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Plasmalogens and oxidative stress: evidence against a major role of plasmalogens in protection against the superoxide anion radical

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Summary: Although ether-linked phospholipids have been known to be constituents of biological membranes for a long time, their physiological function has remained an enigma through the years. Inspired by the suggestion of Zoeller and coworkers that plasmalogens, which are specific types of ether-linked phospholipids characterized by the occurrence of an α,β -unsaturated ether bond at the *sn*-1 position, are involved in the protection of cells against reactive oxygen species, we studied reactive oxygen species metabolism in cultured human skin fibroblasts. Menadione was used as intracellular generator of reactive oxygen species and cytochrome *c* as extracellular indicator for the production of reactive oxygen species. The finding that identical results were obtained in control and plasmalogen-deficient fibroblasts leads us to conclude that plasmalogens do not play a major role in protection against reactive oxygen species.

Plasmalogens are ether linked glycerophospholipids characterized by the presence of an α,β -unsaturated ether bond at the *sn*-1 position of the glycerol backbone. These phospholipids are found in all mammalian cells, being especially abundant in heart (Gross 1984), brain (Scott et al 1967), neutrophils (Mueller et al 1984) and macrophages (Sugimura et al 1983). In human skin fibroblasts, plasmalogens make up about 15% of the total amount of phospholipids (Heymans et al 1984; Hermetter et al 1989). Peroxisomes are obligatorily involved in the biosynthesis of ether-phospholipids since the first two enzymes of the ether phospholipid synthesizing pathway, i.e. acyl-CoA: dihydroxyacetonephosphate acyltransferase (DHAP-AT, EC 2.3.1.42) and alkyldihydroxyacetonephosphate synthase (alkyl DHAP synthase, EC 2.5.1.26) are located in peroxisomes (Hajra et al 1979; Hardeman and van den Bosch 1988).

The biological functions of ether-phospholipids in general and plasmalogens in particular have remained an enigma through the years except for platelet activating factor (PAF) (Hanahan et al 1980), which has been implicated in a variety of pathological processes (Chung 1992). Plasmalogens may play a role in the temporary storage of arachidonic acid

since plasmalogens are especially enriched in arachidonic acid at the *sn*-2 position when compared to the corresponding diacyl phosphoglycerides (Ford and Gross 1989). Importantly, Zoeller and coworkers suggested that plasmalogens may protect the animal cell membranes against damage caused by reactive oxygen species (ROS) (Zoeller et al 1988). This was concluded from experiments in which animal cell mutants lacking plasmalogens due to a deficiency of peroxisomes were found to be hypersensitive to irradiation with long-wavelength ultraviolet light when photosensitized with 12 (1' pyrene)dodecanoic acid (Zoeller et al 1988). This treatment led to a rapid and selective destruction of plasmalogens (Morand et al 1988). Hoefler and coworkers (1991) performed similar experiments in human cultured skin fibroblasts and found increased photosensitized killing both in fibroblasts from patients with a complete deficiency of peroxisomes and in fibroblasts from patients with a defect in the peroxisomal β -oxidation.

In the studies by Raetz and coworkers (Zoeller et al 1988; Morand et al 1988) the nature of the reactive oxygen species responsible for the observed fragmentation of plasmalogens could not be identified unequivocally. One model implies cycloaddition of singlet oxygen itself to the vinyl ether linkage of plasmalogens, generating a dioxetane intermediate. However, since this mechanism could not explain the decomposition products found, the authors concluded that a more reasonable scenario would appear to be a radical-mediated oxidation of plasmalogens via, for instance, the superoxide anion radical (Morand et al 1988, scheme II). This would be especially important since evidence for the existence of singlet oxygen in biological systems other than those involving photosensitization is not very persuasive (Naqui et al 1986; see also discussion by Morand et al 1988).

Resolution of the physiological functions of ether-glycerophospholipids is of utmost importance since there are a number of inherited diseases in man in which there is an impairment in ether-glycerophospholipid biosynthesis. In some of these disorders (such as Zellweger syndrome; ZS, McKusick 214100) peroxisome biogenesis is defective, leading to a generalized loss of peroxisomal functions including ether-phospholipid synthesis. In another disorder, rhizomelic chondrodysplasia punctata (RCDP, McKusick 215100) multiple peroxisomal abnormalities have been described, including a deficiency of DHAP-AT and alkyl DHAP synthase, although peroxisomes are normally present at least in cultured human skin fibroblasts (Lazarow and Moser 1989). Recently, two new disorders of ether phospholipid synthesis have been identified at the level of a deficiency of DHAP-AT and alkyl DHAP synthase, respectively (Wanders et al 1992, 1994).

To shed more light on the physiological functions of plasmalogens, we studied the sensitivity of control and plasmalogen-deficient human skin fibroblasts towards oxidative stress induced by addition of menadione (vitamin K₃, 2-methyl-1,4-naphthoquinone), which produces superoxide anion radicals intracellularly. The results are described in this paper.

MATERIALS AND METHODS

Materials: Ham's F10 nutrient mixture was obtained from Flow Laboratories (Irvine, UK). Bovine calf serum was purchased from Hyclone Laboratories (Cramlington, UK). Ammonium iron(III) citrate and nitrilotriacetic acid (disodium salt) were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). All other chemicals were of analytical grade.

Cell culture: Human skin fibroblasts from healthy control individuals and patients with an established deficiency in plasmalogen biosynthesis (ZS, GOM85AD as described in Wanders et al (1987); RCDP, MCH85AD as described in Heikoop et al (1992); DHAP-AT deficiency as described in Wanders et al (1992)) were routinely cultured in Ham's F10 medium supplemented with 15% bovine calf serum and 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Determination of plasmalogens in human skin fibroblasts: Plasmalogens were determined according to the method of Björkhem et al (1986). Briefly, a lipid extract of fibroblasts is subjected to methyl transesterification and plasmalogens are determined by means of gas-liquid chromatography.

Preparation of a ferric nitrilotriacetate solution: The ferric nitrilotriacetate solution was prepared according to the method of White and Jacobs (1978).

Measurement of menadione-induced cytochrome c reduction: Fibroblasts were harvested using a solution containing 500–750 U/ml trypsin, 0.53 mmol/L EDTA and 145 mmol/L sodium chloride, pH 7.4, reseeded in a 24-well tissue culture plate (growth surface 2 cm² per well; Costar Europe Ltd, Badhoevedorp, The Netherlands), and grown to confluence in the standard culture medium (2 ml per well). The culture medium was subsequently removed by suction and the cells were washed with 1 ml prewarmed Krebs–Henseleit buffer containing 25 mmol/L Hepes and 10 mmol/L L-glutamine. After removal of the washing buffer the cells were incubated in 2 ml prewarmed reaction medium containing Krebs–Henseleit buffer pH 7.4, 25 mmol/L Hepes, 10 mmol/L L-glutamine, 25 µmol/L cytochrome c and 0–5 µmol/L menadione. At various time intervals a 1 ml sample was taken from the incubation medium and transferred to a 1.5 ml Eppendorf tube. After centrifugation for 1 min in an Eppendorf centrifuge at maximum speed (12000g) at room temperature, the supernatant was used for absorbance measurement at 550 nm using a Uvikon 932 spectrophotometer (Kontron instruments S.p.a., Milano, Italy). After absorbance measurement the medium was returned to the incubation. All incubations were carried out at 37°C. In every experiment the average value of four incubations (4 wells) was taken. In order to find out whether all cytochrome c was completely reduced, a 10-fold molar excess of sodium hypochlorite was added to the medium. The absorbance was subsequently measured at 550 nm and this value was taken as 100%. At the end of the incubations the amount of protein in the wells was determined using bicinchonic acid according to Smith and coworkers (1985).

Measurement of TBARS: Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS), using the thiobarbituric acid procedure essentially as described by Wasowicz and coworkers (1993) with some modifications. Briefly, 20 µl of a 23 mmol/L 2,6-di-*t*-butyl-4-methylphenol (butylated hydroxytoluene, BHT) solution (in ethanol) was added to 1 ml sample in order to prevent progression of lipid peroxidation during the assay. Then 0.5 ml 7.7 mol/L HCl was added in order to denature proteins, followed by addition of 0.5 ml thiobarbituric acid (TBA) solution (69.4 mmol/L TBA in

Figure 1 Menadione-induced superoxide production in control fibroblasts. Fibroblasts from a healthy control were incubated in the absence (○) and presence of 0.2 (●), 0.4 (□) and 5.0 $\mu\text{mol/L}$ (■) menadione as described in Materials and Methods. Superoxide production was measured by means of cytochrome *c* reduction and detected spectrophotometrically at 550nm

50 mmol/L NaOH). After heating at 95°C for 30 min and cooling to room temperature, the chromogen was extracted with 2 ml 1-butanol and the fluorescence in the organic layer was measured ($\lambda_{\text{ex}} = 515 \text{ nm}$, $\lambda_{\text{em}} = 553 \text{ nm}$) using an Aminco Bowman series 2 luminescence spectrometer (SLM Aminco, Urbana, IL, USA). Freshly prepared tetramethoxypropane solutions yielding malonaldehyde in the assay were used as standards.

RESULTS

Menadione-induced superoxide production in cultured skin fibroblasts: In order to study the effect of a deficiency of ether phospholipids including plasmalogens on the sensitivity of cells towards oxidative stress, we incubated cultured human skin fibroblasts in a standard reaction medium containing menadione (vitamin K_3 , 2-methyl-1,4-naphthoquinone) as an intracellular generator of reactive oxygen species. Earlier studies with various cell types, including hepatocytes (Thor et al 1982; Orrenius and Nicotera 1987) and Hep G2 cells (Duthie and Grant 1989), have shown that menadione undergoes redox cycling, thus producing reactive oxygen species such as the superoxide anion radical ($\text{O}_2^{\cdot-}$).

To monitor the production of ROS, the reaction medium was supplemented with oxidized cytochrome *c*. Figure 1 shows an experiment in which human skin fibroblasts from a healthy control were incubated in the standard reaction medium in the absence and presence of different concentrations of menadione. The results show a low rate of cytochrome *c* reduction in the absence of menadione, whereas increased rates of cytochrome *c* reduction were found upon addition of 0.2–5.0 $\mu\text{mol/L}$ menadione, rates of reduction increasing with the concentration of menadione added. The rates of cytochrome *c* reduction were found to proceed linearly with time until most of the added cytochrome *c* was reduced

Figure 2 Superoxide release induced by redox cycling of menadione in control fibroblasts. Fibroblasts from a healthy control were incubated in the absence and presence of various amounts of menadione as described in Materials and Methods. Cytochrome *c* reduction was measured by means of the change in absorbance at 550 nm at the start (○), after 3 h (●) and 24 h (□) of incubation

(Figure 1). The cells remained viable when incubated in the presence of menadione up to 5 μmol/L. This is concluded from the fact that incubation in the presence of menadione did not lead to release of lactate dehydrogenase or of phosphoglucose isomerase, two cytosolic marker enzymes, from the cells. When higher concentrations of menadione were used (up to 50 μmol/L), the cells detached from the growth surface and high amounts of the cytosolic marker enzymes were released into the medium (data not shown). The results obtained show that the cytochrome *c* detection system can be used to monitor superoxide production by fibroblasts. In most studies, especially when subcellular fractions or membranes are studied, acetylated cytochrome *c* is used to detect superoxide, which is based on the fact that acetylated cytochrome *c* is relatively inert towards cytochrome *c* oxidase (EC 1.9.3.1) activity (Azzi et al 1975). When acetylated cytochrome *c* was used in our incubations containing intact cells, the same results were obtained when compared to incubations in which non-acetylated cytochrome *c* was used (data not shown). We therefore preferred the use of cytochrome *c* in all subsequent experiments.

The production of superoxide radicals is the result of redox cycling of menadione, at least in hepatocytes (Thor et al 1982; Orrenius and Nicotera 1987) and Hep G2 cells (Duthie and Grant 1989). We studied whether this is also true for human skin fibroblasts. If the superoxide production is a result of redox cycling of menadione, theoretically the production must continue in time after addition of small amounts of menadione to the incubation mixture. Figure 2 shows the result of a typical experiment in which fibroblasts were incubated in the presence and absence of increasing concentrations of menadione. Incubations were allowed to proceed for 3 and 24 h, respectively. The results show that the concentration of reduced cytochrome *c* as measured after 3 and 24 h, respectively, exceeds

Figure 3 Menadione-induced superoxide release in fibroblasts from control as well as plasmalogen deficient cell lines. Fibroblasts from a healthy control (○), RCDP (●), DHAP-AT-deficient (□) and ZS (■) cell lines were incubated in the absence and presence of various amounts of menadione as described in Materials and Methods. Superoxide release was monitored by means of spectrophotometric detection of reduced cytochrome *c* at 550 nm

the concentration of menadione manyfold at all concentrations of menadione. This is especially clear if the results with menadione at 0.1 μmol/L are considered. Indeed, at this menadione concentration, ratios of [reduced cytochrome *c*]/[menadione] of 84 and 218 are found at 3 and 24 h, respectively. These results indicate that redox cycling of menadione is the most likely source of superoxide production by the fibroblasts.

Menadione induced superoxide production of plasmalogen-deficient fibroblasts: In the next series of experiments we studied the role of plasmalogens as radical scavengers by incubating cells with increasing amounts of menadione to induce oxidative stress. Three types of plasmalogen deficient cell lines were used. First, cells from a ZS patient with a deficiency of peroxisomes were used in which there is a generalized loss of peroxisomal functions including plasmalogen synthesis (Lazarow and Moser 1989). The second cell line used was from a patient affected by the rhizomelic form of chondrodysplasia punctata with multiple biochemical abnormalities including deficient synthesis of plasmalogens (Hoeftler et al 1988). The third cell line used was from a patient with an isolated DHAP-AT deficiency (Wanders et al 1992). Since plasmalogens are thought to play a role as radical scavengers, reacting with the superoxide anion radical, one would expect an enhanced superoxide release (giving rise to increased rates of cytochrome *c* reduction) into the incubation medium of plasmalogen-deficient fibroblasts as compared to control fibroblasts. Figure 3 shows the cytochrome *c* reduction in the incubation medium (superoxide release) of plasmalogen-deficient cell lines and a control as induced by different amounts of

Table 1 Plasmalogen levels in human skin fibroblasts incubated for 4 h in the absence and presence of 1 $\mu\text{mol/L}$ menadione^a

Fibroblasts analysed	Menadione			
	0 $\mu\text{mol/L}$		1 $\mu\text{mol/L}$	
	16:0-DMA/ methylpalmitate	18:0-DMA/ methylstearate	16:0-DMA/ methylpalmitate	18:0-DMA/ methylstearate
Control ($n=7$)	11.9 $\pm$ 1.7	14.8 $\pm$ 2.6	11.4 $\pm$ 0.6	14.2 $\pm$ 2.9
ZS ($n=4$)	4.9 $\pm$ 0.5	2.6 $\pm$ 0.4	6.0 $\pm$ 1.4	2.8 $\pm$ 0.3
RCDP ($n=4$)	3.7 $\pm$ 0.2	1.7 $\pm$ 0.1	3.8 $\pm$ 0.3	1.7 $\pm$ 0.1
DHAP-AT deficiency ($n=4$)	3.5 $\pm$ 1.1	0.8 $\pm$ 0.1	3.6 $\pm$ 0.8	1.0 $\pm$ 0.2

^aPlasmalogens were measured as described by Björkhem et al (1986) and are expressed as the ratio between C_{16:0}-dimethylacetal (DMA) and methylpalmitate and the ratio between C_{18:0}-dimethylacetal and methylstearate. Values represent the mean $\pm$ SD with the number of observations given in parentheses in column 1.

menadione after a 3 h incubation period. The various plasmalogen-deficient cell lines showed a somewhat reduced rate of cytochrome *c* reduction as compared to the control cell line.

If plasmalogens were indeed involved in protection against reactive oxygen species such as the superoxide anion radical, one would expect that plasmalogen levels of skin fibroblasts would decrease upon addition of menadione. Therefore, the plasmalogen content of fibroblasts from control and patients with different peroxisomal diseases characterized by a deficiency in plasmalogen synthesis (compare Figure 3) was measured after incubation in the reaction medium in the absence and presence of 1 $\mu\text{mol/L}$ menadione. The results show that menadione had no effect on the plasmalogen levels of control fibroblasts as measured by the method of Björkhem et al (1986) (Table 1). In accordance with the impaired *de novo* synthesis of ether-linked phospholipids in the different mutant cell types used in Figure 3, plasmalogen levels were much lower in the three types of mutant fibroblasts studied. Menadione also had no effect on these low plasmalogen levels (Table 1).

Thiobarbituric acid reactive species (TBARS) in fibroblasts from plasmalogen-deficient patients: Although the true significance of TBARS measurement remains disputed, this parameter is generally regarded as an indicator for lipid peroxidation. In order to establish whether there was an increased level of lipid peroxidation in plasmalogen-deficient fibroblasts, we measured TBARS in fibroblast homogenates. The results in Table 2 show comparable TBARS levels in plasmalogen-deficient cells independently of which type of mutant cells were used. When control fibroblasts were incubated with 100 $\mu\text{mol/L}$ ferric nitrilotriacetate, a known inducer of lipid peroxidation (Carini et al 1992), elevated levels of TBARS were found, thus showing that measurement of TBARS in the cells used in this study truly reflects lipid peroxidation.

DISCUSSION

Although it has been known for many years that ether-linked glycerophospholipids are present in biological membranes, their physiological function has remained an enigma. The identification of patients suffering from a defect in the synthesis of these phospholipids due to an isolated deficiency of dihydroxyacetonephosphate acyltransferase (Wanders

Table 2 TBARS measurements in human skin fibroblasts incubated for 4 h in the absence and presence of 1 $\mu\text{mol/L}$ menadione or 100 $\mu\text{mol/L}$ ferric nitrilotriacetate^a

<i>Fibroblasts analyzed</i>	<i>Menadione</i>	
	0 $\mu\text{mol/L}$	1 $\mu\text{mol/L}$
RCDP	0.9 $\pm$ 0.1 (<i>n</i> = 4)	0.6 $\pm$ 0.2 (<i>n</i> = 4)
ZS	1.1 $\pm$ 0.2 (<i>n</i> = 4)	0.9 $\pm$ 0.1 (<i>n</i> = 4)
Controls	1.0 $\pm$ 0.3 (<i>n</i> = 10)	0.9 $\pm$ 0.3 (<i>n</i> = 10)
Control + 100 $\mu\text{mol/L}$ ferric nitrilotriacetate	4.4 $\pm$ 0.4 (<i>n</i> = 3)	n.d.

^aTBARS were measured as described in Materials and Methods. TBARS are expressed in nmol per mg protein. Values represent the mean $\pm$ SD with the number of observations in parentheses.

n.d. = not determined

et al 1992) or alkylldihydroxyacetonephosphate synthase (Wanders et al 1994) and the fact that these patients show a great variety of clinical abnormalities including neurological and skeletal aberrations, suggests that ether-phospholipids do indeed play an important role *in vivo*. In this paper we investigated the intriguing suggestion put forward by Zoeller and coworkers (1988) that plasmalogens may protect cells against reactive oxygen species (ROS) by means of the characteristic vinyl-ether linkage which would participate directly in scavenging reactive oxygen species. The results obtained show that plasmalogen deficient fibroblasts do not display increased sensitivity towards oxidative stress as generated intracellularly by menadione. In agreement with these results, plasmalogen-deficient cells showed normal TBARS levels, suggesting that there was no imbalance between the production and removal of ROS in which plasmalogens were supposed to play a role. Taken together, these results suggest that plasmalogens do not play an important role in the protection of cells, at least human skin fibroblasts, against ROS. This may be different under conditions in which other systems involved in the detoxification of ROS, such as catalase and superoxide dismutase, are reduced in capacity.

Recently it has been found that the lipid portion of certain phosphatidyl-inositol-glycan-anchored proteins (GPI-proteins) are ether-linked containing a 1-alkyl, 2-acylglycerol unit in their GPI anchor (e.g. acetylcholinesterase) (see Englund 1993 for review). More importantly, a murine lymphoma cell line unable to express such a GPI-linked protein (Thy-1) is deficient in ether-lipid biosynthesis, suggesting that a functional ether-lipid synthesizing system is required for proper localization of Thy-1 and presumably a variety of additional proteins (Stevens and Ractz 1990). Current research is directed towards elucidation of whether certain GPI-linked proteins would be deficient in patients as a consequence of the impairment in ether-phospholipid biosynthesis. These studies may provide important information on the functional importance of ether-phospholipids.

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