Plasmalogens as endogenous antioxidants: somatic cell mutants reveal the importance of the vinyl ether

Raphael A. ZOELLER*[1], Andrew C. LAKE*, Narasimhan NAGAN*[2], Daniel P. GAPOSCHKIN*, Margaret A. LEGNER* and Wilfred LIEBERTHAL†

*Department of Biophysics, Boston University School of Medicine, 715 Albany Street, Boston, MA 02118, U.S.A., and †Renal Section, Boston Medical Center, 88 East Newton Street, Boston, MA 02118, U.S.A.

Exposure of plasmalogen-deficient variants of the murine cell line RAW 264.7 to short-term (0–100 min) treatment with electron transport inhibitors antimycin A or cyanide (chemical hypoxia) resulted in a more rapid loss of viability than in the parent strain. Results suggested that plasmalogen-deficient cells were more sensitive to reactive oxygen species (ROS) generated during chemical hypoxia; the mutants could be rescued from chemical hypoxia by using the antioxidant Trolox, an α-tocopherol analogue, and they were more sensitive to ROS generation by plumbagin or by rose bengal treatment coupled with irradiation. In addition, the use of buffers containing ^2H_2O greatly enhanced the cytotoxic effect of chemical hypoxia, suggesting the involvement of singlet oxygen. We used the unique enzymic deficiencies displayed by the mutants to differentially restore either plasmenylethanolamine (the major plasmalogen species normally found in this cell line) or its biosynthetic precursor, plasmanylethanolamine. Restoration of plasmenylethanolamine, which contains the vinyl ether, resulted in wild-type-like resistance to chemical hypoxia and ROS generators, whereas increasing levels of its precursor, which bears the saturated ether, had no effect on cell survival. These findings identify the vinyl ether double bond as a crucial element in cellular protection under these conditions and support the hypothesis that plasmalogens, through the vinyl ether, act as antioxidants to protect cells against ROS. These phospholipids might protect cells from ROS-mediated damage during events such as chemical hypoxia.

Key words: antimycin, chemical hypoxia, ischaemia, reactive oxygen species, singlet oxygen.

INTRODUCTION

Mammalian phospholipids can be divided into three subclasses based on the substitution found at the *sn*-1 position of the glycerol backbone [1]. These are depicted in Figure 1, with ethanolamine phospholipids as examples. Phosphatidylethanolamine (diacyl) is characterized by acyl chains attached, through ester linkages, to the *sn*-1 and *sn*-2 positions of the glycerol backbone. The other two subclasses, plasmanylethanolamine (alkylacyl) and plasmenylethanolamine (alkenylacyl), each contain an alkyl chain attached at the *sn*-1 position through an ether linkage. In plasmenylethanolamine, a *cis* double bond is adjacent to the ether oxygen in the alkyl chain, forming an alkenyl or 'vinyl ether'. Phospholipids bearing the vinyl ether are classified as plasmalogens. Plasmalogens are most often found as components of the ethanolamine phospholipids [1–5] except for those in the heart, in which they make up a major portion of the choline phospholipids as well [1]. In the human heart one of every three phospholipids is a plasmalogen [1].

The cellular functions of plasmalogens are not yet established. A series of inherited human genetic disorders have been described in which patients' tissues lack plasmalogens [6,7] but the primary defect in most of these patients is a defect in peroxisome assembly and function. Because of the combined loss of peroxisomal function and plasmalogen deficiency, it has not been possible to determine which clinical manifestations are due to a plasmalogen deficit. Patients with a defect in ether lipid biosynthesis and normal peroxisome function have also been described [6,8]. However, because these patients display a general loss of ether lipids, it has been impossible to differentiate between the importance of the alkylacyl-phospholipids and the alkenylacyl-phospholipids (plasmalogens).

We have reported the isolation of mutants, RAW.12 and RAW.108, derived from the macrophage-like cell line, RAW 264.7 [9], that are defective in the biosynthesis of ether lipids [10]. These mutants contain intact, functional peroxisomes. RAW.12 is a particularly useful mutant in which the alkylacyl or the alkenylacyl forms of the ethanolamine phospholipids can be selectively restored through supplementation of the growth medium with different bypass lipids. These mutants provide an ideal model system with which to examine the cellular functions of both ether lipid species independently of one another.

We have compared the sensitivity of wild-type (RAW 264.7) and plasmalogen-deficient mutants to a combination of mitochondrial electron transport inhibitors and glucose-free buffer (chemical hypoxia). Under these conditions, cells undergo physiological events that are observed in cells during hypoxia and anoxia/reperfusion (ischaemic stress), including phospholipase A_2 activation, ATP depletion, induction of stress-activated protein kinase and cell death [11–13]. Also, owing to the interruption of the mitochondrial electron transport system, electrons are prematurely transferred to molecular oxygen, generating superoxide [11] and possibly other radical species [14] that contribute to cellular damage. We found that plasmalogen-deficient mutants were much more sensitive to chemical hypoxia. We also found that the vinyl ether in plasmalogens was a critical

Abbreviations used: AEG, *sn*-1-alkenylglycerol; AG, *sn*-1-alkylglycerol; AM, acetoxymethyl ester; DHAPAT, dihydroxyacetone phosphate acyltransferase; EH, ethidium homodimer; G3P, *sn*-glycerol 3-phosphate; HBS, HEPES-buffered saline; ROS, reactive oxygen species.

[1] To whom correspondence should be addressed (e-mail zoeller@med-biophd.bu.edu).
[2] Present address: Department of Medical Laboratory Sciences, Medical University of South Carolina, Charleston, SC 29425, U.S.A.

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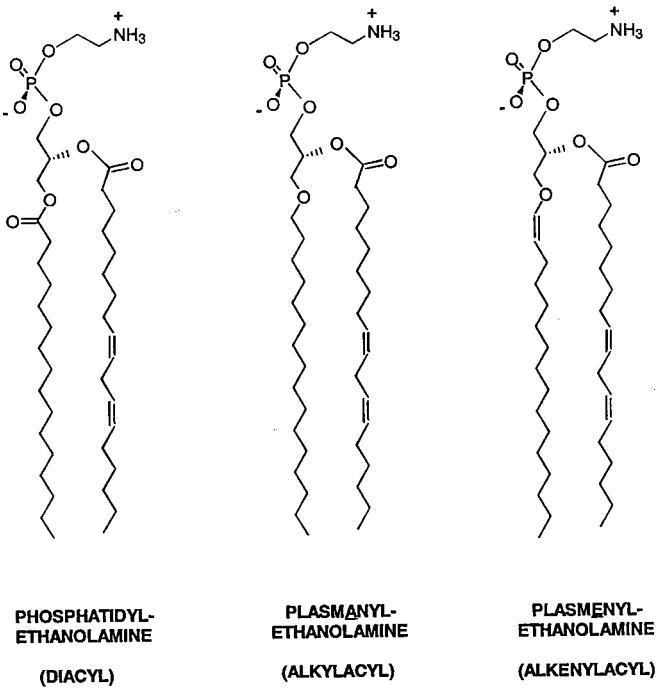

Figure 1 Ethanolamine head-group phospholipid species found in animal cells

Phosphatidylethanolamine (diacyl), plasmanylethanolamine (alkylacyl) and plasmenylethanolamine (alkenylacyl) are depicted. These species vary with respect to the substitution at the *sn*-1 position of the glycerol backbone. Plasmenylethanolamine is commonly referred to as a plasmalogen, characterized by a *cis* double bond adjacent to the oxygen of the ether linkage; it is a vinyl ether. The major plasmalogen species in the wild-type cell lines used in this study (RAW 264.7) was plasmenylethanolamine, which makes up 35% of the ethanolamine phospholipids (8% of the total phospholipid); the remainder is phosphatidylethanolamine. There is little or no plasmenylcholine, the plasmalogen form of the choline phospholipids in RAW 264.7 cells.

factor in determining the resistance of these cells to the cytotoxic effects of chemical hypoxia and the generation of reactive oxygen species (ROS). These results suggest that plasmalogens protect cells against ROS generated during chemical hypoxia.

EXPERIMENTAL

Materials

Bovine heart phosphatidylcholine and alkylglycerol (AG) (purchased as *sn*-1-hexadecylglycerol) were purchased from Doosan-Serdary Research Laboratories (Englewood Cliffs, NJ, U.S.A.). *sn*-1-Alkenylglycerol (AEG) was produced by vitride reduction [15] of bovine heart phosphatidylcholine (which is approx. 60% in the plasmalogen form). The resulting AEG was purified by elution on a silicic acid column with freshly distilled diethyl ether so that the final product was more than 95% pure as judged by TLC. [1-^{3}H]Ethanolamine (30 Ci/mmol) was purchased from Amersham Corp. (Arlington, IL, U.S.A.). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Aldrich (Milwaukee, WI, U.S.A.). Plumbagin (5-hydroxy-2-methyl-1,4-napthoquinone) and all other biochemicals, unless otherwise specified, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cells and culture conditions

The RAW 264.7 cell line [9] was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Plasmalogen content was decreased by approx. 90% in the mutant cell lines RAW.12 and RAW.108 owing to enzymic deficiencies in the biosynthetic pathway [10]. Cells were maintained as suspension cultures in Petri dishes (Valmark, Gloucester, Ontario, Canada) with Ham's F12 (BioWhittaker; Walkersville, MD, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (BioWhittaker), penicillin and streptomycin in an air/CO_2 (19:1) atmosphere at 37 °C. For experiments, cells were treated in suspension or plated on tissue culture plastic or glass, to which they adhered.

Chemical hypoxia

RAW cells were harvested from suspension cultures and pelleted. The cells were washed once in Hepes-buffered saline (HBS) [20 mM Hepes (pH 7.4)/115 mM NaCl/3.6 mM KCl/1.3 mM KH_2PO_4/1 mM $CaCl_2$/1 mM $MgCl_2$]. The final pellet was resuspended at 2.5×10^6 cells/ml in HBS containing 2 μM antimycin A or 5 mM KCN and incubated at 37 °C. At specific time points, a 50 μl aliquot (1.25×10^5 cells) was plated into 2 ml of growth medium in a 24-well tissue culture dish. The cells were left to attach for 2 h at 37 °C, the medium was removed and replaced with 1 ml of fresh medium, and the surviving cells were left to grow for 48 h (approx. four doublings) at 37 °C. Medium was removed and the cells were washed once with PBS. Cellular protein was quantified by the Lowry method [16] after solubilization in 0.5 M NaOH.

Measurement of mitochondrial function after chemical hypoxia

Cells were plated into 12-well tissue culture dishes at 5×10^5 cells per well and left to attach overnight. Monolayers were exposed to 5 mM cyanide in glucose-free buffer for the indicated times. The cyanide-containing medium was removed; the maximal (state 3) mitochondrial respiratory activity of cell monolayers was then determined immediately by measuring the rate of ATP production in permeabilizing cell monolayers by using a modification [12] of the method of Harris et al. [17]. In brief, after exposure to, and removal of, cyanide, cell monolayers were washed with PBS (to remove residual metabolic inhibitors) and incubated in 1 ml of a buffer [120 mM KCl/5 mM KH_2PO_4/10 mM Hepes/2 mM EGTA/5 mM glutamate/5 mM malate/1 mM butyrate (pH 7.4)] containing 100 μM ouabain (to inhibit Na^+/K^+ ATPase), 90 μg/ml digitonin (to permeabilize the plasma membrane) and 2.5 mM ADP. Before the assay, the buffer was bubbled with 100% oxygen for 20 min. After 10 min at 37 °C in an air incubator, an aliquot of medium was removed, diluted in a Tris/HCl buffer (200 mM, pH 8.2) and kept on ice until the determination of ATP levels with the luciferase assay.

Exposure of cells to ROS

To examine the effects of superoxide, cell suspensions were treated with plumbagin. The experiment was performed as above (for chemical hypoxia), with the exception that the cells were suspended in serum-free F12 medium containing 2 μM plumbagin. Plumbagin was added from a 10 mM stock solution in ethanol. Aliquots (50 μl) were plated into 24-well dishes and the protein in each well was determined by using the Lowry method [16] after 48 h at 37 °C.

To examine the effects of singlet oxygen, cell monolayers were treated with rose bengal (4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodo-

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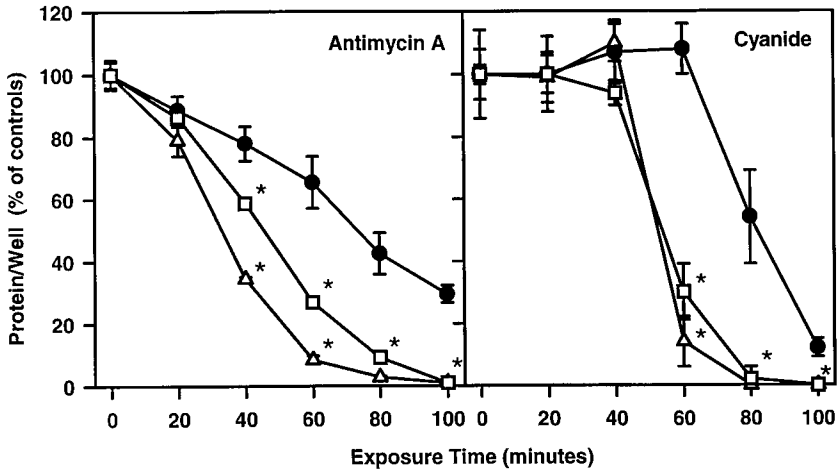

Figure 2 Sensitivity of wild-type and mutant cells to antimycin A and KCN

Cells were exposed to 2 μM antimycin A or 5 mM KCN in glucose-free buffer for the indicated times; surviving cells were left to grow for 48 h in a 24-well dish as described in the Experimental section. Protein in each well was quantified by the Lowry method [16] after solubilization of the cellular protein with 0.5 M NaOH. Values are expressed as a percentage of the zero-time controls. The control values for all cell lines were similar within a given experiment. Results are means $\pm$ S.D. for three wells. Symbols: ●, RAW 264.7; △, RAW.12; □, RAW.108. Incubation of the cells in buffer without an electron-transport inhibitor left the wild-type and mutant cell populations unaffected. An asterisk indicates that the value (or group of closely clustered values) is significantly different ($\alpha < 0.01$) from the value obtained for RAW 264.7 with Tukey's HSD multiple comparison procedure.

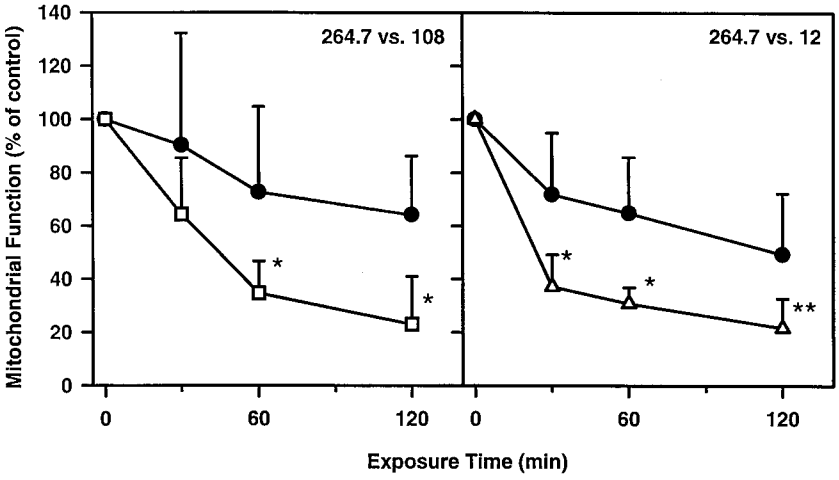

Figure 3 Loss of mitochondrial function during chemical hypoxia

Cells were exposed to 5 mM cyanide in glucose-free buffer for the indicated times; maximal (state 3) mitochondrial respiratory activity of cell monolayers was determined immediately after the removal of cyanide as described in the Experimental section. Results are means $\pm$ S.D. for several samples. Left panel: a comparison between RAW 264.7 cells (●) ($n = 9$) and RAW.108 cells (□) ($n = 9$). Right panel: a comparison between RAW 264.7 cells (●) ($n = 6$) and RAW.12 cells (△) ($n = 6$). $^{*}P < 0.01$; $^{**}P < 0.05$, as compared with RAW 264.7 at the same time point (as determined by Student's t test).

fluorescein) and light. Cells were plated into 24-well dishes at a density of 1.25×10^5 cells per well and left to attach overnight. Medium was removed and the cells were washed once with serum-free medium, then 1 ml of serum-free medium containing 2 μM rose bengal was added at room temperature. Cells were irradiated immediately by placement of the dish on a glass plate 1 mm thick suspended 17 cm above twin 20 W fluorescent light bulbs (Sylvania F20T12/GRO/WS). After irradiation, the cells were left to recover for 2 h at 37 °C. Medium was removed, growth medium was added and cells were left to grow at 37 °C for 2 days before protein determinations as described above. All manipulations involving rose bengal-exposed cells were conducted under red light. Failure to irradiate the cells with the fluorescent light resulted in no cell death.

Determination of plasmalogen content by using ethanolamine labelling

Cells were plated into sterilized 20 ml glass scintillation vials (10^5 cells per vial) for growth as monolayers. Growth medium containing 1 μCi of [1-^{3}H]ethanolamine was added together with vehicle [0.1 % (v/v) ethanol; no supplementation], 20 μM AG or 20 μM AEG. After 48 h at 37 °C, medium was removed and 3.8 ml of chloroform/methanol/PBS (1:2:0.8, by vol.) was

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added to solubilize the lipids. The lipids were extracted by using the method of Bligh and Dyer [18] and the phospholipids were separated on silica gel 60 (Merck) with the use of a double development technique [19] in which the vinyl ether group was cleaved with $HgCl_2$ and the resulting lysophospholipid was separated from the intact diacyl and alkylacyl subclasses. The TLC plates were exposed to X-ray film at -80 °C after the plate had been sprayed with EN^{3}HANCE. Bands containing the large majority of label were resolved: band 1 contained plasmenylethanolamine and band 2 contained plasmanylethanolamine and phosphatidylethanolamine (which were not separated with this technique). These fractions were scraped into scintillation vials containing 1 ml of methanol. After the addition of 8 ml of scintillation cocktail, the radioactivity associated with each band was determined by liquid-scintillation spectrometry. This technique overestimated the contribution that plasmenylethanolamine made towards the ethanolamine phospholipids but it was an accurate reflection of the relative plasmenylethanolamine content when comparing the different strains [10,19].

Viability staining

Viability was monitored with the method of Levesque et al. [20]. Cells were plated on glass coverslips that been placed in 12-well dishes, at a density of 5×10^4 cells/cm^2, and left to attach overnight. The fluorescent dye calcein acetoxymethyl ester (calcein AM) was added to the medium 30 min before chemical hypoxia. Calcein AM enters live cells and is enzymically modified to become plasma-membrane-impermeant. The coverslips were rinsed with buffer and then incubated in glucose-free buffer containing 5 mM NaCN for 1 h. During the last 10 min of this period, another fluorescent dye, ethidium homodimer (EH), was added. EH, a DNA-binding fluorogenic substance, is impermeant to live cells, but enters through the compromised membranes of non-viable cells and stains the nuclear DNA orange. The modified calcein diffuses out of the compromised cell with other soluble cytosolic components. After incubation of cells with calcein AM and EH, they were washed once with PBS and fixed with 4% (w/v) paraformaldehyde. The fixed cells were then mounted on a slide and viewed at a magnification of $\times 400$ by using epifluorescence microscopy with an excitation filter of 500 nm and emission filter of 640 nm. Under these conditions the live, calcein AM-containing cells fluoresced green, whereas dead cells displayed an orange fluorescence due to EH.

Statistical analysis

Data in most of the graphs were analysed with the NCSS60 statistical program (Jandel Scientific). Multiple groups were analysed by ANOVA, followed by Tukey's HSD multiple comparison procedure. The data for Figure 3 were analysed with Student's t test.

RESULTS

Plasmalogen-deficient cells are hypersensitive to chemical hypoxia

We examined the effects of chemical hypoxia on wild-type cells and the two plasmalogen-deficient mutants RAW.12 and RAW.108. Exposure to two different electron-transport inhibitors, antimycin A or cyanide, compounds that inhibit mitochondrial respiration at different locations within the electron transport process [11], caused a loss of cell viability as determined by growth after exposure (Figure 2). Both mutant cell lines were more sensitive to these conditions than the wild-type cells, displaying a loss of viability approx. 30 min earlier than the wild-type cells. The effects on cellular function were rapid. Mitochondrial function, when measured immediately after the removal of cyanide, was decreased in all cell lines (Figure 3). As with cell growth, there was a more rapid decrease in the mitochondrial function in the mutants.

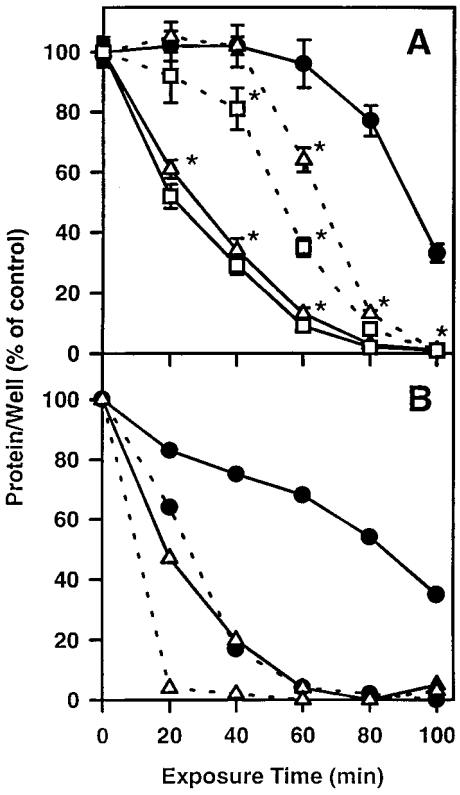

Figure 4 Reactive oxygen species play a role in the cytotoxicity of chemical hypoxia

(**A**) Protection of cells by Trolox. Cytotoxicity assays, with the use of antimycin A (2 μM), were performed and analysed as described in the Experimental section. Cells were preincubated for 3 h at 37 °C in growth medium containing no additions (solid lines) or medium containing 0.5 mM Trolox (dotted lines) before exposure to antimycin A. Trolox was also in the incubation buffer during exposure. At these levels, Trolox had no effect on cell survival. Results are means $\pm$ S.D. for triplicate determinations. Symbols: ●, RAW 264.7; △, RAW.12; □, RAW.108. An asterisk indicates that the value (or group of closely clustered values) is significantly different ($\alpha < 0.01$) from the value obtained for RAW 264.7 with Tukey's HSD multiple comparison procedure. (**B**) Enhancement of the cytotoxic effects of chemical hypoxia by ^2H_2O. Chemical hypoxia survival curves were performed as described in the legend to Figure 2. Cells were exposed to 2 μM antimycin A in HBS made with water (solid lines) or 90% (v/v) ^2H_2O (dotted lines). Results are averages for duplicate samples and did not vary by more than 10% of the average. Incubation of cells in HBS with 90% ^2H_2O, without antimycin A, had no effect on cell survival. Symbols: ●, RAW 264.7; △, RAW.12.

Involvement of ROS

The generation of ROS in cells during chemical hypoxia has been well established. In accord with these reports, a portion of the toxic effect of chemical hypoxia on the RAW cells could be attributed to the generation of ROS. When the cells were treated with Trolox [21], an analogue of α-tocopherol, mutant cells were partly rescued (Figure 4A). Characteristically for events that are mediated through singlet oxygen [22], ^2H_2O greatly exacerbated the cytotoxic effect of antimycin A (Figure 4B) in both wild-type and mutant cells.

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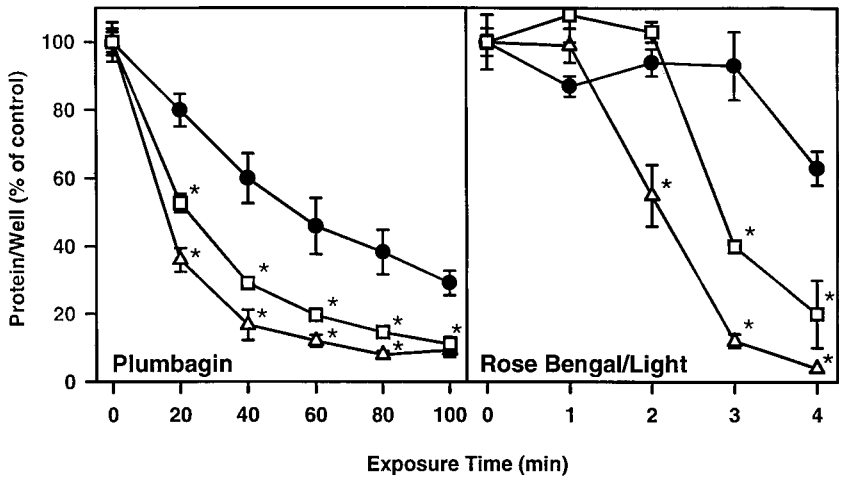

Figure 5 Cell survival after short-term exposure to ROS

Left panel: cells (2×10^6/ml) were suspended in HBS containing 2 μM plumbagin. At 20 min intervals, 50 μl aliquots were removed and transferred to 24-well dishes containing 2 ml per well. After attachment of the cells, the medium was changed and the surviving cells were left to grow and fill the wells. Cytotoxicity was determined as described in the Experimental section. Right panel: cells were plated into 24-well dishes in serum-free medium and left to attach for 2 h. Rose bengal was added to each well to a final concentration of 10 μM and the cells were irradiated immediately as described in the Experimental section. Medium was removed and replaced with normal growth medium containing no rose bengal 2 h after irradiation. The surviving cells were left to grow for 2 days before protein determinations. Failure to irradiate the cells resulted in no loss of viability. Results are means $\pm$ S.D. for three samples. Symbols: ●, RAW 264.7; △, RAW.12; □, RAW.108. An asterisk indicates that the value (or group of closely clustered values) is significantly different ($\alpha < 0.01$) from the value obtained for RAW 264.7 with Tukey's HSD multiple comparison procedure.

Short-term exposure (0–100 min) of the cells to ROS resulted in cell death (Figure 5). Both RAW.12 and RAW.108 cells were more sensitive to plumbagin, a menadione analogue that generates superoxide within the cell [23]. We also exposed the cells to rose bengal and light [24], which generates singlet oxygen. Again, there was a pronounced hypersensitivity displayed by the mutant cells. In this case the cells were irradiated immediately after the addition of rose bengal so that singlet oxygen was produced externally. However, the same results were obtained if the cells were irradiated after preincubation of the cells with rose bengal for 2 h at 37 °C before irradiation, so that singlet oxygen was generated internally (results not shown). In the latter case, lower concentrations of rose bengal and longer exposures to light (0–100 min) were used.

Restoration of plasmalogens reverses the hypersensitivity phenotype

To define further the relationship between ether lipids and the ability of the cells to survive chemical hypoxia, we made use of our ability to restore plasmalogens to the cells selectively through supplementation of the growth medium with different precursor lipids. Although both RAW.12 and RAW.108 display an ether lipid deficiency [10], their enzymic lesions within the biosynthetic pathway are different (Scheme 1). Both mutants display a deficiency in dihydroxyacetone phosphate acyltransferase (DHAPAT), the enzyme that catalyses the first reaction in the pathway, but RAW.12 has an additional deficiency in the final step in the pathway, plasmanylethanolamine desaturase (Δ1′-desaturase). In this latter step, the vinyl ether double bond is introduced, forming the plasmalogen (plasmenylethanolamine).

When AG (see Scheme 1) was added to the growth medium, it was able to enter the biosynthetic pathway downstream of the first three steps through the action of a kinase [25], forming the intermediate 1-alkyl-2-lyso-*sn*-glycerol 3-phosphate (1-alkyl-G3P). This bypassed the DHAPAT lesion: plasmalogen levels returned to slightly above normal in RAW.108 cells, as judged by long-term [1-^{3}H]ethanolamine labelling (Table 1). Because RAW.12 cells have the additional loss of Δ1′-desaturase activity, only a small portion of the plasmenylethanolamine was restored to these cells. These data agreed with previous results that showed that RAW.12 cells, instead, accumulated the immediate biosynthetic precursor of the plasmalogen, plasmanylethanolamine (alkylacyl *sn*-glycerol 3-phosphoethanolamine) during AG supplementation [10]. The ether linkage is present in this precursor but the vinyl ether double bond is not.

Supplementation of the medium with AEG also resulted in increased levels of plasmalogens in wild-type and RAW.108 cells. However, AEG was able to restore plasmalogen levels in RAW.12 cells (Table 1). AEG, like AG, bypassed the lesion in the first step of the pathway, entering as 1-alkenyl-2-lyso-*sn*-G3P (1-alkenyl-G3P; Scheme 1). Because it contained the vinyl ether group it was also able to bypass the Δ1′-desaturase lesion. Thus, by using AG or AEG supplementation of RAW.12 cells, we were able to restore the ether (alkyl) or the vinyl ether (alkenyl) selectively at the *sn*-1 position.

Supplementation of cells with AG, which restored plasmalogens to RAW.108 only, rescued only these cells during antimycin A exposure (Figure 6). The supplemented RAW.108 cells were slightly more resistant to chemical hypoxia than the unsupplemented wild-type cells. Similar results were observed with cyanide (results not shown). Supplementation of RAW.12 cells with AG, which restored only plasmanylethanolamine, but not the vinyl ether-containing plasmalogen (plasmenylethanolamine) had no effect upon the sensitivity of these cells to either antimycin A or cyanide. However, supplementation of the growth medium with AEG, which restored plasmalogens to both mutant cell lines, restored wild-type-like resistance to antimycin A in both RAW.108 and RAW.12 (Figure 7). Also, restoration of plasmalogens, and not the alkylacyl phospholipids, completely restored wild-type-like resistance of the mutants to both ROS generators, plumbagin and rose bengal (results not shown).

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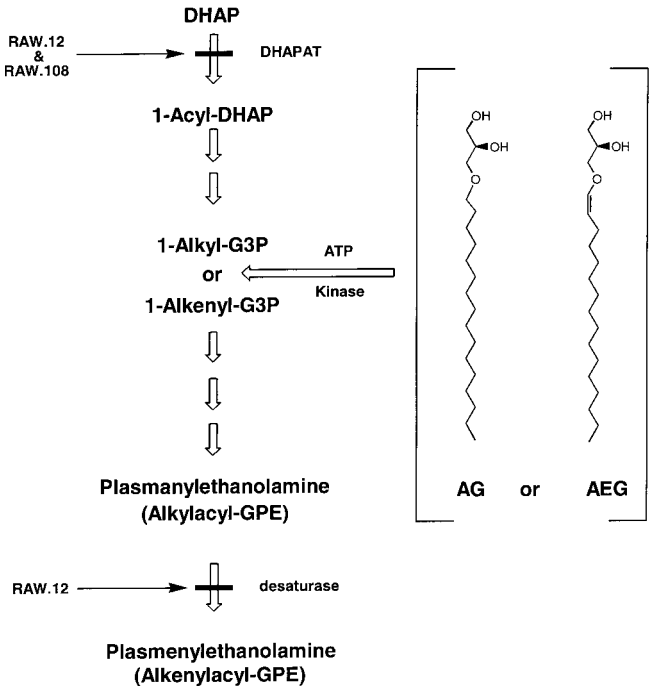

Scheme 1 Biosynthetic pathway for plasmenylethanolamine in animal cells and entrance of AG or AEG into the pathway

DHAPAT (glycerone-3-phosphate acyltransferase; EC 2.3.1.42) is missing from both RAW.12 and RAW.108. The plasmanylethanolamine desaturase ($\Delta 1'$-desaturase; EC 1.14.99.19) is lacking from RAW.12 only. The kinase, ATP:1-alkyl-*sn*-glycerol phosphotransferase (EC 2.7.1.93), is important for introductions of AG and AEG into the biosynthetic pathway. The biosynthetic lesions associated with RAW.12 and RAW.108 are indicated by the bars. Note that RAW.12 displays two lesions within the biosynthetic pathway. Also note that plasmanylethanolamine is the direct biosynthetic precursor to the plasmalogen, plasmenylethanolamine. The introduction of AG into the pathway results in the initial formation of 1-alkyl-*sn*-glycerol 3-phosphate, whereas AEG results in the initial formation of 1-alkenyl-G3P. AEG bypasses the requirement for $\Delta 1'$-desaturase activity because it contains the vinyl ether group and plasmanylethanolamine would not be formed as an intermediate.

Table 1 Recovery of plasmenylethanolamine levels by supplementation with AG and AEG

Cells were labelled for 48 h with [1-^{3}H]ethanolamine in medium containing no supplements, 20 μM AG or 20 μM AEG. The values are given as percentages of chloroform-soluble radioactivity found in the plasmenylethanolamine band. Most of the remaining radioactivity was recovered in a fraction that contained both phosphatidylethanolamine and plasmanylethanolamine; these species could not be separated with the TLC system used [19]. Results are means ± S.D. for three determinations.

	[1-^{3}H]Ethanolamine in plasmenylethanolamine (% of total)		
Cell line	No additions	+ AG	+ AEG
RAW 264.7	58.5 ± 2.8	79.5 ± 2.8	78.1 ± 1.4
RAW.12	12.6 ± 2.5	29.2 ± 2.6	75.0 ± 2.1
RAW.108	12.7 ± 2.6	78.9 ± 1.2	77.5 ± 1.8

The results obtained with long-term survival assays were confirmed by using viability staining during a 60 min exposure to either cyanide (Figure 8) or antimycin A (results not shown). Most wild-type (RAW 264.7) cells were viable (green) after treatment with cyanide. In contrast, most of the unsupplemented RAW.108 and RAW.12 cells were dead (orange). Supplementation with AG rescued RAW.108 cells, whereas RAW.12 cells were still killed by cyanide. Supplementation with AEG rescued both RAW.108 and RAW.12 cells.

DISCUSSION

We observed a marked hypersensitivity of the two plasmalogen-deficient mutants RAW.12 and RAW.108 to chemical hypoxia. Because these mutants contained intact, functional peroxisomes, we were better able to interpret changes in cell function, by using plasmalogens as the only variable. The fact that RAW.12 had two lesions in the biosynthetic pathway (DHAPAT and $\Delta 1'$-desaturase) gave us a unique opportunity to restore phospholipids containing the ether bond (plasmanylethanolamine) or the vinyl ether (plasmenylethanolamine) to these cells, depending on the supplement (AG or AEG). These studies revealed that recovery of the vinyl ether, not the ether bond, resulted in cellular protection under these conditions.

Any reasonable mechanism explaining the protective function of plasmalogens must take into account the events, triggered during chemical hypoxia, that can cause cellular damage. Phospholipase A_2 activity increases [26–28], resulting in the generation of membrane-disrupting non-esterified fatty acids and lysophospholipids. Evidence for the activation of a plasmalogen-specific phospholipase A_2 has been reported in hypoxic rabbit proximal tubules [26] and in ischaemic myocardium [28]; it has been suggested that this has a role in causing cell damage. We did observe the rapid release of arachidonate from the phospholipid pools during chemical hypoxia and there was a slight decrease in the rate of arachidonate release in the mutants (D. P. Gaposchkin and R. A. Zoeller, unpublished work). On the basis of these results, we would have expected the plasmalogen-deficient cells to be damaged somewhat more slowly than their plasmalogen-containing counterparts during chemical hypoxia. Instead we found the opposite: the mutant cells were damaged more rapidly.

It is possible that plasmalogens impart structural properties to a membrane that make it less susceptible to perturbations by fatty acids, lysophospholipids or other membrane-disrupting agents. However, we could not find any differences between the mutants and wild-type cells in respect of their sensitivities toward these compounds (N. Nagan and R. A. Zoeller, unpublished work). Studies by Han and Gross [29] have shown that the presence of plasmalogens in model membranes makes them more susceptible to perturbations by lysophospholipid. This would suggest that the plasmalogen-deficient mutants should be less sensitive to such compounds and therefore more resistant to their generation during chemical hypoxia. Again, this was contrary to our observations.

Several lines of evidence suggest that plasmalogens, by virtue of their vinyl ether, act as antioxidants. The generation of ROS during chemical hypoxia has been well established [11,30,31]. Our ability to partly rescue cells with the antioxidant Trolox demonstrated that reactive oxygen generation had a role in the chemical hypoxia-induced cytotoxicity. The enhanced cytotoxicity of antimycin A in the presence of ^2H_2O is consistent with the involvement of singlet oxygen as a mediating agent in the cell damage [22,32,33]. In agreement with these results, we observed a marked hypersensitivity of the plasmalogen-deficient mutants to exposure to the generation of singlet oxygen and superoxide (which can be converted into a variety of ROS, including singlet oxygen [34–36]). Restoration of wild-type-like resistance to the ROS generators also required the restoration of the vinyl ether-containing phospholipids (plasmalogens) to the membranes; the recovery of the saturated ether linkage to membrane phospho-

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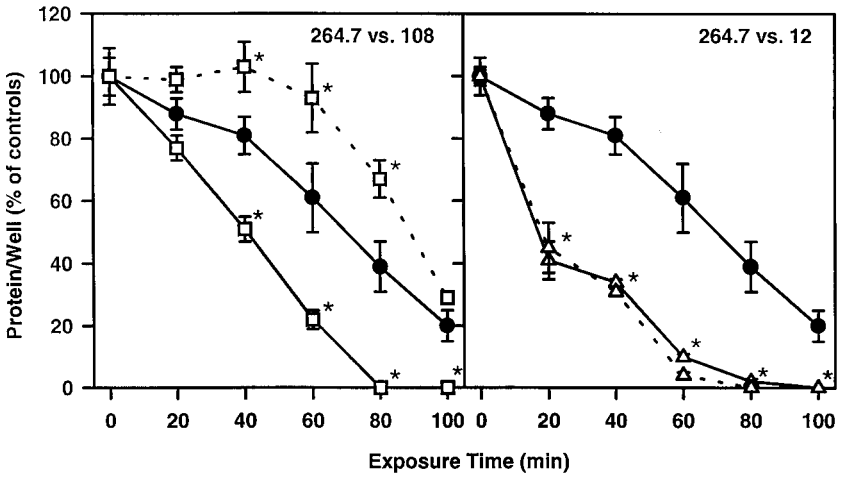

Figure 6 Rescue of mutants by supplementation with AG

Cells were grown for 48 h in unsupplemented medium (solid lines) or medium that had been supplemented with 20 μM AG (dotted lines). After this they were exposed to antimycin A and the sensitivity of the cells was determined as described in the legend to Figure 2. Values are expressed as percentages of the zero-time controls. The control values for all cell lines were similar within a given experiment. Results are means $\pm$ S.D. for three wells. Symbols: ●, RAW 264.7; △, RAW.12; □, RAW.108. In separate experiments, supplementation of RAW 264.7 with AG resulted in a slight improvement in the cells' ability to survive chemical hypoxia. This result was not included in the graphs for the purpose of clarity. An asterisk indicates that the value (or group of closely clustered values) is significantly different ($\alpha < 0.01$) from the value obtained for RAW 264.7 with Tukey's HSD multiple comparison procedure.

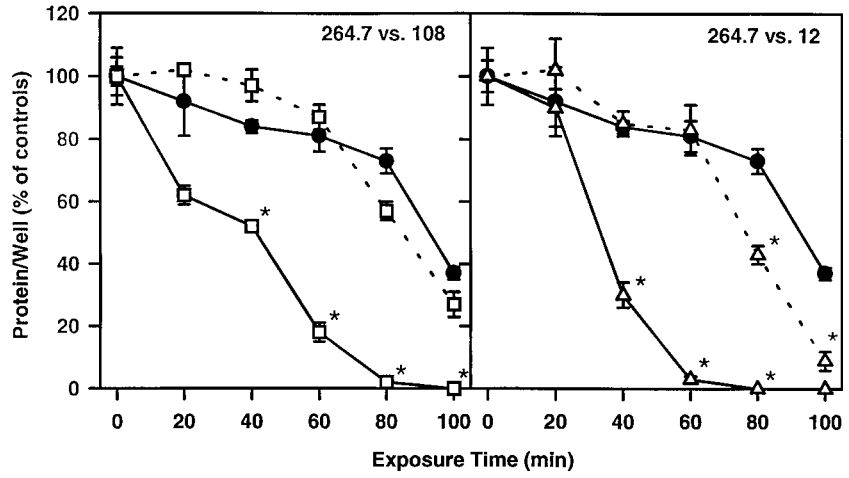

Figure 7 Rescue of mutants by supplementation with AEG

Cells were grown for 48 h in unsupplemented medium (solid lines) or medium that had been supplemented with 20 μM AEG (dotted lines). After this they were exposed to antimycin A and the sensitivity of the cells was determined as described in the legend to Figure 2. Values are expressed as percentages of the zero-time controls. Results are means $\pm$ S.D. for three wells. Symbols: ●, RAW 264.7; △, RAW.12; □, RAW.108. An asterisk indicates that the value (or group of closely clustered values) is significantly different ($\alpha < 0.01$) from the value obtained for RAW 264.7 with Tukey's HSD multiple comparison procedure.

lipids was not effective in cell protection. Saturated ethers are not particularly reactive with ROS. Finally, these results are consistent with previous studies with intact cells [37,38] and model systems [39–41], suggesting that plasmalogens are serving as scavengers of ROS. A number of studies have demonstrated plasmalogen-mediated protection and selective degradation of plasmalogens when lipoproteins [39,40] or cells [37] are exposed to ROS. For example, Jurgens et al. [39] found that enrichment of isolated low-density lipoproteins with choline or ethanolamine plasmalogen resulted in a delay in Cu^{2+}-catalysed oxidation of low-density lipoproteins from several different human donors. The interaction of plasmalogen with ROS results in the accumulation of predicted breakdown products such as α-hyroxyaldehydes [42] and fatty aldehydes [38]. In preliminary studies we have observed increases in fatty aldehyde, a product predicted from the singlet-oxygen-mediated breakdown of plasmalogens, in wild-type cells during chemical hypoxia (N. Nagan and R. A. Zoeller, unpublished work). A full characterization of these products will aid in identifying the types of ROS that plasmalogens are exposed to, and react with, during chemical hypoxia or anoxia/reperfusion.

In summary, we have made a clear link between the presence of a specific chemical group, the vinyl ether, found in plasmalogens, with the cell's ability to survive chemical hypoxia and ROS generation. Our ability to regulate the presence of the ether and/or the vinyl ether groups within the phospholipids of a

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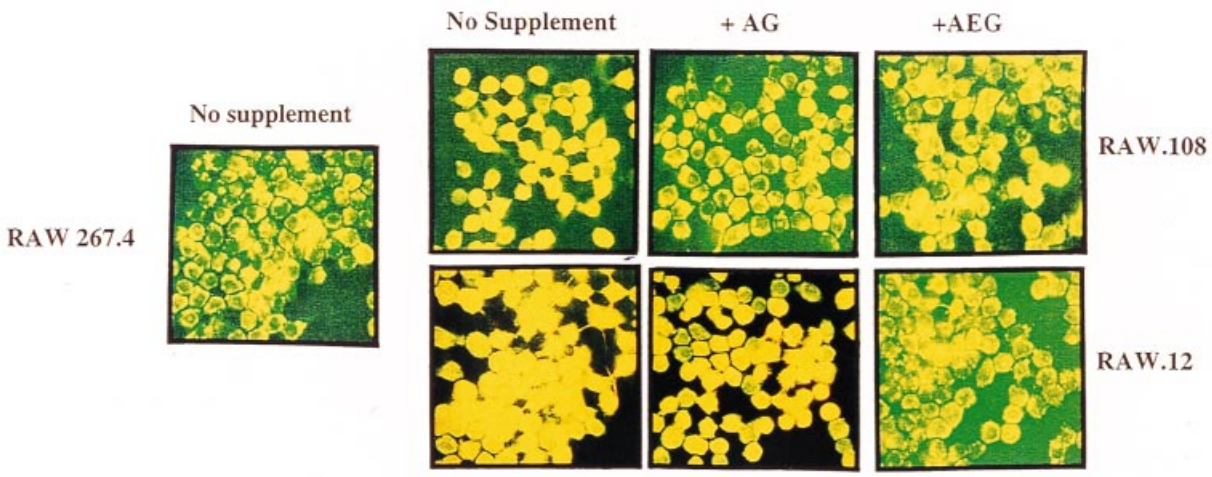

Figure 8 Mutant cell plasma membrane is compromised more rapidly than wild-type plasma membrane during chemical hypoxia and this can be prevented by restoration of plasmalogens

Cells were grown for 48 h in unsupplemented medium or medium that had been supplemented with either 20 μM AG or 20 μM AEG. Cells were left to attach to coverslips, then exposed to chemical hypoxia (cyanide) for 60 min while in the presence of ethidium homodimer and calcein AM to determine viability (see the Experimental section). The live, calcein AM-containing cells fluoresce green, whereas dead cells display an orange fluorescence due to ethidium homodimer.

single cell line, RAW.12, has allowed us to pinpoint the vinyl ether double bond as the crucial element in the protective effect. We suggest that plasmalogens, through the vinyl ether, act as antioxidants, protecting cells under conditions in which ROS cause cell damage. These findings might have some relevance to pathological events such as stroke or myocardial infarction.

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REFERENCES

1 Horrocks, L. A. and Sharma, M. (1982) in Phospholipids (Hawthorne, J. N. and Ansell, G. B., eds.), pp. 51–100, Elsevier, Amsterdam
2 Sugiura, T. and Waku, K. (1987) in Platelet Activating Factor and Related Lipid Mediators (Snyder, F., ed.), pp. 55–58, Plenum, New York
3 Sugiura, T., Onuma, Y., Sekiguchi, N. and Waku, K. (1982) Biochim. Biophys. Acta **712**, 515–522
4 Mueller, H. W., O'Flaherty, J. T., Greene, D. G., Samuel, M. P. and Wykle, R. (1984) J. Lipid Res. **25**, 383–388
5 Gross, R. W. (1984) Biochemistry **23**, 158–165
6 Moser, A. B., Rasmussen, M., Naidu, S., Watkins, P. A., McGuiness, M., Hajra, A. K., Chen, G., Raymond, G., Liu, A., Gordon, D. et al. (1995) J. Pediatr. **127**, 13–22
7 Lazarow, P. B. and Moser, H. W. (1989) in The Metabolic Basis of Inherited Disease, 6th edn. (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.), pp. 1479–1509, McGraw-Hill, New York
8 Wanders, R. J. A., Schumacher, H., Heikoop, J., Schutgens, R. B. H. and Tager, J. M. (1992) J. Inher. Metab. Dis. **15**, 389–391
9 Raschke, W. C., Baird, S., Ralph, P. and Nakoinz, I. (1978) Cell **15**, 261–268
10 Zoeller, R. A., Rangaswamy, S., Herscovitz, H., Rizzo, W. B., Hajra, A. K., Das, A. K., Moser, H. W., Moser, A., Lazarow, P. B. and Santos, M. J. (1992) J. Biol. Chem. **267**, 8299–8306
11 Dawson, T. L., Gores, G. J., Nieminen, A.-L., Herman, B. and Lemasters, J. J. (1993) Am. J. Physiol. **264**, C961–C967
12 Sheridan, A. M., Schwartz, J. H., Kroshian, V. M., Tercyak, A. M., Laraia, J., Masino, S. and Lieberthal, W. (1993) Am. J. Physiol. **265**, F342–F350
13 Pombo, C. M., Bonventre, J. V., Avruch, J., Woodgett, J. R., Kyriakis, J. M. and Force, T. (1994) J. Biol. Chem. **269**, 26546–26551
14 Halliwell, B. and Gutteridge, J. M. C. (1989) Free Radical in Biology and Medicine, 2nd edn., Clarendon, Oxford
15 Snyder, F., Blank, M. L. and Wykle, R. L. (1971) J. Biol. Chem. **246**, 3639–3645
16 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
17 Harris, S. I., Balaban, R. S., Barrett, L. and Mandel, L. J. (1981) J. Biol. Chem. **256**, 10319–10328
18 Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–917
19 Zoeller, R. A. and Raetz, C. R. H. (1992) Methods Enzymol. **209**, 34–51
20 Levesque, A., Paquet, A. and Page, M. (1995) Cytometry **20**, 181–184
21 Rubinstein, J. D., Lesnefsky, E. J., Blyer, R. M., Fennessey, P. V. and Horwitz, L. D. (1994) Free Radical Biol. Med. **13**, 627–634
22 Nilsson, R. and Kearns, D. R. (1973) Photochem. Photobiol. **17**, 65–68
23 Floreani, M., Forlin, A., Pandolfo, L., Petrone, M. and Bellin, S. (1996) J. Pharmacol. Exp. Therap. **278**, 763–770
24 Strassle, M. and Stark, G. (1992) Photochem. Photobiol. **55**, 461–463
25 Snyder, F. (1992) Methods Enzymol. **209**, 211–215
26 Portilla, D., Shah, S. V., Lehman, P. A., and Creer, M. H. (1994) J. Clin. Invest. **93**, 1609–1615
27 Portilla, D. and Creer, M. H. (1995) Kidney Int. **47**, 1087–1094
28 Ford, D. A., Hazen, S. L., Saffitz, J. E. and Gross, R. W. (1991) J. Clin. Invest. **88**, 331–335
29 Han, X. and Gross, R. W. (1991) Biochim. Biophys. Acta **1069**, 37–45
30 Borle, A. B. and Barsic, M. (1995) Cell Calcium **17**, 307–315
31 Hagar, H., Ueda, N. and Shah, S. V. (1996) Am. J. Physiol. **271**, F209–F215
32 Foote, C. S. (1976) in Free Radicals in Biology, vol. 2 (Pryor, W. A., ed.), pp. 85–133, Academic Press, New York
33 Wei, H., Cai, Q., Rahn, R. and Zhang, X. (1997) Free Radical Biol. Med. **23**, 148–154
34 Pryor, W. A. (1986) Annu. Rev. Physiol. **48**, 657–667
35 Corey, E. J., Mehrotra, M. M. and Kahn, A. V. (1987) Science **236**, 68–69
36 Mao, Y., Zang, L. and Shi, X. (1995) Biochem. Mol. Biol. Int. **36**, 227–232
37 Zoeller, R. A., Morand, O. H. and Raetz, C. R. H. (1988) J. Biol. Chem. **263**, 11590–11596
38 Morand, O. H., Zoeller, R. A. and Raetz, C. R. H. (1988) J. Biol. Chem. **263**, 11597–11606
39 Jurgens, G., Fell, A., Ledinski, G., Chen, Q. and Paltauf, F. (1995) Chem. Phys. Lipids **77**, 25–31
40 Engelmann, B., Brautigam, C. and Thiery, J. (1994) Biochem. Biophys. Res. Commun. **204**, 1235–1242
41 Reiss, D., Beyer, K. and Engelmann, B. (1997) Biochem. J. **323**, 807–814
42 Felde, R. and Spiteller, G. (1995) Chem. Phys. Lipids **76**, 259–267

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