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Peripheral ethanolamine plasmalogen deficiency: a logical causative factor in Alzheimer's disease and dementia

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J. Lipid Res., November, 2007; 48 (11): 2485-2498.

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Neutrophil-mediated accumulation of 2-CIHDA during myocardial infarction: 2-CIHDA-mediated myocardial injury

Arun K. Thukkani, Bradley D. Martinson, Carolyn J. Albert, George A. Vogler and David A. Ford

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Increasing plasmalogen levels protects human endothelial cells during hypoxia

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Zoeller, Raphael A., Todd J. Grazia, Peter LaCamera, James Park, Daniel P. Gaposchkin, and Harrison W. Farber. Increasing plasmalogen levels protects human endothelial cells during hypoxia. *Am J Physiol Heart Circ Physiol* 283: H671–H679, 2002. First published March 14, 2002; 10.1152/ajpheart.00524.2001.—Supplementation of cultured human pulmonary arterial endothelial cells (PAEC) with *sn*-1-*O*-hexadecylglycerol (HG) resulted in an approximately twofold increase in cellular levels of plasmalogens, a subclass of phospholipids known to have antioxidant properties; this was due, primarily, to a fourfold increase in the choline plasmalogens. Exposure of unsupplemented human PAEC to hypoxia (P_O₂ = 20–25 mmHg) caused an increase in cellular reactive oxygen species (ROS) over a period of 5 days with a coincident decrease in viability. In contrast, HG-supplemented cells survived for at least 2 wk under these conditions with no evidence of increased ROS. Hypoxia resulted in a selective increase in the turnover of the plasmalogen plasmenylethanolamine. Human PAEC with elevated plasmalogen levels were also more resistant to H₂O₂, hyperoxia, and the superoxide generator plumbagin. This protection was seemingly specific to cellular stresses in which significant ROS were generated because the sensitivity to lethal heat shock or glucose deprivation was not altered in HG-treated human PAEC. HG, by itself, was not sufficient for protection; HG supplementation of bovine PAEC had no effect upon plasmalogen levels and did not rescue these cells from the cytotoxic effects of hypoxia. This is the initial demonstration that plasmalogen content can be substantially enhanced in a normal cell. These data also demonstrate that HG can protect cells during hypoxia and other ROS-mediated stress, likely due to the resulting increase in these antioxidant phospholipids.

sn-1-hexadecylglycerol; antioxidant; hyperoxia; heat shock; glucose deprivation; reactive oxygen species

CELLS, TISSUES, AND ORGANISMS are often exposed to decreases in environmental oxygen concentration. The ability to tolerate acute hypoxia and to adapt to chronic hypoxia is critical to their survival. Vascular endothelial cells are directly exposed to decreases in blood oxygen tension; in particular, pulmonary artery endothelial cells (PAEC) are continually exposed to the

low oxygen content of mixed venous blood (P_O₂: 35–45 mmHg) and, during pathological states, may be exposed to even lower levels. However, these cells are more resistant than other cell types to the cytotoxic effects of acute and chronic hypoxia (22). Mechanisms by which endothelial cells, particularly PAEC, maintain cellular and functional integrity under these physiological and pathological conditions are likely complex and multiple. Identifying and enhancing these protective mechanisms is of critical importance in prevention of hypoxic damage to the cell.

Numerous studies link hypoxic injury, particularly if combined with reperfusion, to generation of reactive oxygen species (ROS) (38, 47, 54, 58). In addition, ROS have been implicated as second messengers in the pathway linking detection of changes in oxygen concentration with functional responses (8, 12). Thus the presence of endogenous antioxidant systems is important in the prevention of hypoxic injury. Plasmalogens are a unique subset of phospholipids in which the *sn*-1 carbon of the glycerol backbone contains a vinyl ether-linked (a *cis* double bond adjacent to an ether bond) long-chain hydrocarbon instead of the typical ester-linked fatty acid (43). Studies using animal cell lines (40, 55, 56), model membrane systems (45), and lipoproteins (13, 29) suggest that plasmalogens, by virtue of this vinyl ether, act as endogenous antioxidants, protecting cells and membranes from ROS.

sn-1-*O*-hexadecylglycerol (HG) is a simple lipid compound containing a 16-carbon, saturated alkyl chain attached to glycerol through an ether linkage. This compound readily enters mammalian cells and enters the biosynthetic pathway for plasmalogen biosynthesis after its phosphorylation (43). This compound has been used to restore normal plasmalogen levels to somatic cells displaying defects in the early steps in plasmalogen biosynthesis (55, 57). We have found that supplementation of human PAEC (HPAEC) with this compound resulted in a significant increase of plasmalogen levels. This increase was associated with an increased tolerance of HPAEC not only to hypoxia, but also to other cellular stresses in which generation of ROS

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contributes significantly to cytotoxicity. This is the first demonstration that plasmalogen content can be substantially enhanced in a normal cell and suggests that HG supplementation, by elevating plasmalogen levels, can protect human EC during exposure to hypoxia.

MATERIALS AND METHODS

Materials. Tissue culture plastic was from Falcon Plastics (Los Angeles, CA) and Costar (Cambridge, MA). $^{32}\text{P}_i$ (9,000 Ci/mmol) and [*methyl*- ^3H]thymidine (74 GBq/mmol) were from Perkin-Elmer/New England Nuclear (Boston, MA). Phospholipid standards were from Avanti Polar Lipids (Alabaster, AL). HG was from Serdary Research Laboratories (Englewood, NJ). All other reagents were from Sigma (St. Louis, MO) unless otherwise noted.

Cell culture. HPAEC were obtained from Clonetics (San Diego, CA) and maintained in growth media (EBM, Clonetics). Bovine PAEC (BPAEC) were harvested by lightly scraping the luminal surface of longitudinally opened pulmonary arteries (24). Cultures were maintained in MEM (GIBCO-BRL; Grand Island, NY), supplemented with 15% heat-inactivated fetal bovine serum (Hyclone Laboratories; Logan, UT), at 37°C in a humidified incubator in 5% CO_2 -95% air. EC purity was confirmed by a typical cobblestone appearance, factor VIII immunofluorescence, and uptake of fluorescent acetylated low-density lipoprotein (24). All experiments were performed using 80–90% confluent PAEC monolayers of passage numbers 3–10, and all cells were fed within 24 h of each experiment. Control and experimental conditions for an individual experiment were performed in parallel on identical cell lines of identical passage number. In experiments in which HPAEC were exposed to cellular stresses, the cells were incubated with or without HG for 4–5 days before experimentation. Neither 20 μM HG nor the vehicle caused cellular toxicity or significantly slowed cell growth (data not shown).

Phospholipid analysis. The phospholipid composition was determined by steady-state labeling with $^{32}\text{P}_i$ (14) or by phosphorous analysis (46). For steady-state labeling, EC were grown in sterile glass scintillation vials for 6 days at 37°C in medium containing $^{32}\text{P}_i$ (5 $\mu\text{Ci}/\text{ml}$) and either 20 μM HG or vehicle (0.1% ethanol). At the end of the labeling period, the medium was removed, and 3.8 ml of chloroform-methanol- H_2O (1:2:0.8) and 200 μg of carrier lipid (total lipid from the bovine heart) were added. Lipids were extracted (5), and phospholipids were separated by two-dimensional (2-D) thin-layer chromatography (TLC) (15) using silica gel 60 chromatography plates (EM Science; Gibbstown, NJ). Plates were sprayed with 10 mM HgCl_2 in acetic acid and allowed to dry for 45 min between dimensions to cleave the vinyl ether bond of the plasmalogen; the resulting lysophospholipids could then be separated from the unaffected diacyl phospholipid in the second dimension. Plates were exposed to GBX-2 X-ray film at -80°C after preflash. ^{32}P -labeled phospholipids were scraped into scintillation vials for quantification using liquid scintillation spectrometry.

For phosphorous analysis, EC were grown for 6 days in 100-mm tissue culture dishes containing 15 ml medium alone or medium containing 20 μM HG. Medium was changed every 2 days. EC were harvested using trypsin, pelleted by centrifugation at 600 g for 7 min, and resuspended in PBS (0.01 M phosphate, 0.0027 M KCl, and 0.137 M NaCl). Cells were washed twice with PBS, and the final cell pellet was resuspended in 1 ml PBS. Lipids were extracted (5) and phospholipids were separated on silica gel G plates (Analtech; Newark, DE) using the 2-D TLC system described

above. Phospholipids were visualized by spraying with 50% sulfuric acid followed by heating; areas corresponding to the various phospholipid species were scraped into acid-washed glass tubes, and the phosphorus content of each was determined by the method of Rouser et al. (46).

Fatty acid and dimethylacetal analysis. HPAEC were grown for 5 days in unsupplemented growth medium containing 0.1% ethanol or medium containing 20 μM HG. Cells were washed once with PBS and scraped from the dish in PBS using a rubber policeman, and the cellular lipids were extracted as above. The lipids were spotted onto silica gel G TLC plates, and the plates were developed in hexane-ethyl ether-acetic acid (30:70:1 vol/vol/vol). Neutral lipids, including alkylglycerols, migrated in this system, whereas phospholipids remained at the origin. Phospholipids (the origin) were eluted from the plates using chloroform-methanol (2:1 vol/vol). Samples were dried under a nitrogen stream in screw-capped test tubes and transesterified with BF_3 in methanol at 100°C for 1 h (30). After cooling and the addition of 1.5 ml water, the fatty acid methyl esters (FAME) and the dimethylacetals (DMA) were extracted twice using 3-ml portions of *n*-hexane. Solvent was removed using a stream of nitrogen, and the samples were resuspended in chloroform. FAME and DMA species were separated using gas liquid chromatography as described previously (20). Individual FAME and DMA were identified by comparison of retention times to those of authentic standard mixtures.

Hypoxia. Hypoxic conditions were generated by exposing EC monolayers in humidified sealed chambers (Billups-Rothenburg; Del Mar, CA) to 0% O_2 -5% CO_2 -95% N_2 . Oxygen levels in the media have been measured at ~ 20 –25 mmHg during exposure to 0% O_2 (1, 23, 49). The chambers were kept at 37°C and repurged with this gas mixture every 24 h.

EC viability. Cell monolayers were assessed for injury as previously described (24, 41). In the current experiments, phase contrast microscopic appearance, adherent cell counts, trypan blue exclusion, ^{51}Cr release, or [^3H]thymidine labeling of DNA was used. For adherent cell counts, at specified times, the medium was removed and adherent cells were counted by hemocytometer or Coulter counter after trypsinization. For trypan blue exclusion, at specified times, the medium was removed and, after a 5-min incubation period, trypan blue exclusion was assessed by light microscopy. For ^{51}Cr release, at specific times, radioactivity in the medium and EC was measured and the chromium release was expressed as the percent above control. For DNA labeling, cells were pulsed with [*methyl*- ^3H]thymidine for 2 h, and the amount of trichloroacetic acid-insoluble radioactivity produced by the cells was measured (41).

Measurements of ROS production. Generation of ROS was assessed using the fluorescent probe chloromethyl-2,7-dichlorofluorescein diacetate (DCFDA; Molecular Probes) (12). After diffusion into cells, the acetate groups of DCFDA are cleaved by esterases, trapping it inside; subsequent oxidation yields a fluorescent adduct. Before initiation of stress, HPAEC monolayers in 12-well plates were rinsed twice with PBS and then incubated with 5 μM DCFDA in 0.5 ml PBS for 1 h at 37°C. At the end of the incubation, monolayers were rinsed twice with PBS to remove excess probe, fresh medium or buffer was added, the monolayers were exposed to stress, and fluorescence was measured at indicated time points using a Cyto-Fluor 2300 fluorescence plate reader (excitation: 485 nm, emission: 530 nm).

Phospholipid turnover. Cells were labeled for 5 days at 37°C in medium containing $^{32}\text{P}_i$ (5 $\mu\text{Ci}/\text{ml}$) and 20 μM HG in tissue culture flasks. The day before hypoxia, cells were plated into sterile glass scintillation vials in labeling medium

and allowed to attach overnight. The following day, the medium was removed, and the cells were washed once with 3 ml medium and maintained under either normoxic or hypoxic conditions in unlabeled growth medium containing 20 μ M HG. After 4 days, the medium was removed, the cellular lipids were extracted, individual phospholipid species were separated, and radioactivity was quantitated as described above.

Oxidant stresses. Cells were stressed using H₂O₂, hyperoxia (95% O₂), or plumbagin. HPAEC were seeded into 24-well plates and allowed to attach overnight. Medium was changed 1 h before stress. For H₂O₂, a concentrated stock solution of H₂O₂ was added in PBS to the medium at a final concentration of 0.25 mM. For hyperoxia, EC monolayers were placed in humidified, sealed chambers (Billups-Rothenburg), and the chambers were purged with 95% O₂-5% CO₂ for 15 min. Oxygen levels in the media were measured at ~500 mmHg during exposure to 95% O₂. The chambers were kept at 37°C and regassed every 24 h. For plumbagin, the medium was removed, the cells were washed once with HEPES-buffered saline (HBS), and 0.5 ml HBS containing 1 mM CaCl₂, 1 mM MgCl₂, and 2 μ M plumbagin was added at 37°C for times up to 100 min. Buffer was then removed and replaced with growth medium, and the surviving cells were allowed to recover and grow for 24 h before viability determinations.

Heat shock. EC were exposed to sublethal and lethal heat stress as previously described (50). Briefly, dishes containing monolayers were sealed with parafilm and placed in a water bath at specified temperatures (44–60°C) for 15 min, and upregulation of heat shock proteins (HSPs) was determined by [³⁵S]methionine labeling (50).

Glucose deprivation. EC were deprived of glucose as previously described (50). Briefly, monolayers were cultured in glucose-free medium (GIBCO-BRL) for varying times (24 h–15 days) before viability determinations and DCFDA fluorescence measurements, and upregulation of glucose-regulated proteins (GRPs) was determined by [³⁵S]methionine labeling (50).

Statistical analyses. Most experiments were performed 3–12 times, and data are expressed as means \pm SE. Results were qualitatively similar and reproducible in all HPAEC examined and were independent of passage number or primary cell line of HPAEC used. Student's *t*-test or one-way ANOVA, followed by the Student-Newman-Keuls multiple comparison test, was used to compare means.

RESULTS

HG elevates plasmalogen levels in HPAEC. Murphy et al. (42) demonstrated that human EC contained significant amounts of plasmalogen. Most plasmalogen was found within the ethanolamine phospholipid pool, although a small portion of the choline phospholipids was plasmenylcholine. Our analyses, using steady-state labeling with ³²P_i (Fig. 1 and Table 1), were consistent with those data. The plasmalogens (plasmenylethanolamine and plasmenylcholine) represented 11% of the labeled phospholipids in HPAEC (Table 1) with the majority being plasmenylethanolamine.

Supplementing growth medium with the plasmalogen precursor lipid HG during the labeling period resulted in a 1.6-fold increase in plasmalogen content (Table 1). This increase was due to a 3.5-fold increase in plasmenylcholine; plasmenylethanolamine levels remained unchanged (Fig. 1B and Table 1). There was also a decrease in phosphatidylethanolamine labeling. The results in HPAEC using steady-state ³²P_i labeling were in agreement with direct measurement of phospholipid mass using phosphorous analysis (Table 1); there was a 1.9-fold increase in plasmalogens due, in part, to a 4-fold increase in plasmenylcholine levels. By measuring mass, we also detected a 50% increase in plasmenylethanolamine levels after HG supplementation.

Analysis of the fatty acid composition of the phospholipid pools (Table 2) showed a two- to threefold increase in DMA in the HG-supplemented cells. DMA are generated from the vinyl ether-linked alkyl groups. As expected, the increase was the result of a four- to fivefold increase in the 16:0 species; this was due to the 16-carbon alkyl chain associated with HG. There was also a 27% increase in polyunsaturated fatty acids at the expense of monounsaturated and saturated fatty acids.

Analyses of unsupplemented bovine cells, BPAEC, using steady-state ³²P_i, revealed a phospholipid profile almost identical to HPAEC with plasmalogens, primarily plasmenylethanolamine, constituting 13.0% of

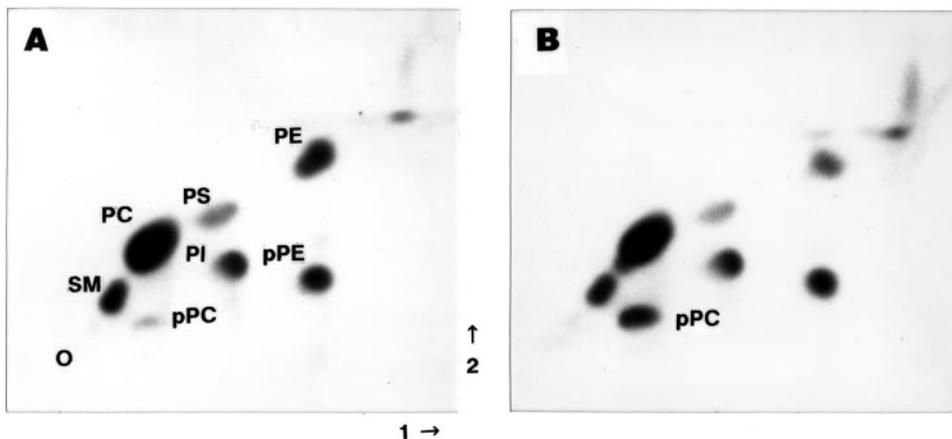


Fig. 1. *sn*-1-*O*-hexadecylglycerol (HG) supplementation increases plasmenylcholine in human pulmonary arterial endothelial cells (HPAEC). HPAEC were labeled with ³²P_i for 6 days in the absence (A) or presence (B) of 20 μ M HG; phospholipids were extracted and separated by two-dimensional thin-layer chromatography (TLC) as described in MATERIALS AND METHODS. The TLC plates were exposed to X-ray film to visualize the labeled phospholipids. SM, sphingomyelin; PC, phosphatidylcholine; pPC, plasmenylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; pPE, plasmenylethanolamine.

Table 1. PL composition of BPAEC and HPAEC: effects of HG supplementation

Phospholipid	Radioactivity in PL Species, %chloroform-soluble counts				Phosphorous Content, %total PL	
	Bovine		Human		Human	
	-HG	+HG	-HG	+HG	-HG	+HG
SM	6.6(0.4)	6.9(0.5)	7.5(0.3)	7.7(0.1)	10.8(1.8)	10.9(1.7)
PC	45.5(1.9)	45.0(0.0)	54.0(2.1)	47.6(1.5)	46.0(4.1)	45.0(6.0)
pPC	1.3(0.1)	1.6(0.0)	2.7(1.0)	9.3(0.1)	1.5(0.0)	6.1(0.2)
PS	2.7(1.5)	3.2(0.8)	4.7(1.4)	4.6(1.9)	8.6(0.6)	7.7(0.5)
PI	11.3(0.2)	10.8(0.8)	8.5(0.5)	8.6(0.9)	7.6(0.1)	7.0(0.0)
PE	9.8(0.2)	8.4(0.3)	9.1(2.1)	5.7(1.1)	17.8(1.2)	12.6(5.5)
pPE	12.2(0.0)	11.4(0.1)	8.7(0.3)	9.2(2.1)	6.4(0.1)	9.6(2.4)
Other PL	10.6(0.5)	12.7(1.7)	4.9(1.0)	7.4(0.7)	1.3(0.3)	1.1(0.4)
pPE + pPC	13.5	13.0	11.4	18.5	7.9	15.7

Values represent the mean of 2 samples; values in parentheses represent the difference between the 2 values. For radioactivity in phospholipid (PL) species, endothelial cells (EC) were labeled with $^{32}\text{P}_i$ for 6 days \pm 20 μM *sn*-1-*O*-hexadecylglycerol (HG); PL were extracted and separated by 2-dimensional thin-layer chromatography (TLC). For phosphorous content, EC were grown for 5 days \pm 20 μM HG; PL were extracted and separated by 2-dimensional TLC, and the phosphorous content in each PL species was determined according to Rouser et al. (46). BPAEC, bovine pulmonary arterial EC; HPAEC, human pulmonary arterial EC; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; pPC, plasménylcholine; pPE, plasménylethanolamine. Other PL: cardiolipin and phosphatidic acid.

the total label. However, supplementation of the growth medium with HG had no effect upon BPAEC phospholipid composition; there was no increase in plasmalogen levels.

HG supplementation imparts hypoxia resistance to HPAEC. Exposure of EC to 0% oxygen is normally lethal in 4–5 days (22). This was apparent in the unsupplemented cells, which displayed a contracted dysmorphic shape after 5 days under hypoxic conditions (Fig. 2). HG-supplemented HPAEC were much more resistant to the toxic effects of hypoxia, displaying normal morphology even after 14 days in hypoxia. By all viability criteria used, unsupplemented HPAEC were significantly damaged after 5 days of hypoxia

Table 2. Analysis of FAME and DMA content of PL pools from unsupplemented and HG-supplemented HPAEC

	FAME and DMA Concentrations, %total mass	
	-HG	+HG
16:0 DMA	1.2 \pm 1.0	5.7 \pm 1.4*
16:1 DMA	1.7 \pm 0.1	2.0 \pm 0.1
18:0 DMA	0.5 \pm 0.6	1.0 \pm 0.7
16:0	18.7 \pm 0.6	13.4 \pm 0.6*
16:1	1.8 \pm 0.2	1.4 \pm 0.1
18:0	16.1 \pm 0.4	13.7 \pm 0.7*
18:1 <i>n</i> - 9	20.8 \pm 0.7	16.8 \pm 0.3*
18:1 <i>n</i> - 7	7.8 \pm 0.2	6.4 \pm 0.2*
18:2 <i>n</i> - 6	2.2 \pm 0.2	2.4 \pm 0.1
20:4 <i>n</i> - 6	15.6 \pm 0.1	19.9 \pm 0.7*
22:4 <i>n</i> - 6	5.0 \pm 0.6	6.4 \pm 0.2*
22:5 <i>n</i> - 3	3.8 \pm 0.2	4.8 \pm 0.2*
22:6 <i>n</i> - 3	4.8 \pm 0.2	6.1 \pm 0.2*

Values are averages \pm SE of 3 samples. HPAEC were grown for 4 days in unsupplemented growth medium or medium supplemented with 20 μM HG. Total cellular PL pools were extracted, isolated, and treated with BF_3 to generate fatty acid methyl esters (FAME) and dimethylacetals (DMA). Analysis was performed by gas-liquid chromatography. *Values for -HG and +HG are significantly different, $P < 0.05$.

(10 \pm 2% viable by trypan blue exclusion); in contrast, HPAEC with increased plasmalogen content remained undamaged (95 \pm 4% viable) after exposure to 0% oxygen for 14 days. After exposure to >5 days hypoxia, the HG-supplemented cells could be passaged to a lower density, and they would continue to grow (data not shown). HG supplementation had no effect on hypoxia-induced cytotoxicity in BPAEC cells (10% viability in both unsupplemented and HG-supplemented cells after 5 days).

Detection of the fluorescent adduct of the probe DCFDA in control HPAEC was used to detect ROS in cells exposed to hypoxia (Fig. 3). In unsupplemented HPAEC, fluorescence increased after 4 days of exposure to hypoxia; this increase in fluorescence correlated with the loss of viability (e.g., ^{51}Cr release). In contrast, in HG-supplemented HPAEC, fluorescence did not increase after 14 days of hypoxia.

Plasmalogen loss during hypoxia. We examined phospholipid turnover during normoxia and hypoxia. Over 4 days, under either condition, 30–50% of the label was lost from the ^{32}P -labeled phospholipids (Table 3). The relative amount of label associated with phosphatidylcholine and phosphatidylinositol decreased, whereas these values increased in sphingomyelin, phosphatidylethanolamine, and phosphatidylserine. Again, these changes occurred whether the cells were maintained under normoxic or hypoxic conditions. The notable exception was plasménylethanolamine; as with phosphatidylethanolamine, label associated with this phospholipid increased in the normoxic cells; however, the relative amount of label did not significantly change during hypoxia. The plasménylethanolamine-to-phosphatidylethanolamine ratio was 1.82 at *time 0* and 1.88 at *day 4* under normoxic conditions, whereas this decreased to 1.08 after 4 days in hypoxia. There was no change in the relative amount of label associated with plasménylcholine after 4 days of hypoxia or normoxia.

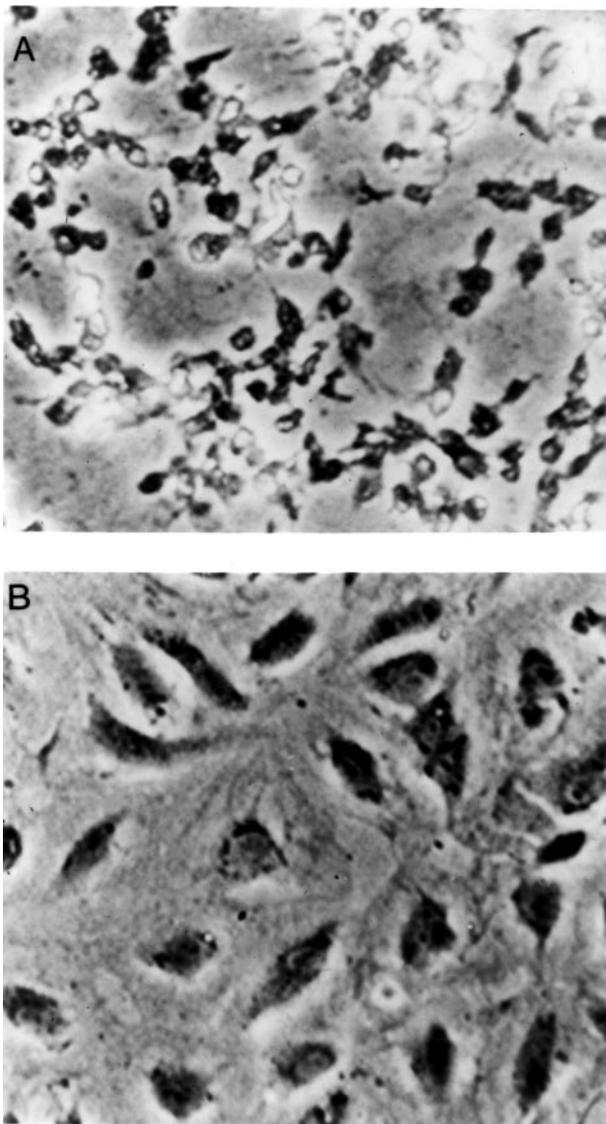


Fig. 2. Hypoxia-induced morphological changes are prevented by plasmalogen enhancement. HPAEC were incubated for 4 days in medium plus vehicle (0.1% ethanol) or medium supplemented with 20 μ M HG. HPAEC maintained in the appropriate medium (\pm HG) for the duration of the experiment were then exposed to hypoxia (0% O_2) for 5 days (A) or 14 days (B). A: nonsupplemented cells; B: HG-supplemented cells.

HG supplementation and HPAEC resistance to other oxidative stresses. Exposure of control (nonsupplemented) cells to 0.25 mM H_2O_2 resulted in a rapid decrease in cell viability over the initial 15 min, followed by a further decline over 7 h (Fig. 4). The cytotoxic effects of H_2O_2 were greatly reduced in HPAEC with increased plasmalogen content at either time point. Exposure to 95% oxygen also decreased HPAEC viability dramatically; increasing the plasmalogen content with HG significantly blunted cell damage under these conditions. Likewise, the damaging effect of plumbagin, which generates intracellular superoxide anion (17), was attenuated in HPAEC with increased plasmalogen content (Fig. 5). Again, HG supplementa-

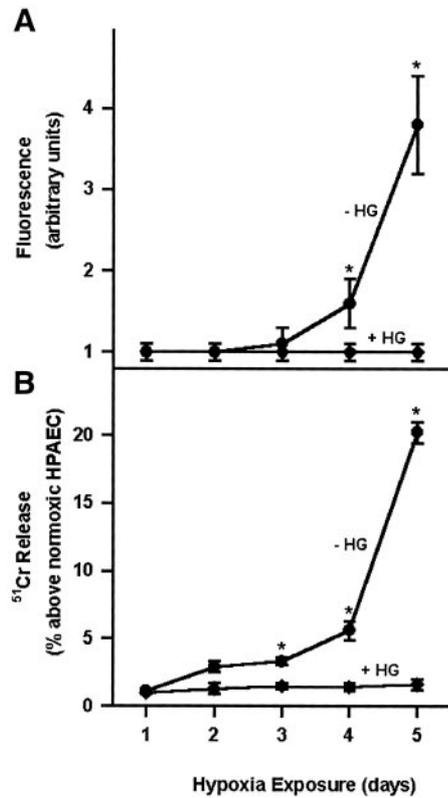


Fig. 3. Increased cellular chloromethyl-2,7-dichlorofluorescein diacetate (DCFDA) fluorescence coincides with ^{51}Cr release during hypoxia. HPAEC were incubated with 0.1% ethanol (-HG) or 20 μ M HG (+HG) for 4 days before and during hypoxia and then labeled with either the fluorescent reactive oxygen species (ROS) probe DCFDA (A) or ^{51}Cr (B) before exposure to hypoxia as described in MATERIALS AND METHODS. Intracellular ROS production during hypoxia was measured using DCFDA fluorescence (12) and is expressed as arbitrary units relative to normoxic cultures. \bullet , Unsupplemented; \blacklozenge , HG supplemented. All values represent the averages \pm SE of 3–6 samples. *Value is significantly different ($P < 0.05$) from control values.

Table 3. PL turnover in HPAEC under normoxic and hypoxic conditions

	0 Days	4 Days Normoxia	4 Days Hypoxia
<i>Total PL-associated label, counts \cdot min$^{-1} \cdot 10^{-3}$</i>			
	21.95 \pm 2.26	11.40 \pm 0.67	14.99 \pm 4.19
<i>^{32}P associated with PL species, %total</i>			
SM	6.32 \pm 0.25	10.58 \pm 0.35	11.42 \pm 0.88
pPC	6.79 \pm 0.62	7.10 \pm 0.54	6.66 \pm 0.18
PC	51.72 \pm 1.55	39.39 \pm 0.92	39.91 \pm 1.18
PS	4.13 \pm 0.60	7.61 \pm 0.70	8.46 \pm 0.85
PI	10.35 \pm 0.69	6.22 \pm 0.44	5.82 \pm 0.15
pPE	10.90 \pm 0.85	15.82 \pm 1.05	11.79 \pm 0.36*
PE	6.00 \pm 0.68	8.40 \pm 1.03	10.96 \pm 0.32*
Other PL	3.78 \pm 0.07	4.87 \pm 0.22	4.96 \pm 0.12
pPE/PE	1.82	1.88	1.08

Each value represents averages \pm SE of 4 samples. Cells were labeled for 6 days using $^{32}P_i$ in medium containing 20 μ M HG. Label was removed, and the cells were incubated in unlabeled medium containing 20 μ M HG under normoxic or hypoxic conditions. Cellular lipids were extracted and separated as described in MATERIALS AND METHODS. Abbreviations are the same as Table 1. *Values for normoxic and hypoxic are significantly different, $P < 0.05$.

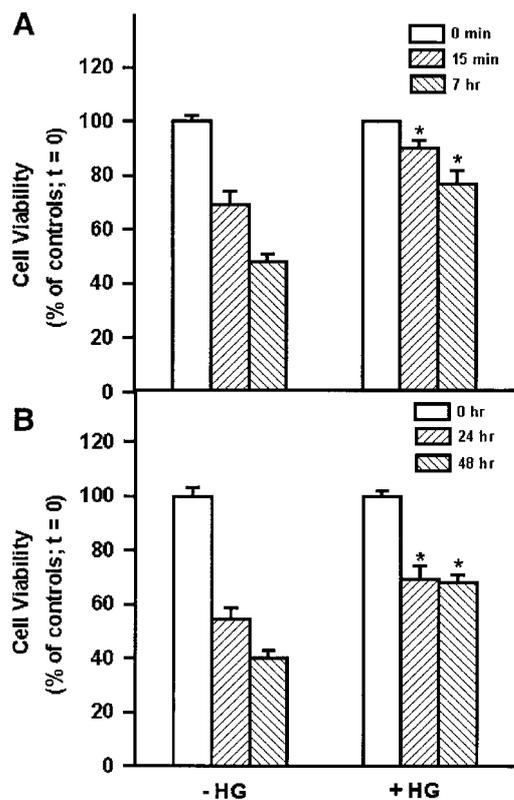


Fig. 4. Plasmalogen enhancement increases resistance of HPAEC to H_2O_2 and hyperoxia. HPAEC were incubated for 4 days in medium plus vehicle (0.1% ethanol) or medium supplemented with 20 μM HG. Cells were then exposed to either 0.25 mM H_2O_2 (A) or hyperoxia (95% O_2) (B), and cell viability was determined by trypan blue exclusion as described in MATERIALS AND METHODS. Values are expressed as the percentage of untreated controls and represent means \pm SE ($n = 3-6$). *Value is significantly different ($P < 0.05$) than values from unsupplemented cells.

tion did not cause any change in BPAEC sensitivity to any of these stresses (data not shown). The rescue of HPAEC with HG supplementation corresponded to a reduction in cellular fluorescence when DCFDA-pretreated HPAEC were exposed to either plumbagin or hyperoxia (Fig. 6).

HG supplementation and EC tolerance to non-ROS-mediated cellular stresses. Lethal heat shock occurred in both control (nonsupplemented) and HG-supplemented HPAEC at 52°C (Table 4). Moreover, there was no difference in cellular damage (Table 4) or production of HSPs (data not shown) between nonsupplemented and HG-supplemented HPAEC at any temperature examined. Cytotoxicity due to glucose deprivation occurred in both nonsupplemented and HG-supplemented HPAEC 12–15 days after the removal of glucose from the medium and was not accompanied by an increase in cellular fluorescence in DCFDA-pretreated HPAEC (Table 5). There was no decrease in cellular damage in HG-supplemented HPAEC. We found no difference in expression of GRPs between the HG-supplemented and nonsupplemented HPAEC at any time point examined (data not shown).

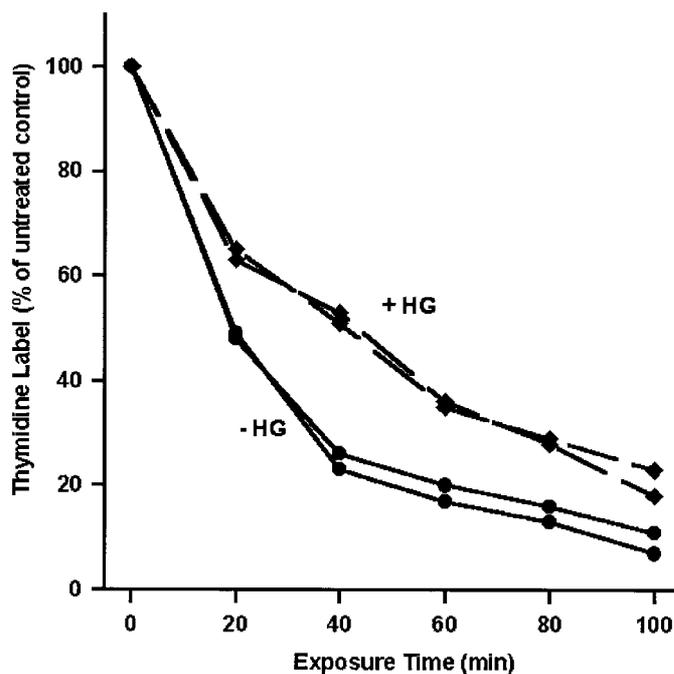


Fig. 5. Plasmalogen enhancement increases resistance of HPAEC to the superoxide generator plumbagin. HPAEC were incubated for 4 days in medium plus vehicle (0.1% ethanol) or medium supplemented with 20 μM HG. EC were then exposed to the superoxide generator plumbagin (2 μM) for the time indicated. Surviving cells were allowed to recover for 24 h before determination of viability by measuring the incorporation of [methyl- 3H]thymidine into DNA (41). Data represent individual values from duplicate experiments. ●, Unsupplemented; ◆, HG supplemented.

DISCUSSION

ROS are formed as a result of normal cellular and physiological processes, whereas increased ROS generation contributes to cellular damage under pathological conditions. ROS generation has been implicated in

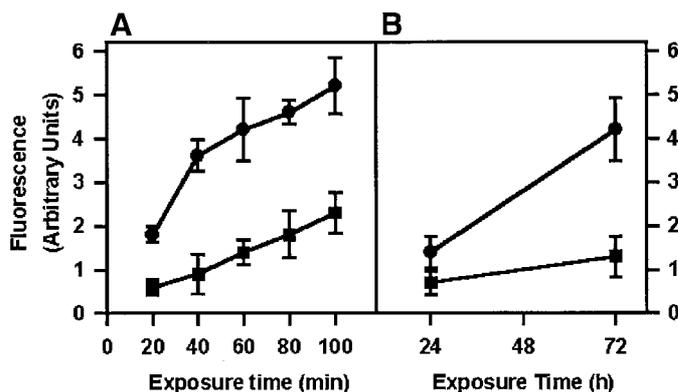


Fig. 6. Plasmalogen enhancement reduces DCFDA fluorescence in HPAEC exposed to plumbagin and hyperoxia. HPAEC were incubated for 4 days in medium plus vehicle (0.1% ethanol) or medium supplemented with 20 μM HG. EC were then exposed to the superoxide generator plumbagin (2 μM) (A) or hyperoxia (95% O_2 -5% CO_2) (B) for the times indicated. Intracellular ROS production was measured using DCFDA fluorescence and expressed as arbitrary units. HG was also present during the 72 h of hyperoxia (not present during plumbagin exposure). Data represent means \pm SE ($n = 6$). ●, Unsupplemented; ◆, HG supplemented.

Table 4. *Effects of heat shock on HPAEC variability*

Temperature	⁵¹ Cr Release, %above unheated cultures	
	-HG	+HG
44°C	1.2 ± 0.1	1.0 ± 0.1
48°C	1.8 ± 0.3	1.5 ± 0.1
52°C	10.3 ± 0.8*	11.1 ± 1.0*
56°C	24.1 ± 1.4*	23.4 ± 0.9*
60°C	61.4 ± 2.6*	63.7 ± 2.4*

Values represent averages ± SE of 3 samples. Heat shock and measurements of cell damage were performed as described in MATERIALS AND METHODS. *Values are significantly different from control values, $P < 0.05$.

a variety of acute and progressive clinical conditions including adult respiratory distress syndrome (21), myocardial infarct (28), stroke (32), Alzheimer's disease (36), atherosclerosis (10), and aging (34, 48). Enhancing or augmenting cellular levels of various antioxidant molecules, such as carotenoids (31), α -tocopherol (16), catalase, superoxide dismutase, or glutathione peroxidase (37), has been shown to ameliorate ROS-mediated cytotoxicity in certain systems.

We were interested in the possibility of raising plasmalogen levels in EC because of their role as antioxidants. We hypothesized that supplementation with HG would result in increased cellular plasmalogen levels and possible protection of the cells against conditions, such as hypoxia, under which ROS are generated. However, previous attempts at elevating plasmalogen levels have not been successful. HG supplementation of established cell lines (7) resulted in only minor increases in plasmalogen content, due to an increase in plasmenylethanolamine. Similarly, in animal models (3, 11), alkylglycerol supplementation of the diet resulted in heavy incorporation of the dietary lipid into plasmalogen pools; however, there were only minor changes in tissue plasmalogen levels (kidney, lung, heart, intestine, erythrocytes, testis, and brain). In agreement with these reports, our attempts to modify plasmalogen levels in bovine EC using HG were unsuccessful. We were, however, able to increase plasmalogen levels in human cells under identical culture conditions, demonstrating, for the first time, that plasmalogen content can be increased significantly above normal levels in certain cell types.

It is unclear why we were able to increase plasmenylcholine levels in HPAEC. The pathway for plasmenylcholine biosynthesis has not been well defined, although plasmenylethanolamine appears to be a precursor (4, 18, 33, 51). Clearly, the enzymatic machinery required for plasmenylcholine biosynthesis is present in the human cells. Further studies, exploiting our ability to modulate plasmenylcholine levels in HPAEC under controlled conditions, may be useful in examining the pathway(s) for choline plasmalogen biosynthesis in EC.

Supplementing HPAEC with HG resulted in a significant increase in the resistance of HPAEC to the cytotoxic effects of hypoxia. This resistance appeared to

be due to the increase in plasmalogen content rather than the presence of HG alone; HG supplementation did not increase plasmalogen content in the BPAEC, and these cells were not protected during hypoxia. All of our observations were consistent with increased plasmalogen content imparting resistance to ROS. First, increased plasmalogen content resulted in resistance, not only to hypoxia, but also to hyperoxia, H_2O_2 , and the ROS generator plumbagin. This protective effect appeared specific for cellular stresses in which ROS generation contributes to cellular toxicity because resistance to either heat shock or glucose deprivation was not affected by plasmalogen enhancement of HPAEC. Second, the increase in ROS associated with hypoxia, hyperoxia, or plumbagin, as judged by DCFDA fluorescence, was prevented or attenuated when plasmalogen content was increased. Although DCFDA has been used widely as evidence of ROS or radical formation, *in vitro* studies have demonstrated that this compound can be oxidized by cytochrome *c* (6). It is possible to interpret the fluorescence increase in hypoxic cells as the release of cytochrome *c* into the cytosol during cell death through apoptosis; however, we observed no fluorescence increases during glucose deprivation, which is known to result in cell death through apoptosis (2, 39). Finally, plasmalogen turnover, specifically plasmenylethanolamine, was increased during hypoxia. The increased plasmenylethanolamine turnover would be consistent with chemical breakdown upon reaction with ROS. The loss of plasmenylethanolamine could also be due to lipase activation; phospholipases demonstrating selectivity for the plasmalogen over the diacyl forms of a phospholipid have been identified (27, 44, 53); however, no lipases demonstrating exclusive preference for plasmalogens have been reported.

In attempting to explain the protective effect of plasmalogens during hypoxia, other possibilities must also be considered. A number of studies have demonstrated biological activity of plasmalogens or their metabolites including possible functions as growth factors (35), in ion exchange across membranes (26), as sources for stimulated arachidonate release (19) and as modulators of intracellular signaling (9, 52). Changes in plasmalogen status may alter these functions, influencing

Table 5. *Effects of glucose deprivation of HPAEC viability*

Day	Trypan Blue Exclusion, %control cultures		DCFDA Fluorescence, arbitrary units	
	-HG	+HG	-HG	+HG
5	95 ± 2	95 ± 3	0.9 ± 0.1	1.0 ± 0.0
10	92 ± 3	90 ± 4	1.1 ± 0.1	1.0 ± 0.1
15	72 ± 2*	70 ± 3*	1.0 ± 0.0	1.1 ± 0.1

Values represent averages ± SE. Glucose deprivation and measurements of cell damage were performed as in MATERIALS AND METHODS. Intracellular reactive oxygen species production during glucose deprivation was measured using chloromethyl-2,7-dichloro fluorescein diacetate (DCFDA). *Values are significantly different from control values, $P < 0.05$.

cellular response to hypoxia. In this study, HG supplementation caused an increase in polyunsaturated fatty acids (PUFA) in the phospholipid pools. Modifying PUFA levels in cultured cells can have different effects on a cells response to hypoxia depending on the PUFA species and the effect being monitored (25). However, using plasmalogen-deficient mutants, we noted that changing the levels of PUFA in the phospholipid pools had no bearing on the sensitivity of the cell to chemical hypoxia; plasmalogen-deficient cells displayed a decrease in phospholipid PUFA levels (20) and an increased sensitivity to chemical hypoxia and ROS (55). However, recovery of saturated ether lipids, not plasmalogens, was sufficient to restore PUFA levels (20), but it was not sufficient to restore resistance to chemical hypoxia (55).

Regardless of the mechanism of protection, we have shown that the plasmalogen content of a human cell type, HPAEC, can be enhanced and that this was associated with an increased resistance to hypoxia as well as to specific ROS or ROS generators. The ability to increase plasmalogen content using a chemically simple, stable precursor lipid has implications for therapeutic use against hypoxia and possibly other pathologies.

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