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Antioxidant survey to assess antagonism to redox stress using a prokaryotic and an eukaryotic system

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Abstract. Using a prokaryote (*Escherichia coli*) and a metazoa-resembling eukaryote (*Ochromonas danica*), we surveyed antioxidants which might overcome redox stress imposed by menadione sodium bisulphite (MD) and buthionine sulphoximine (BSO). BSO oxidant stress was evident only in *O. danica*; MD oxidant stress was evident in both organisms. Glutathione, its precursors, e.g. cysteine, homocysteine, and 2-oxo-4-thiazolidine carboxylic acid, and red blood cells, emerged as prime antioxidants for relieving BSO and MD oxidant stress. BSO and MD oxidant activity and antioxidant-annulling effect in *O. danica* were judged comparable to those found in animal cells whereas the results *E. coli* were not entirely equivalent. The *O. danica* system emerged as a practical, rapid, and useful system for pinpointing oxidant stressors and antioxidants, and shows promise for studies with mammalian systems.
Key words. Glutathione; free radicals; antioxidants; oxidant stressors; buthionine; menadione.

Abbreviations. MD = menadione sodium bisulphite; BSO = buthionine sulphoximine; GSH = gluthathione; GSSG = gluthathione disulphide; NAC = N-acetyl-L-cysteine; OTC = 2-oxo-4-thiazolidine carboxylic acid; DTT = dithiothreitol.

Antioxidants scavenge and minimize the formation of free radicals[1,2]. Most antioxidants have a scantily populated outer electron shell that readily incorporates the unpaired electron characteristic of a free radical – an action which disrupts oxidative stress produced by free radicals[1,2]. Oxidant stressors kill by generating free radicals; buthionine sulphoximine (BSO) and menadione (MD) are such stressors[3–10]. BSO [S-(n-butyl)-homocysteine sulphoximine] inhibits γ-glutamylcysteine synthetase, thereby inhibiting GSH production[3]; MD (2-methyl-1,4-naphthoquinone) can induce oxidant stress by enhancing generation of superoxide, hydroxyl, and peroxyl radicals which can alter DNA[8–10] and conformation of cytoskeletal proteins[7–11].

Here, we tested the activity of various antioxidants often mentioned in the literature[3–10] for their ability to annul cellular redox stress produced by BSO and MD. We compared a prokaryote (bacterium) with a eukaryote (protist) system to determine which more closely resemble reported mammalian cell systems used to delineate oxidant stressors and antioxidants. The eukaryote system has previously been considered equivalent to mammalian models for measuring many vitamin and drug actions[12].

Materials and methods

The system for assessing antioxidants comprised (a) a prokaryote – *Escherichia coli* 113-3, ATCC 11105, and (b) a phagotrophic metazoa-like eukaryote – *Ochromonas danica* (ATCC 30004). Simple axenic growth of both organisms in chemically defined growth media has been described[13,14]. Media components, antioxidants, L-buthionine-(S,R)-sulphoximine (BSO), and menadione sodium bisulphite (MD) were purchased from Sigma Chemical Co. (St. Louis, MO). Test substances added to media were dissolved in hot distilled water; aqueous dilutions were prepared as needed. Blood from 12 healthy volunteers was drawn from an antecubital vein into vacutainers (Becton Dickenson, Rutherford, NJ) containing 14 mg of disodium EDTA powder as anticoagulant. Whole blood was needed to gauge the antagonistic activity of plasma and red blood cells (rbc) against BSO and MD. Plasma was obtained by pipetting it away from rbc after centrifuging whole blood; remaining rbc were washed thrice with physiological saline and retained. Phosphate buffer (0.01 M NaH_2PO_4 with 0.01 M $CaCl_2$) at pH 4.5 served to hydrolyze plasma or rbc. One ml of plasma or rbc was added to 4 ml of buffer and autoclaved for 10 min; debris was centrifuged off and the supernatant analyzed for antioxidant activity. The BSO and MD concentrations used derived from weight and not from their

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molar base equivalents. Organismal growth was used as the index of antioxidant action against the BSO and MD targets. Growth was expressed in absorbance units; a unit of 1.0 is equivalent ot 0.50 to 0.55 g of washed dried organisms per litre[12–14]. Means and standard deviations of results were determined by standard methods.

Results and discussion

All antioxidants tested without addition of BSO or MD to the growth medium permitted full growth of *E. coli* and *O. danica*. BSO did not antagonize *E. coli* growth; it did kill *O. danica* (table 1A). Perhaps BSO did not penetrate *E. coli* cells to produce killing; if it did, it was inactive in producing oxidant stress. Further BSO testing with *E. coli* was discontinued. In contrast to BSO, MD (0.36 mM) did hinder the prokaryote growth; 50 times less MD (0.007 mM) inhibited eukaryote growth. MD might, therefore, be a universal redox stressor for all cells. Table 1B shows results obtained with various antioxidants surveyed that counteracted BSO and MD oxidant stress. GSH (γ-glutamylcysteinylglycine) counteracted growth inhibition by BSO and MD for both organisms; GSSG overcame BSO inhibition of *O. danica* but not MD toxicity. Apparently, MD inhibits glutathione reductase more effectively than BSO, thereby impeding GSH production from GSSH. The best studied antioxidant in natural substances in GSH[3]. GSH is found in most cells; it can scavenge free radicals directly[2,3]. Many sulphydryl-containing antioxidants, notably cysteine, oppose oxidant stress by serving as precursors for GSH synthesis[3–5]. GSH is synthesized from glutamate, cysteine, and glycine by the conjoined actions of γ-glutamylcysteine synthetase and glutathione synthetase[3]. Interference with these catalysts by oxidant initiators interrupts GSH synthesis and cellular antioxidant protection. Some compounds were tested to determine whether they could overcome oxidant stress, possibly through GSH synthesis. NAC completely negated MD growth inhibiton with *O. danica*; BSO

Table 1. Effect of antioxidants in annulling buthionine sulphoximine (BSO) and menadione Na bisulphite (MD) redox stress.

A. Addition (mM)	Mean growth units (±SD)*	
	E. coli	*O. danica*
None	0.72 (±0.08)	1.24 (±0.16)
MD (0.007)	0.72 (±0.08)	0
MD (0.36)	0	0
BSO (0.45)	0.72 (±0.07)	0

B. Addition (mM)	*E. coli*	*O. danica*	
	MD (0.36)	MD (0.007)	BSO (0.45)
Glutathione (1.6)	0.72 (±0.06)	1.24 (±0.18)	1.24 (±0.20)
Glutathione disulphide (1.6)	0.16 (±0.03)	0.04 (±0.01)	1.32 (±0.25)
N-acetyl-L-cysteine (4.5)	0.55 (±0.08)	1.24 (±0.21)	0.80 (±0.13)
S-carboxymethyl-L-cysteine (2.3)	0.20 (±0.04)	0.12 (±0.04)	0.14 (±0.04)
L-homocysteine (3.3)	0.38 (±0.06)	1.28 (±0.20)	1.24 (±0.22)
L-homocystine (1.2)	0.30 (±0.05)	0	0.20 (±0.03)
L-cysteine (8.3)	0.44 (±0.06)	1.20 (±0.15)	1.00 (±0.09)
L-cystine (2.1)	0.50 (±0.09)	1.10 (±0.13)	0.50 (±0.08)
2-oxo-4-thiazolidinecarboxylic acid (7.0)	0.32 (±0.05)	1.16 (±0.16)	1.22 (±0.18)
DL-methionine (1.5)	0.16 (±0.04)	0	1.18 (±0.15)
Dithiothreitol (3.3)	0.70 (±0.10)	1.24 (±0.15)	0.60 (±0.10)
Na metabisulphite (0.8)	0.54 (±0.07)	1.3 (±0.21)	0.80 (±0.08)
Cystathionine (2.3)	0.34 (±0.06)	0	0
Thiomalic acid (1.4)	0.74 (±0.12)	1.18 (±0.16)	0.24 (±0.04)
Na_2 thiosulphate (0.4)	0.32 (±0.05)	0.44 (±0.07)	0.78 (±0.11)
Thiourea (1.3)	0.20 (±0.06)	0	0.20 (±0.05)
Red blood cells (4 ml %)	0.50 (±0.09)	1.24 (±0.16)	1.24 (±0.19)
Plasma (4 ml %)	0.14 (±0.04)	0.26 (±0.08)	0.82 (±0.09)

*Values (±SD) listed for each reagent tested for growth annulment or reversal of growth inhibition is an average of triplicate values, each verified 4 times.

inhibition was less opposed. Cysteine, cystine, and homocysteine slightly suppressed MD inhbition of *E. coli* but completely annulled MD and BSO inhibition of *O. danica*. Methionine did not antagonize MD inhibition but completely overcame BSO toxicity for *O. danica*. Methionine, a precursor of cysteine, countered BSO toxicity for *O. danica* probably by enhancing GSH production (table 1B). The sulphur of methionine is incorporated into GSH and permits methionine to maintain GSH levels for counteracting toxic oxi-free radical stress[15]. OTC, an alternative source of cysteine, countered BSO and MD stressor action against *O. danica* – an effect also seen in mammalian cells[3,16]. In contrast, OTC was relatively inactive against MD killing of *E. coli* (table 1B). OTC is converted to cysteine in mammalian cells via 5-oxoprolinase[17]. Thus, it may be that prokaryotes, unlike metazoa-like cells, lack sufficient 5-oxyprolinase for GSH synthesis. A reactive SH-group, as in cysteine, seems necessary for antioxidant action. DTT, an SH-group protector, helped protect against MD toxicity. However, not all cysteine-containing compounds necessarily have an antioxidant action. We found that cystathionine (homocysteinylserine), homocystine, and S-carboxymethylcysteine did not annul BSO and MD action even though they contain derivatives of cysteine (table 1B); the reasons for this are not obvious. On the other hand, Na metabisulphite strongly overcame MD toxicity as in other cell systems, which may be attributable to its strong antiperoxide properties[18] and not necessarily to a role in GSH synthesis. Interestingly, some simple SH-containing compounds such as thiomalic acid annulled MD toxidity; thiosulphate slightly reversed inhibition whereas thiourea did not. Glutamic acid, glycine, and low concentration cysteine, alone or in combination, did not enable the organisms to synthesize enough GSH to overcome stress inhibition. Rbc had enough antioxidant potency to overcome MD and BSO oxidant stress for *O. danica* completely, whereas plasma was slightly active (table 1B). Rbc are more potent than plasma in completely preventing BSO and MD toxicity for *O. danica* (table 1B) probably because of higher thiol content and relatively slow GSH turnover rate[19–21]. Rbc also have the capacity to synthesize large amounts of GSH from amino acid precursors; 98% of whole blood GSH is contained in rbc[22]. In contrast, plasma has a destructive effect on GSH[21], which presumably may have contributed to the lessened activity of plasma compared to rbc for counteracting oxidant effect (table 1B).

Our results with BSO and MD indicate that the *O. danica* system, rather than the bacterial system, compares favourably with laborious and expensive mammalian cell systems for pinpointing antioxidants as well as oxidant stressors[17]. Possibly such results (table 1B) derive from the metabolic resemblance of *O. danica* to metazoan cells[12–14].

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