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Modulation of Gardos channel activity by oxidants and oxygen tension: effects of 1-chloro-2,4-dinitrobenzene and phenazine methosulphate

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Abstract

We compare the effects of 1-chloro-2,4-dinitrobenzene (CDNB) and phenazine methosulphate (PMS) on Gardos channel activity in normal human red cells. Both stimulate channel activity, both are dependent on the presence of extracellular Ca^{2+}, and neither is affected by inhibitors of protein (de)phosphorylation. Of the two, PMS has a considerably greater effect. In addition, a major difference is that whilst CDNB has a greater stimulatory effect in oxygenated cells, by contrast, PMS is more effective in deoxygenated cells. These actions are correlated with ca. 30% inhibition of the plasma membrane Ca^{2+} pump (PMCA) and an increased sensitivity of the Gardos channel to Ca^{2+} (EC_{50} falling to about 150 nM). These findings are important in understanding how oxidants alter red cell cation permeability and may be relevant to the abnormal permeability phenotype shown by deoxygenated sickle cells.
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Keywords: Gardos channel; 1-Chloro-2,4-dinitrobenzene; Phenazine methosulphate; Oxygen

1. Introduction

The effect of oxidants on red cell membrane permeability is important for two main reasons. First, they have been used experimentally to study mechanisms of ion permeation [1]. Second, they may accumulate in vivo, for example, in various haemoglobinopathies and oxidant toxicities (e.g. onion poisoning in dogs: Ref. [2]). In this context, they have been associated with the increase in cation permeability observed in red cells from sickle cell patients [3] and may be involved in the pathophysiology of sickle cell disease (SCD, e.g. Ref. [4]). It is therefore relevant to understand their action as fully as possible.

1-Chloro-2,4-dinitrobenzene (CDNB) and phenazine methosulphate (PMS) are two reagents which have been shown to increase passive K^+ permeability in human red cells [5–9]. Effects are mediated via both of the major passive K^+ pathways, the K^+–Cl^- cotransporter (KCC, probably KCC1 isoform: Refs. [10,11]) and the Ca^{2+}-activated K^+ channel (Gardos channel, probably IK1/SK4: Refs. [12,13]). Many red cell membrane transporters are also affected by O_2 tension [14] and the effect of oxidants may vary with this parameter. The interaction of these compounds with changes in O_2 tension, however, has been largely ignored.

In this paper, we consider the effects of CNBD and PMS on Gardos channel activity, at different O_2 tensions. We correlate activation of the channel with changes in passive and active Ca^{2+} transport, and changes in Ca^{2+} sensitivity of the channel.

2. Materials and methods

2.1. Chemicals

Bumetanide, CDNB, MOPS, *N*-ethylmaleimide (NEM), ouabain, PMS, salts, Tris base and vanadate were purchased from Sigma (Poole, Dorset, UK). Calyculin A,

Abbreviations: ATP, adenosine triphosphate; CDNB, 1-chloro-2,4-dinitrobenzene; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GSH, reduced glutathione; Hct, haematocrit; KCC, K^+–Cl^- cotransporter; MAPTAM, 1,2-bis(*o*-amino-5′-methylphenoxy)ethane-N,N,N′,N′-tetraacetic acid tetraacetoxymethyl ester; MBS, MOPS-buffered saline; MOPS; NEM, *N*-ethylmaleimide; PMS, phenazine methosulphate; SCD, sickle cell disease.

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clotrimazole and 1,2-bis(*o*-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethyl ester (MAPTAM) were purchased from Calbiochem (Nottingham, UK) and $^{86}Rb^{+}$ was from DuPont-NEN (Stevenage, UK). $^{45}Ca^{2+}$ was purchased from Amersham Biosciences (Little Chalfont, UK).

2.2. Solutions

The standard saline (MBS) comprised (in mM): 80 KCl, 70 NaCl, 2.5 $Ca(NO_3)_2$, 0.15 $MgCl_2$, 10 inosine and 10 MOPS (pH 7.4 at 37 °C; 290 ± 5 mosM kg^{-1} H_2O). Where required, low K^+ salines were used and contained (in mM): 4 KCl, 145 NaCl, 2.5 $Ca(NO_3)_2$, 0.15 $MgCl_2$, 10 inosine and 10 MOPS (pH 7.4 at 37 °C; 290 ± 5 mosM kg^{-1} H_2O). Stock solutions of bumetanide (1 mM) were prepared daily in 100 mM Tris base and used at a final concentration of 10 μM. Stock solutions of NEM (100 mM) were prepared daily in distilled water and used at 1 mM; those of ouabain (10 mM) were prepared in distilled water and used at a final concentration of 100 μM. Stocks of calyculin A (10^{-5} M) and clotrimazole (1 mM) were prepared in DMSO, frozen until required, and used at final concentrations of 100 nM and 5 μM, respectively. CDNB and PMS (both 500 mM) were dissolved in DMSO and water, respectively, and used at a final concentration of 1 mM; in all cases, oxygenated cells were treated with CDNB or PMS (1 mM) at 4% haematocrit (Hct) for 60 min before washing (twice with MBS). They were then placed in tonometers (at 40% Hct) to equilibrate at the requisite O_2 tension before measurement of transport activity or other cell parameters (again in the presence of the oxidant). In all experiments, controls and cells treated with reagents were exposed to the same concentrations of solvents.

2.3. Sample collection and handling

Blood samples were obtained by venepuncture of healthy donors (HbAA) and collected into heparinized syringes. Buffy coats were removed by centrifugation and aspiration. Red cell samples were placed on ice and used within 36 h.

2.4. Tonometry

Red blood cell suspensions were incubated at about 40% haematocrit in glass tonometers (Eschweiler, Kiel, Germany) flushed with gas mixtures with the appropriate O_2 tension using a Wösthoff gas mixing pump. The gases were warmed up to 37 °C and fully humidified through three humidifiers.

2.5. ATP, GSH, metHb, cell volume and intracellular pH

For all these assays, cells were first incubated for 60 min ± CDNB or PMS (1 mM) at 4% Hct. ATP was determined by using an NADH-based commercial assay from Sigma using the method of Beutler and Duron [15]. GSH was assayed following the procedure of Beutler [16] based on reaction with DTNB. Determination of metHb content was carried out following the procedure of Hegesh et al. [17] based on its conversion to cyanmetHb and absorption of light at 632 nm. Cell volume was measured using the method of Borgese et al. [18] in samples swollen anisotonically by 10%. Intracellular pH was measured by centrifuging cells through dibutyl phthalate oil, lysing the cells by freeze-thawing and measuring the pH with a micropH probe—these values, coupled with those for extracellular pH, were used to calculate r values (where $r=[H^+]_i/[H^+]_o$, a value of 1.6 was determined). In the presence of A23187, $[Ca^{2+}]_i=[Ca^{2+}]_o \times r^2$.

2.6. K^+ influx

To determine the activity of the K^+ transport pathways, K^+ influx was measured at 37 °C using $^{86}Rb^{+}$ as a congener for K^+ [19]. Cells were taken from the tonometers and diluted 10-fold into saline, pre-equilibrated at the appropriate O_2 tension, at 260 mosM kg^{-1} and pH 7. The high $[K^+]$ (80 mM) in the salines was chosen to prevent cell dehydration following transporter activation. $^{86}Rb^{+}$ was added in distilled water. Except for experiments on the Na^+/K^+ pump, ouabain (100 μM) and bumetanide (10 μM) were present in all experiments to obviate any K^+ transport through the Na^+–K^+-ATPase and the NaK2Cl cotransporter, respectively. Either microhaematocrit determination or the cyanohaemoglobin method was used to measure Hct. Gardos channel activity was calculated as the clotrimazole-sensitive, 5 μM, K^+ influx, and Na^+/K^+ pump activity as the ouabain-sensitive K^+ influx.

2.7. Ca^{2+} efflux and influx

In these experiments, transport determinations were carried out at 37 °C and $^{45}Ca^{2+}$ as used as a tracer for Ca^{2+}. Plasma membrane Ca^{2+} pump activity (PMCA) was assayed following the method of Tiffert et al. [20]. Briefly, cells were washed twice with MBS lacking Ca^{2+} and containing 100 μM EGTA (MBS-0Ca-E—to remove contaminant Ca^{2+}), then twice further with MBS-0Ca (without EGTA). They were then suspended at 10% Hct and loaded with $^{45}Ca^{2+}$ using the ionophore A23187 (10 μM) at $[Ca^{2+}]_o$ of 120 μM. The ionophore was then blocked using Co^{2+} (0.4 mM), after which Ca^{2+} is progressively pumped out of the cells by PMCA. Serial aliquots (50 μl) of cells taken during these procedures were washed twice with ice-cold MBS-0Ca-E plus 0.2 mM Co^{2+} (1.3 ml). The maximum negative slope of the curve (Ca^{2+} content vs. time—see Fig. 2) after addition of Co^{2+} was used as a measure of PMCA V_{max} [20]. For influx experiments, the saline (MBS-0Ca-P) comprised (in mM): 80 KCl, 65 NaCl, 5 Na-pyruvate, 0.15 $MgCl_2$, 10 inosine and 10 MOPS (pH 7.4 at 37 °C; 290 ± 5 mosM kg^{-1} H_2O). Pyruvate was added

to protect cells from ATP depletion by formaldehyde released following ester hydrolysis [21,22]. Cells were preloaded with MAPTAM, a Ca^{2+} chelator, for 90 min (0.25 mM MAPTAM at 20% haematocrit) before incubating with CDNB (60 min, 1 mM, 4% Hct—as previously). They were taken to 4% Hct, treated with vanadate (1 mM) to inhibit PMCA, prior to addition of $^{45}Ca^{2+}$ (final $[Ca^{2+}]_o$ of 1 mM). Serial aliquots (100 μl) were taken and added to 10 ml ice-cold MBS-0Ca-P, pelletted, and washed once more (see Ref. [19]).

2.8. Statistics

Results are presented as single observations representative of at least three others, or as means ± S.E.M. of *n* observations. Where appropriate, comparisons were made using paired Student's *t*-tests.

3. Results

3.1. Cell parameters

Neither PMS nor CDNB altered cell volume or intracellular pH. Cell ATP remained constant with CDNB but was slightly elevated with PMS (from 3.6 ± 0.5 to 5.4 ± 0.4 μmol g^{-1} haemoglobin in deoxygenated cells; all means ± S.E.M., $n = 3$ or 4). Reduced glutathione levels (GSH) were completely depleted with either reagent, falling from ca. 3 to 0 mM. Methaemoglobin accumulated from < 1% in control cells, to 12 ± 0% with CDNB and, with PMS, to 89 ± 5% in N_2 and 77 ± 3% with PMS in air. Na^+/K^+ pump activity was unaltered by CDNB, but decreased from 2.5 ± 0.3 to 1.1 ± 0.2 mmol (l cells h)$^{-1}$ with PMS.

3.2. O_2 tension and activation of the Gardos channel by CDNB and PMS

Red cells treated with CDNB or PMS (both 1 mM, 60 min) showed elevated Gardos channel activity (Fig. 1a and b). Stimulation was approximately 10-fold higher with PMS than with CDNB. Comparing the effects of changing O_2 tension, for CDNB, stimulation was greater in oxygenated cells compared to deoxygenated ones (by 4 ± 1-fold). By contrast, in the case of PMS, Gardos channel activity was higher in deoxygenated cells (by 20 ± 3-fold).

3.3. Dependence on extracellular Ca^{2+} and modulators of protein phosphorylation

The dependence on extracellular Ca^{2+} was examined by comparing Gardos channel activity in the presence of 2.5 mM Ca^{2+} or with nominally Ca^{2+}-free saline with the addition of 100 μM EGTA. CDNB was examined in air, PMS in N_2. In the case of CDNB, Gardos channel activity was 4.1 ± 0.2 mmol (l cells h)$^{-1}$ in the presence of Ca^{2+} and

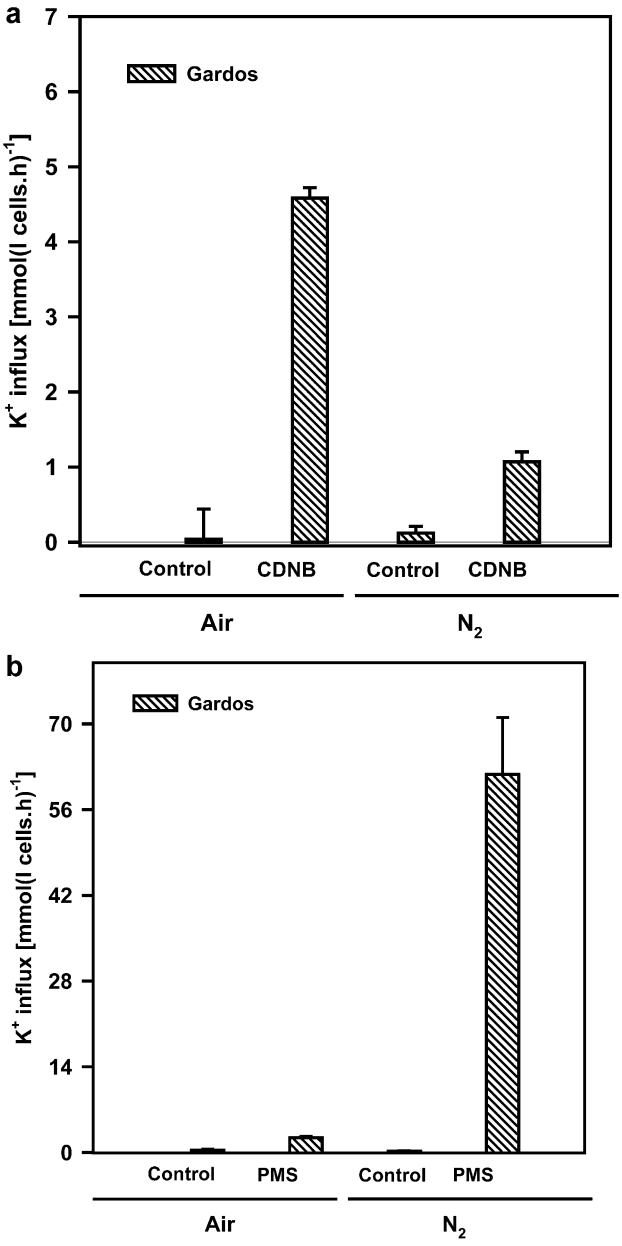

Fig. 1. The effect of 1-chloro-2,4-dinitrobenzene (CDNB) and phenazine methosulphate (PMS) treatment on Gardos channel activity in human red cells. Cells were treated with (a) CDNB or (b) PMS (1 mM) for 60 min prior to measurement of the activity of the Gardos channel (as clotrimazole-sensitive K^+ influx, 5 μM) in fully oxygenated (air) or deoxygenated (N_2) cells. Ouabain (100 μM) and bumetanide (10 μM) were present to prevent transport via Na^+/K^+ pump and $Na^+–K^+–Cl^-$ cotransporter. Transport is given as mmol K^+ (l cells h)$^{-1}$, means ± S.E.M., $n = 3$ different experiments.

was fully inhibited (to 0) by its removal; for PMS, the activities were 47.3 ± 2.0 and 0 mmol (l cells h)$^{-1}$, respectively. Thus for both reagents, effects on the Gardos channel were abolished by removal of extracellular Ca^{2+}, consistent with the requirement for Ca^{2+} influx. We went on to examine the effects of treatment with the phosphatase inhibitor calyculin A (100 nM) or the alkylating agent *N*-ethylmaleidmide (1 mM). Neither reagent altered Gardos

channel activity. For example, Gardos channel activities were 4.1 ± 0.2 and 78.9 ± 12.4 mmol (l cells h)$^{-1}$ for CDNB and PMS in the absence of calyculin A, 4.3 ± 0.7 and 80.0 ± 11.5 when cells were pretreated with calyculin A.

3.4. Effect of CDNB and PMS on Ca^{2+} influx and efflux and Ca^{2+} sensitivity

Passive Ca^{2+} influx was unaltered or reduced by treatment with CDNB and PMS. In control cells, values were typically 10–50 μmol (l cells h)$^{-1}$, falling by about $25 \pm 30\%$ with CDNB in air and by $65 \pm 9\%$ with PMS in N_2. The effect of CDNB and PMS was investigated on the activity of the plasma membrane Ca^{2+} pump (PMCA). In air, CDNB inhibited PCMA by $29 \pm 3\%$ from 6.6 ± 1.2 to 4.8 ± 1.1 mmol (l cells h)$^{-1}$ (Fig. 2). In N_2, CDNB had minimal effect (data not shown). For PMS, the effect on PMCA in oxygenated cells was minimal (10.6 ± 1.1 and 10.2 ± 1.2 mmol (l cells h)$^{-1}$ ± PMS, respectively). In deoxygenated conditions, however, PCMA was inhibited by $28 \pm 3\%$, from 9.0 ± 1.3 to 6.6 ± 1.2 mmol (l cells h)$^{-1}$ (Fig. 2). In air, CDNB increased the Ca^{2+} sensitivity of the channel (EC_{50} for $[Ca^{2+}]_i$ reduced from 260 ± 26 to 175 ± 15 nM). For PMS, in N_2, Gardos channel EC_{50} was reduced by $65 \pm 2\%$ relative to control values (to 152 ± 57 nM; Fig. 3). Maximal activity of the Gardos channel showed some changes. With CDNB in air, activity decreased by

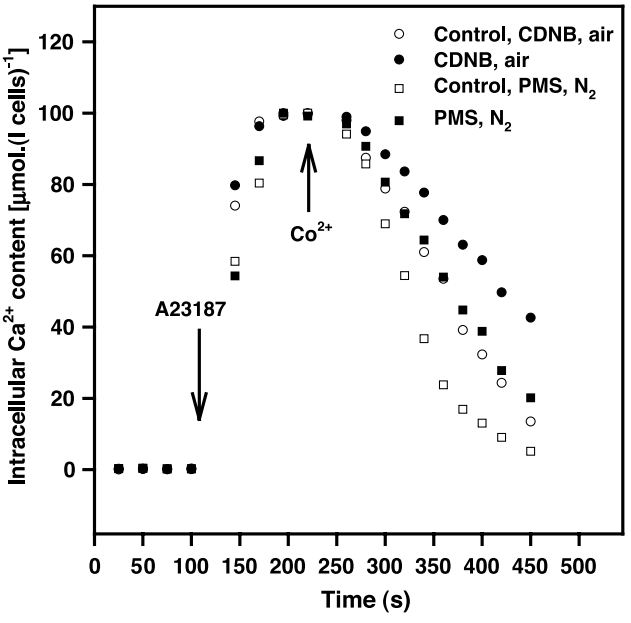

Fig. 2. Effect of 1-chloro-2,4-dinitrobenzene (CDNB) and phenazine methosulphate (PMS) treatment on Ca^{2+} efflux from human red cells. Plasma membrane Ca^{2+} pump activity (PMCA) was measured following the method of Tiffert et al. [20]. Cells were loaded with $^{45}Ca^{2+}$ using the ionophore A23187 (10 μM) before addition of Co^{2+} (0.4 mM) to inhibit ionophore permeability. Because control values of PMCA vary, the effects of oxidants were always compared simultaneously in paired samples. Symbols represent single determinations representative of four further experiments.

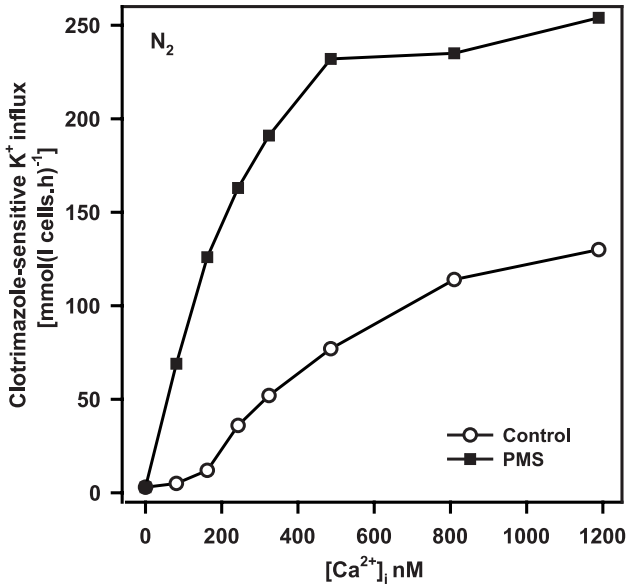

Fig. 3. Effect of phenazine methosulphate (PMS) treatment on Ca^{2+} sensitivity of the Gardos channel in human red cells. Cells were incubated for 60 min in the presence or absence of PMS (1 mM). They were then divided into six aliquots and treated with A23187 and combinations of EGTA and different $[Ca^{2+}]_o$ to provide the $[Ca^{2+}]_i$ indicated, $r^2 = 1.6$. Gardos channel activity was determined as the clotrimazole-sensitive (5 μM) K^+ influx (mmol (l cells h)$^{-1}$). Symbols represent triplicate means of three experiments on samples from different donors.

$35 \pm 19\%$. With PMS, it was also decreased in air, falling by $32 \pm 6\%$. By contrast, however, in N_2, maximal activity increased by $95 \pm 32\%$. Finally, deoxygenation per se had modest effects on Gardos channel activity which were not significant, causing a reduction in Ca^{2+} sensitivity ($18 \pm 19\%$) and a fall in maximal activity ($14 \pm 23\%$).

4. Discussion

This paper compares the effects of CDNB and PMS on Gardos channel activity in normal human red cells. We show that both stimulate channel activity, both are dependent on the presence of extracellular Ca^{2+}, and neither is affected by inhibitors of protein (de)phosphorylation. Of the two, PMS has a considerably greater effect. In addition, a major difference is that whilst CDNB has a greater stimulatory effect in oxygenated cells, by contrast, PMS is more effective in deoxygenated cells. These actions are correlated with ca. 30% inhibition of the plasma membrane Ca^{2+} pump (PMCA) and an increased sensitivity of the Gardos channel to Ca^{2+} (EC_{50} falling to about 150 nM). These findings are important in understanding how oxidants alter red cell cation permeability and may be relevant to the abnormal permeability phenotype shown by deoxygenated sickle cells.

Human red cells have two main passive K^+ permeability pathways, KCC and the Gardos channel. The former is regulated by a phosphorylation cascade [23–25], whilst for the Gardos channel Ca^{2+} is the important stimulus [12]. We

have presented elsewhere findings on the effect of these oxidants on KCC activity [9,26]. We show here that the activation of the Gardos channel by CDNB and PMS is independent of changes in protein phosphorylation, as prior treatment of cells with either NEM or calyculin A has no effect. Rather, as expected, Ca^{2+} plays a central role. For both CDNB and PMS, extracellular Ca^{2+} is required for activity, implying that entry of Ca^{2+} from outside the cell is critical. In addition, the action of both oxidants correlated with inhibition of PMCA (by about 30%) and an increase in sensitivity of the Gardos channel to Ca^{2+} (by about threefold). For CDNB, these changes occurred in oxygenated cells, whilst for PMS, they were observed for deoxygenated cells. CDNB stimulation of the Gardos channel has been previously noted for human sickle cells [8]. Previous studies with PMS, however, show some discrepancy, with stimulation of the Gardos channel present [5] or absent [6]. The difference is probably explained by the presence or absence of extracellular Ca^{2+}, which we show here to be necessary.

Amongst oxidants that we have studied, PMS is unique in having a greater effect in deoxygenated cells. The resulting phenotype shows some similarities with the increased cation permeability shown by deoxygenated sickle cells [27]. It is interesting to consider why PMS has this effect. PMS functions as a hydrogen acceptor and donor [5,28]. It is therefore able to generate oxygen free radicals in the presence of hydrogen donors, such as ascorbate, GSH, NADH and NADPH. Of these, GSH is absent whilst ascorbate is not obviously affected by O_2 tension. On deoxygenation, however, the red cell metabolism switches from flux of glucose through the pentose phosphate pathway to increased flux through the glycolytic pathway [29]. As this occurs, (re)generation of NADH will increase. We speculate that increased availability of NADH, in the presence of PMS, leads to greater formation of oxygen free radicals and hence a greater effect on K^+ permeability. The increased formation of metHb in deoxygenated cells treated with PMS is consistent with this hypothesis. We are currently investigating the action of these oxidants on different cell populations and the ability of antioxidants to protect cells against damage.

Acknowledgements

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