorescent dyes HE for superoxide anion and DCFH-DA for other general ROS and hydrogen peroxide and analyzed on a Coulter EPICS XL-MCL flow cytometry (Coulter Corporation, Miami, FL, USA).

2.9. Subcellular fractionation and Western blot analysis of mt-associated death signaling

Proapoptotic and antiapoptotic signaling of Bcl-2, translocation of Bax and Bak to mitochondria and cytochrome *c* release into the cytosol were investigated by Western blot analysis and the protocols for subcellular fractionation were described elsewhere in detail [12,32]. For analysis of caspases activation, levels of Procaspase 3 and 9 were measured. The gene for Caspase 3, also known as CPP32, codes for a 32-kDa protein which is activated by cleavage events at Asp-28/Ser-29 and Asp-175/Ser-176 to generate subunits with caspase activity. The anti-Caspase 9 antibody recognized a region composed of residues at the N-terminus of human Procaspase-9. For analysis of Procaspase 3 and 9, cells were lysed in lysis buffer [50 mmol Tris-HCl/L (pH 8·0), 150 nmol NaCl/L, 1 g sodium dodecyl sulfate/L, 1 mmol sodium ethylene glycol tetraacetic acid/L, 1 mmol dithiothreitol/L, 250 mmol sucrose/L and a mixture of protease inhibitors for 30 min on ice and then centrifuged at 4°C (14 000 g for 15 min)]. The protein concentration was measured by Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as the standard. Proteins were probed with polyclonal antibodies to detect pro-form of Caspase 3 and Caspase 9 (BD Biosciences, San Jose, CA, USA), then with peroxidase-labeled antimouse antibodies, and autoradiographed in an Enhanced Chemiluminescence detection kit (Amersham, Little Chalfont, England).

2.10. Analysis of apoptotic cells

The Annexin-V-Fluos kit was used to measure the apoptotic cells with membrane phosphatidylserine (PS) translocation (Roche Diagnostics, Mannheim, Germany). Cells were suspended in incubation buffer that contained 1 mg/L PI and a 1:50 dilution of Annexin-V-Fluos labeling solution. The mixed solution was incubated for 15 min on ice, and green Annexin-V and red PI fluorescence intensities were analyzed on an EPICS XL-MCL flow cytometer (Coulter, Miami, FL). Cells with Annexin-V-positive and PI-negative fluorescence were defined as apoptotic. Cells with Annexin-V-negative and PI-positive fluorescence (PI permeable) were defined as necrotic. Agarose gel electrophoresis of apoptotic DNA fragmentation was performed as described [21].

2.11. Statistical analyses

All data are presented as means±S.D. One-way analysis of variance and Duncan's test were used for comparisons among groups using the General Linear Model of SAS (SAS Institute, Cary, NC, USA). A significant difference was indicated at $P<.05$.

3. Results

3.1. Intracellular oxidative stress in the absence/presence of tBH

Before tBH exposure, glyB possessed 50% more intracellular superoxide than glyA and CHOK1 ($P<.05$) (Fig. 1A), but there was no significant difference in hydrogen peroxide levels among the lines (Fig. 1B). Treatment with tBH caused slight oxidative stress in wild-type CHOK1 cells (Fig. 1). In contrast, superoxide and hydrogen peroxide levels of glyB reached, respectively, 2.5× and 1.9× the untreated counterpart control values ($P<.05$) at 24 h. The significant rise in ROS levels of tBH-treated glyB cells compared with either the wild-type cells or their untreated controls persisted at 48 and 72 h. In glyA, the superoxide levels were moderately increased to 1.5–1.8×, but hydrogen peroxide levels were not significantly different from CHOK1 at 72 h.

3.2. Antioxidant self-defense systems of cell lines

To further understand why mutant cells were susceptible to elevated oxidative stress, we assayed the self-defense antioxidant systems (Table 1). In the absence of tBH treatment, activities of antioxidant enzymes (Cu-Zn SOD, catalase, GPx) and levels of reduced glutathione did not differ between CHOK1 and mutant cells. tBH increased the specific activity of all measured antioxidant enzymes and levels of reduced glutathione in CHOK1 cells. The data suggest an adaptive response of CHOK1 cells to the oxidative stress induced by tBH. In contrast, activities of catalase and GPx and levels of reduced glutathione, but not Cu–Zn SOD, were lower in tBH-treated glyA and glyB cells than in CHOK1 cells ($P<.05$).

Table 1
Activities of antioxidant enzymes and levels of reduced glutathione in cells in the absence/presence of tBH challenge[1]

Antioxidant defenses	CHOK1	glyA	glyB
Cu–Zn SOD[2], U/(mg protein)/min			
−tBH	6.62±0.1[a]*	7.4±1.7[a]*	8.1±0.1[a]*
+tBH	17.7±1.8[b]	21.3±5.6[c]	52.7±3.9[a]
Catalase[3], units/(mg protein)/min			
−tBH	4.69±1.67[a]*	4.24±1.52[a]	4.87±0.73[a]
+tBH	14.1±1.67[a]	5.87±0.73[b]	2.83±1.58[c]
GPx[4], units/(mg protein)/min			
−tBH	14.5±0.75[a]*	15.6±0.46[a]	17.1±0.46[a]
+tBH	32.9±1.2[a]	15.7±0.5[b]	16.8±0.47[b]
Reduced glutathione, nmol/mg protein			
−tBH	12.4±1.23[a]*	16.2±2.47[a]*	12.2±1.62[a]
+tBH	31.1±1.24[a]	8.56±0.84[c]	13.3±0.35[b]

* Pairs of values in the same column differ between –tBH and +tBH at $P<.05$.

[1] Cells were cultured in the absence/presence of 50 μM tBH for 72 h. Data are means±S.D., $n=3$. Values in a row with different superscript letters differ at $P<.05$.

[2] One unit of Cu–Zn SOD activity was arbitrarily defined as the amount of enzyme required to inhibit 50% autoxidation of pyrogallol.

[3] One unit of catalase activity was arbitrarily defined as the amount of enzyme required to decompose 1 mmol H_2O_2/min.

[4] One unit of glutathione peroxidase (GPx) activity was defined as the amount of enzyme catalyzing the oxidation of 1 nmol NADPH/min.

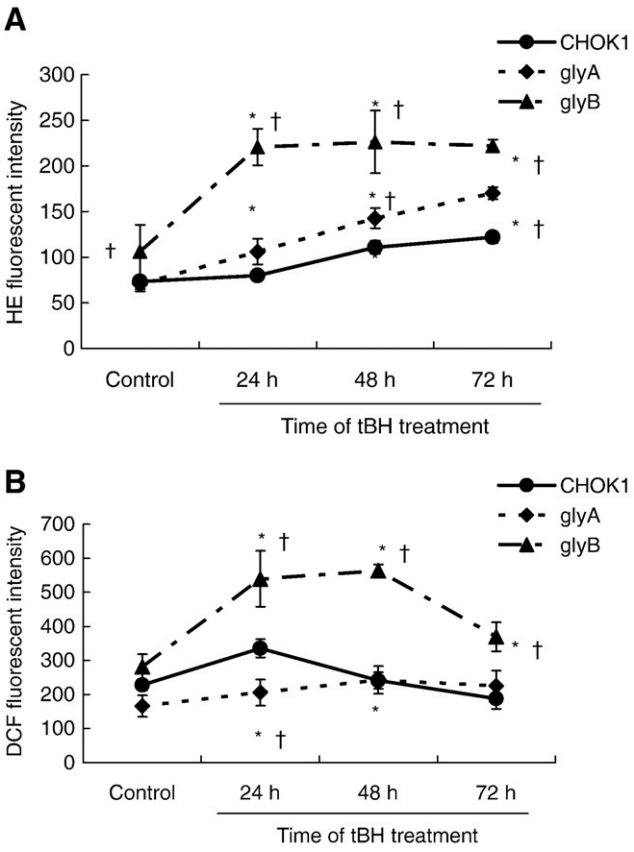

Fig. 1. Levels of reactive oxygen species in CHO cells in the absence/presence of 50 μM tBH. The intracellular levels of superoxide (A) (labeled by hydroethidine [HE] fluorescence) and hydrogen peroxide (B) [labeled by dichlorofluorescein (DCF) fluorescence] were analyzed by flow cytometry. Values are means±S.D., $n=3$. *Values of tBH-treated cells were significantly different from those of untreated counterpart control ($P<.05$). †Values of mutants were significantly different from those of wild-type at each time point ($P<.05$).

3.3. Changes in compartmental folate levels

Since folate deficit has been associated with elevated oxidative stress and impaired antioxidant defenses [34], we measured the compartmental folate levels of cells. GlyB possessed the lowest folate levels in the cytosol (Fig. 2A) and mitochondria (Fig. 2B) both before and after tBH exposure ($P<.05$). tBH-Depleted cytosolic folate did not differ between CHOK1 and glyA. tBH did not alter mt folate levels in CHOK1, but it significantly depleted folate levels in the mt compartment of both glyA and glyB (Fig. 2B).

3.4. Detection of oxidative $mtDNA^{4834}$ deletion

To demonstrate oxidative injuries inside the mitochondria, the frequencies of oxidative stress–associated mtDNA deletions in the absence/presence of tBH were assessed (Table 2). In the absence of tBH, glyB carried 2.25 times as many mtDNA large deletions ($P<.05$) as the wild-type untreated levels at 72 h. In the presence of tBH, the frequencies rose to 5.5× in glyB, 3.5× in glyA and 1.75× in CHOK1.

3.5. Respiratory chain activity in mitochondria in the absence/presence of tBH

To determine whether elevated oxidative stress of two mutants was associated with mt dysfunction, we measured the activities of respiratory complexes I–IV. Only trace activity of glutamate dehydrogenase (mt matrix maker enzyme) was detectable in cytosol, indicating that mitochondria remained intact during isolation (Table 3). Activities of NADH–cytochrome *c* oxidoreductase (respiratory complex I+III) did not differ among the cell lines either before or after tBH treatment. In the absence of tBH, activity of CcOX (Complex IV) in glyA and glyB was significantly less than that in CHOK1 ($P<.05$). tBH Treatment for 72 h reduced succinate–cytochrome *c*

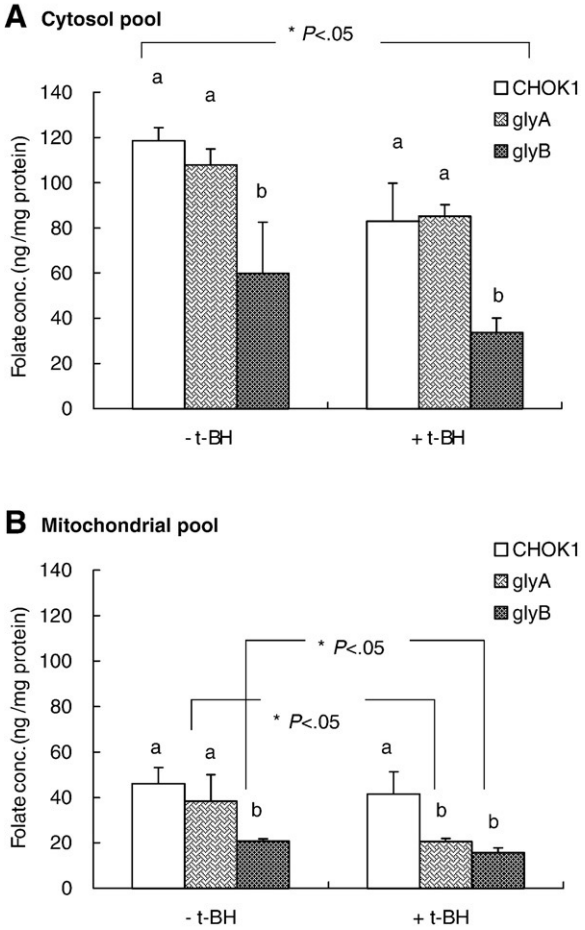

Fig. 2. Changes in the compartmental folate levels of cell lines in the absence/presence of 50 μM tBH for 72 h. Folate levels in compartment fractions were assayed. Data are means±SD, $n=3$. Means without a common letter differ at $P<.05$. *Values of tBH-treated cells were significantly different from those of untreated counterpart control ($P<.05$).

oxidoreductase (complex II+III) and CcOX activities in all cell lines. GlyA and glyB levels were lower in the CcOX activities than tBH-treated CHOK1 levels, glyB levels significantly so.

3.6. mt-Associated death signaling in the absence/presence of oxidant challenge

Significantly higher fractions of glyA and glyB cells had depolarized mt membrane potential, indicating respiratory dysfunction, than wild-type CHOK1 cells (Fig. 3A). tBH Aggravated membrane potential dissipation in glyA and glyB, but not in CHOK1. Basal expression of the antiapoptotic protein Bcl-2 in glyA [relative density (RD) of 18] and glyB (RD: 21) was reduced to 45% and 52%, respectively, of the wild-type value (RD: 40). Compared with the tBH-treated wild type (RD: 21), tBH remarkably lowered the levels of Bcl-2 protein in glyB (RD: 0.06), whereas glyA slightly increased its basal level (RD: 26). Expression of proapoptotic protein Bak was not affected by tBH treatment in any cell line. Translocation of proapoptotic protein Bax to mitochondria by the decrease in cytosolic Bax (change of RD from 12 to 6 before and after tBH treatment) was evidenced in tBH-treated glyB but not in glyA. Release of cytochrome *c* was detected in tBH-treated glyA at a RD of 2.6 and in glyB at a RD of 4.8.

3.7. Apoptotic propensity of cells upon tBH challenge

Procaspase 9 and Procaspase 3 were activated in tBH-treated glyB (Fig. 4A). The proportion of apoptotic cells displaying membrane PS translocation was increased from 1% (untreated) to 30% in tBH-treated glyB (Fig. 4B). Electrophoresis revealed DNA laddering in tBH-treated glyB, a typical sign of apoptotic damage to DNA (Fig. 4C). No obvious activation of Procaspase 9 or 3 (Fig. 4A) or induction of apoptosis (Fig. 4B and C) was observed in tBH-treated wild-type cells. Procaspase 9 was activated in tBH-treated glyA, yet no activation of Procaspase 3 as well as apoptotic promotion was observed.

3.8. Effects of folate supplementation on tBH-induced oxidative stress and apoptosis

To investigate whether folate-associated oxidative stress and mt dysfunction mediated tBH-induced apoptosis of glyB, we preincubated glyB with 0–1000-μM folate before tBH treatment, and assayed the parameters of oxidative stress and apoptosis (PS membrane exposure). Folate at 100 and 1000 μM significantly reduced tBH-elicited overproduction of superoxide by 20% and 60%, respectively (Fig. 5A). Hydrogen peroxide levels were decreased by 20%. These reductions in ROS levels by folate supplementation were associated with enhanced catalase activities and normalized SOD activities (Fig. 5B). Both mtDNA oxidative deletions and apoptotic lesions were reduced by 20–40% by 10 μM folate (Fig. 5C), but higher levels of folate did not provide additional protection against either.

3.9. Effects of folate supplementation on tBH-induced mt membrane depolarization and folate enrichment in compartmental pools

The effect of folate on mt oxidative damage and apoptosis in glyB appeared to correlate with normalization of the membrane potential and the enrichment of folate levels in the compartmental pool (Fig. 6). Folate supplementation at 10 μM significantly corrected mt membrane depolarization, mimicking the effect of antioxidant vitamin C, but not of vitamin E or NAC (Fig. 6A). The reversal of mt dysfunction and apoptosis by folate supplementation coincided with enrichment of folate levels in the compartmental pools, which increased by up to

Table 2
Accumulation of $mtDNA^{4834}$ deletions in cells in the absence/presence of tBH challenge[1, 2]

Culture interval	Control treatment			tBH Treatment, 50 μM		
	CHOK1	glyA	glyB	CHOK1	glyA	glyB
24 h	0.14 ± 0.03^{c} (100)	0.18 ± 0.02^{b} (129)	0.16 ± 0.04^{b} (114)	0.20 ± 0.01^{b} (143)	0.24 ± 0.01^{b} (171)	0.39 ± 0.08^{a} (278)
48 h	0.32 ± 0.03^{d} (100)	0.46 ± 0.04^{bcd} (125)	0.38 ± 0.06^{c} (118)	0.57 ± 0.13^{ab} (178)	0.51 ± 0.05^{ab} (159)	0.60 ± 0.16^{a} (188)
72 h	0.16 ± 0.02^{c} (100)	0.20 ± 0.03^{c} (125)	0.36 ± 0.06^{b} (225)	0.28 ± 0.06^{c} (175)	0.56 ± 0.14^{b} (350)	0.88 ± 0.27^{a} (550)

[1] Cells were cultured in the absence/presence of 50 μM tBH for 72 h. Data are means±S.D., $n=3$. Means in a row without a common letter differ at $P<.05$. The values in parentheses of a row represent for percentage of the wild-type values.

[2] The ratio of $mtDNA^{4834}$ deletion to mt D-loop DNA was calculated as $2^{-\Delta Ct}$, where delta Ct=mt Ct_{del} – mt $Ct_{D\text{-}loop}$.

Table 3
Activities of mitochondrial respiratory complexes of cells in the absence/presence of tBH challenge[1]

Cell lines	Glutamate dehydrogenase[2] (ΔA/min per mg protein)				Mitochondrial enzyme complex[3] (nmol/min per mg mitochondrial protein)					
	Cytosol		Mitochondria		NCCR (I+III)		SCCR (II+III)		CcOX (IV)	
	−tBH	+tBH	−tBH	+tBH	−tBH	+tBH	tBH	+tBH	−tBH	+tBH
CHOK1	0.07±0.01	0.07±0.009	1.87±0.16	1.79±0.14	128±9.0	124±7.4	36.3 ± 3.2^{x}	22.9 ± 4.8^{y}	187 ± 22.6^{ax}	130 ± 13.4^{ay}
glyA	0.08±0.009	0.08±0.01	1.85±0.08	1.82±0.08	119±4.1	115±5.1	32.4 ± 2.1^{x}	23.5 ± 1.5^{y}	136 ± 10.6^{bx}	115 ± 9.7^{aby}
glyB	0.07±0.01	0.08±0.01	1.86±0.09	1.85±0.07	120±5.8	116±9.4	34.3 ± 3.6^{x}	21.7 ± 1.9^{y}	107 ± 5.8^{bx}	92.9 ± 10.2^{by}

[1] Data are means±S.D. $n=3$. Values in a column with different letters differ at $P<.05$. Values between −tBH and +tBH groups with different letters differ at $P<.05$.Cells were cultured in the absence/presence of 50 μM tBH for 72 h.
[2] Specific enzyme activity of glutamate dehydrogenase, a mitochondrial marker enzyme. ΔA=change in absorbance.
[3] CcOX, cytochrome *c* oxidase; NCCR, NADH–cytochrome-*c* oxidoreductase; SCCR, succinate–cytochrome *c* oxidoreductase.

350% by 10 μM folate and became saturated at 100 μM folate (Fig. 6B). The absolute level of mt folate in folate-supplemented glyB was significantly lower than that of folate-supplemented wild type (58±15 vs. 72±5 ng/mg protein). A similar effect of folate against mt dysfunction was observed in tBH-treated glyA (data not shown).

4. Discussion

Despite decades of experimental work [1–7], the functional role of mt folate-associated proteins in glyA and glyB cells is not clearly understood. Our data demonstrate for the first time that these two mutants carry dysfunctional mitochondria characterized by reduced respiratory complex IV activity and increased rates of mt membrane depolarization (Table 3, Fig. 3A). Why biochemical defects in distinctly different mt folate-associated proteins cause a common respiratory aberration in the two mutants is currently not known. In glyA, defective mt SHMT activity inhibited the conversion of THF to 5,10-methylene-THF, redistributing mt folate coenzymes with accumulated THF and reducing levels of 10-formyl-THF [35]. In glyB, a defect in mt folate transporter blocked the transport of folate to mitochondria, depleting the overall mt folate level and reducing 10-formyl-THF levels in particular [35]. 10-Formyl-THF, a major mt folate coenzyme [36], has been proposed to stabilize respiration of complex IV in isolated rat liver mitochondria [37]. Mt folate deficiency in liver tissue or folate supplementation to primary hepatocytes could modify CcOX (Complex IV) activity and mt membrane depolarization [20]. Alternatively, it has been suggested that 10-formylTHF provided one carbon unit for the synthesis of formylmethionyl-tRNA used in mitochondrial protein synthesis or to produce one carbon unit for purine synthesis [38]. Although the glycine auxotrophs of mammalian fibroblasts with disturbed mt folate metabolisms did not affect mt protein synthesis [38], it is not known if the reduced respiratory activity of glyA and glyB mutants may be due to the impaired mt protein synthesis. Regardless of the mechanisms, these and our results suggest that the disturbed distribution of mt folate coenzymes due to defects in mt SHMT or mt folate transporter leads to common mt respiratory dysfunction.

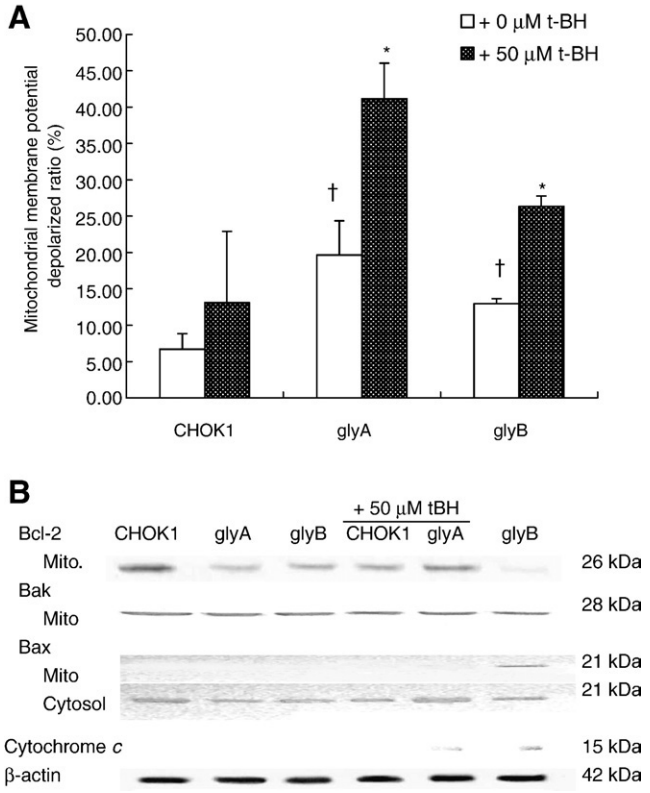

Fig. 3. mt-Associated death signaling in cells in the absence/presence of 50 μM tBH for 72 h. (A) Fraction of cells with depolarized membrane potential. †Values of mutants were significantly different from those of wild-type ($P<.05$). *Values of tBH-treated mutants were significantly different from those of tBH-treated wild type ($P<.05$). (B) Western blot of mt-associated death signals: mt Bcl-2, translocation of Bax and Bak to mitochondria and cytosol and cytochrome *c* release to cytosol.

Respiratory dysfunction is considered to promote electron leakage and elevate oxidative stress [8,9]. Increased levels of superoxide and accumulated mtDNA large deletions in glyB confirmed elevated oxidative genotoxic stress inside the mitochondria (Table 2). The oxidative phenotype of glyB may not be due to impaired antioxidant defenses, since activities of antioxidant enzymes and reduced GSH levels were similar between wild type and glyB (Table 1). The increased mt genotoxicity of glyB could be partially, if not entirely, attributed to the shortage of mt folate owing to the defective mt folate transporter [2–4]. Accumulated mtDNA oxidative deletions depended upon decreasing mt folate content [31]. Lack of mt folate was associated with mt protein oxidative injuries and superoxide overproduction in cells and tissues [20,31]. This ROS overproduction from mt oxidative decay could further consume folate in other compartments on account of the radical-scavenging capability of folate [39]. This speculation is supported by the fact that cytosolic folate in glyB was significantly depleted relative to wild-type cells with normal mitochondrial function (Fig. 2). Both folate depletion and mt dysfunction could lead to inefficient energy production and impaired cell proliferation, consistent with the previously reported slower growth rate of glyB cells [3].

One of the major findings here is that the depleted folate pool and dysfunctional mitochondria sensitized glyB to oxidant-induced oxidative injury and apoptosis. tBH is a model hydroperoxide, which cellular catabolism by GSH peroxidase results in enhanced GSSG formation through oxidation of GSH and a decreased GSH/GSSG redox state. High doses of tBH disturb redox homeostasis,

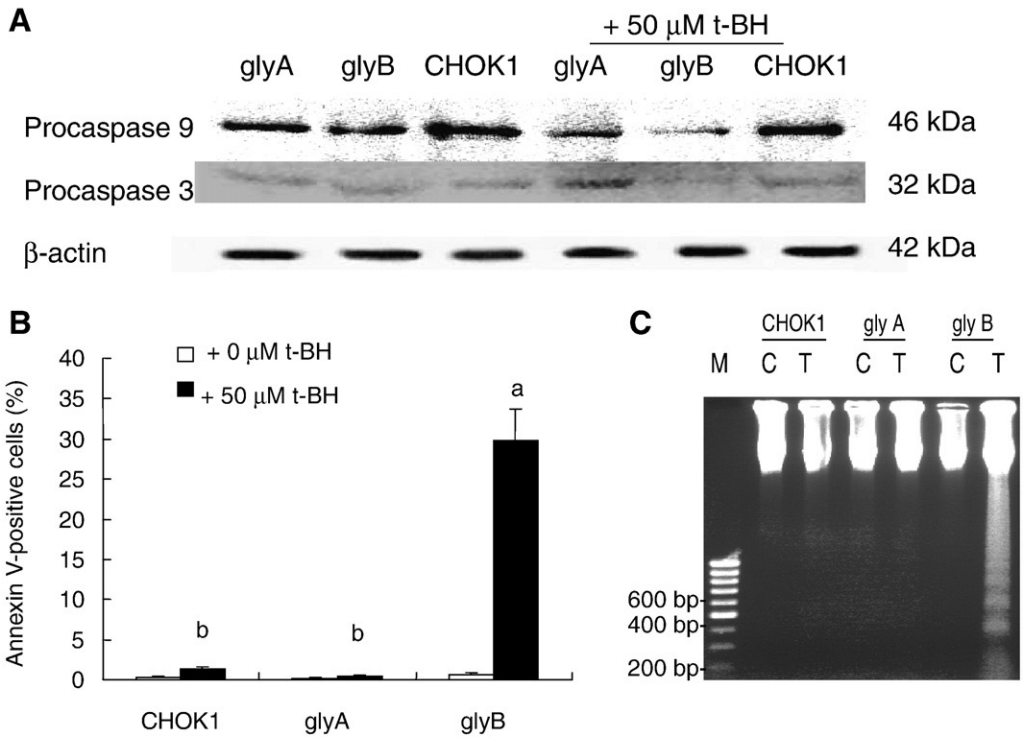

Fig. 4. Apoptosis of cells in the absence/presence of 50 μM tBH for 72 h. (A) Effects of tBH on activation of procaspases 9 and 3. (B) Proportion of apoptotic cells with membrane phosphatidylserine exposure as measured by Annexin-V-Fluos labeling and analyzed by flow cytometry. Values are means±SD, $n=3$. Means without a common letter differ at $P<.05$. (C) Agarose gel electrophoresis of DNA extracted from cells cultured in the absence (control, C)/presence of 50 μM tBH for 72 h (T). Lane M: DNA markers.

induce mt dysfunction, elevate ROS levels and promote apoptosis in several cell lines [40,41]. Elevated oxidative stress and ROS production were associated with folate depletion [34] as a result of antioxidant action of folate. Consistently, low dose of tBH elicited the antioxidant response of wild-type cells but impaired the antioxidant enzymatic activities and depleted folate levels in the compartments of glyB (Table 1). The impaired antioxidant defense of tBH-treated cells have been associated with decreased expression of Bcl-2 [42], an antioxidant mt protein that protects against ROS release and mt dysfunction [43,44] and an anti-apoptotic protein that blocks cytochrome *c* release from mitochondria by regulating opening of the mt permeability transition pore [45]. Suppression of Bcl-2 protein, together with translocation of Bax, a component of permeability transition pore opening, to mitochondria, could lead to collapse of the mt inner transmembrane potential and start of the postmitochondrial execution phase of apoptosis [46]. As shown in tBH-treated glyB, the elevated ratio of mt Bax/Bcl-2 coincided with a doubling of the fraction of depolarized cells, release of cytochrome *c* to cytosol, activation of Procaspases 9 and 3, and subsequently induction of apoptotic DNA fragmentation (Figs. 3 and 4). The oxidative state and apoptotic phenotype of tBH-treated glyB could be partially corrected by folate supplementation (Figs. 5 and 6), suggesting that lack of folate was in part responsible for the increased susceptibility of glyB to tBH-induced apoptosis.

The molecular mechanisms by which folate supplements could counter tBH-induced oxidative stress and apoptosis of glyB are largely unknown. Several potential protective mechanisms are plausible. First, direct scavenging of radicals by folate could preserve the cellular antioxidant defenses and spare the oxidation of macromolecules. The reduced intracellular ROS levels and restoration of catalase activity in folate-treated glyB under tBH exposure support this antioxidant action of folate [39,47,48]. Secondly, folate could enrich one-carbon folate coenzymes in the compartment pool to protect against tBH-induced mtDNA damage. Accumulation of mtDNA deletions, a large-scale deletion of mtDNA removing 30% of mtDNA molecules encoded for respiratory complex subunits, was associated with mt functional decline, elicited vicious cycles in superoxide generation and apoptosis induction [23,49]. By enriching the mt folate pool, folate supplementation may strengthen respiratory function, the impairment of which leads to superoxide production and abnormal mt permeability transition during apoptosis [50]. This postulation is partially supported by the fact that alleviation of tBH-induced mt membrane potential dissipation coincided with enrichment of the compartmental folate pool, reduced superoxide levels and reduced apoptosis. It should be noted that further increase in folate at 100 or 1000 μM was not followed by a linear elevation of mt folate levels in glyB and offers little additional protection against tBH-induced apoptosis. After folate supplementation by 10 μM, glyB had significantly lower levels of mt folate than wild type cells, confirming the limiting capacity of glyB to accumulate mt folate upon high levels of folate supplementation. These observations thus suggest that the defective mt folate transporter in glyB presents an obstacle for mt folate enrichment. Thus, the restriction in mt folate enrichment following folate supplementation may limit the rescuing effect of folate against tBH-induced mt dysfunction and apoptosis. The findings were consistent with the point mutation identified in mt folate transporter (*mft*) cDNA of the glyB, which resulted in the impaired folate transporting function [3,4]. Alternatively, the defect in mt folate transporter may exist an irreversible lesion for the high-dose folate supplementation to rescue mt-associated death signaling. The lesion may involve the disturbance of proapoptotic and antiapoptotic proteins, as shown by the reduced basal levels of Bcl-2 in glyB cells (Fig. 3). The mechanisms affecting the apoptotic signaling in glyB mutant, however, remain to be studied.

The oxidative status and apoptotic phenotype of glyA were distinct from those of glyB. Despite carrying dysfunctional mitochondria, glyA did not show the elevated oxidative stress that glyB displayed. Similarly, tBH-treated glyA had less mt dysfunction and oxidative injury than tBH-treated glyB. As the antioxidant enzymatic responses of both mutants were similar both before and after tBH treatment, the moderate oxidative stress in tBH-treated glyA may be attributable to a less-depleted folate pool than in tBH-treated glyB (Fig. 2). It was hypothesized that defective mt SHMT activity may deviate one-carbon flow to accumulated THF and 5-methyl-THF in mitochondria of glyA [35], providing stronger antioxidant action of folate in ROS scavenging. Reduced forms of folate coenzymes such as THF and 5-methyl-THF have been shown to possess strong antioxidant ability in scavenging free radicals in vitro [51] and alleviating oxidative stress in vivo [48]. Accumulation of THF and 5-methyl-THF in mitochondria of glyA cells due to the mt SHMT defect may benefit glyA via the reduction of dysfunctional mt-associated ROS release and tBH-induced oxidative stress (Fig. 1, Table 2). This oxidative stress buffering effect may also prevent glyA from reaching the oxidative death signaling threshold that induces apoptosis. On the other hand, disturbed one carbon coenzyme distribution by reduced mt 10-formyl THF of glyA may affect the supplied flow of formate, a major one carbon unit for cytosolic purine and pyrimidine synthesis. A decrease of 5-methylTHF in the cytosolic pool of glyA [35] may have impact on DNA methylation and deregulation of nuclear-encoded gene expressions for mt respiratory submits or/and for apoptotic signaling. Folate deficiency has been showed to decrease expression levels of Bcl-2 in human T lymphocytes [22]. The fact that glyA cells possessed a lower basal level of Bcl-2 than wild type cells may support this speculation. Further studies are warranted to elucidate the contributing mechanisms of disturbed one carbon metabolism to the altered phenotype of glyA mutant.

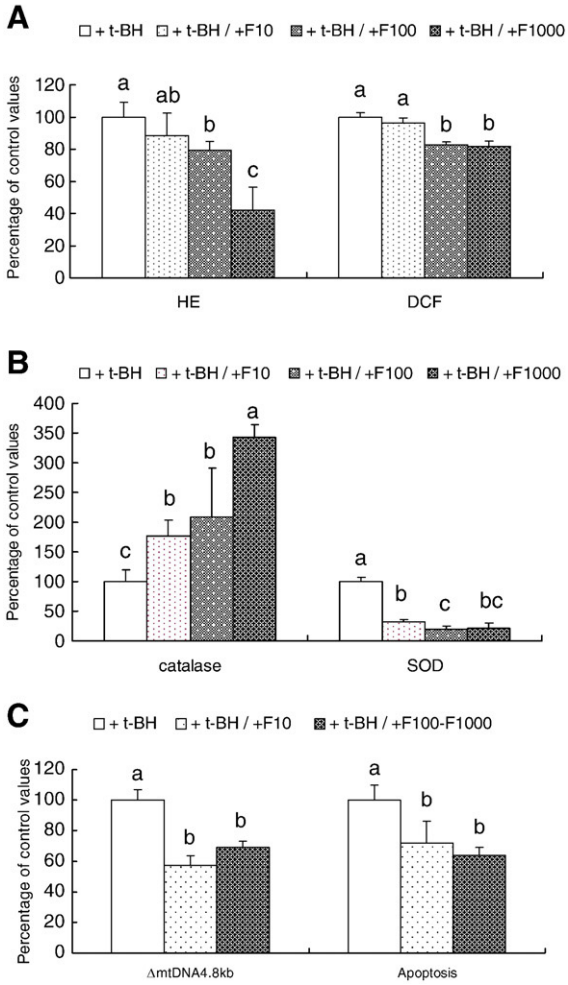

Fig. 5. Effects of folate supplementation on ROS release (A), antioxidant enzymatic activities (B) and (C) mtDNA^{4834} deletions and apoptosis upon tBH treatment. Cells were preincubated with various levels of folate for 3 d before 50 μM tBH treatment for 72 h. Cells were collected and the parameters were assayed as described in "Materials and Methods." Apoptotic cells with membrane phosphatidylserine exposure were measured by Annexin-V-Fluos labeling and analyzed by flow cytometry. Effects of folate on tBH-induced mtDNA deletions were assayed at the supplemental levels of F10 and F100 μM. Effects of folate on tBH-induced apoptosis were measured at the supplemental levels of F10 and F1000 μM. Data are expressed as the percentage of the control values (means±S.D., n=3). For each parameter, means without a common letter differ at P<.05.

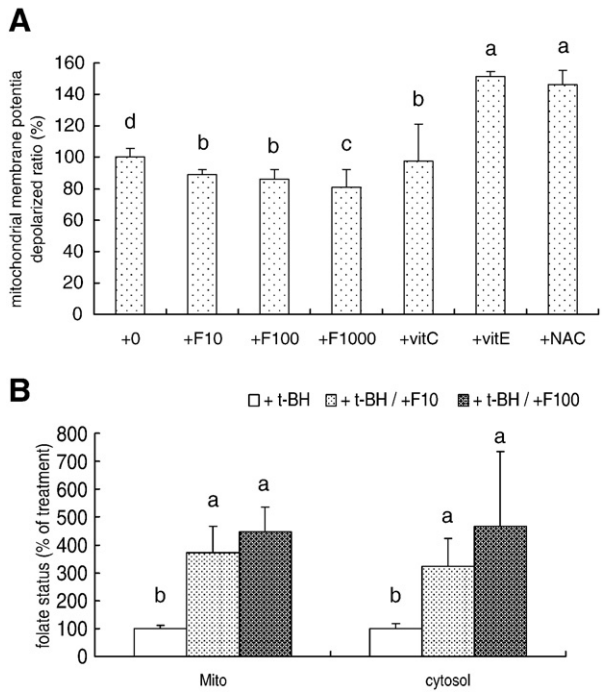

Fig. 6. Effect of folate supplement on membrane depolarization (A) and folate levels (B) in the cytosolic and mitochondrial compartments in glyB cells upon tBH treatment. (A) GlyB cells were preincubated with folate (0, 10, 100 or/and 1000 μM), vitamin C (100 μM), vitamin E (100 μM) or NAC (5 mM) for 3 d before 50 μM tBH treatment for 72 h. Cells were collected and membrane depolarization was assayed. Values are means±S.D., n=3. Means without a common letter differ at P<.05. (B) GlyB cells were preincubated with folate (0, 10, 100 μM) for 3 d before 50 μM tBH treatment for 72 h. Folate status of cells was assayed. Values are means±S.D., n=3. Means without a common letter differ at P<.05.

In summary, our data disclose the indispensable role of mt SHMT and mt folate transporter in the maintenance of functional mitochondria. Mutant cells carrying defective mt SHMT or mt folate transporter are susceptible to oxidant-induced oxidative stress and mt dysfunction. Defective mt folate transporter sensitizes CHO cells to tBH-induced the mt-associated death signaling and apoptosis, mediated in part by depleted mt folate. Defective mt SHMT sensitized glyA to respiratory dysfunction and tBH-induced oxidative injury without promoting apoptosis.

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