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Menadione-induced cytotoxicity: effects on human erythrocyte membranes studied by electron paramagnetic resonance

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Abstract

menadione increases protein-protein interactions ($P < 0.001$) of cytoskeletal proteins, (2) there is a slightly significant increase in the rotational motion of spin-labeled sialic acid ($P < 0.05$), while (3) the physical state of galactose residues was unaffected by menadione. Since glycophorin is coupled to the major cytoskeletal protein, spectrin, by protein 4.1, we suggest that menadione-induced oxidation could alter the conformation of protein 4.1. As a consequence, single or multiple sites of weakness could be induced leading to the alteration of the

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1. Introduction

Chemicals such as menadione (2-methyl-1,4-naphtoquinone) induce cytotoxicity in hepatocytes [1] and other cellular systems. The toxic action of such chemicals in

Menadione possesses an electrophilic carbon center and hence may bind cellular soft nucleophiles such as protein thiols [3]. It has been suggested [4] that singlet oxygen band hydroxyl radicals ($OH^{\bullet}$) are formed during the redox cycling of menadione in isolated hepatocytes. Both O^{-}_{2} and more active forms of oxygen are toxic and are known to cause DNA strand breaks [5], enzyme inhibition [6], and oxida-

induced surface blebbing. Mirabelli et al. [9,10] have shown that the metabolism of quinones in rat hepatocytes and in human platelets is associated with the oxidation of sulfhydryl groups in actin. These findings suggest that cytoskeletal structures are targets in quinone- and oxidative stress-induced cell injury. Oxidation leading to disulfide bond formation may lead to the redistribution of several cytoskeletal and

munocytochemical investigations performed on menadione-exposed cells [11] revealed that some surface proteins like sialoglycoproteins, beta 2 microglobulin and others underwent changes in their expression over the bleb surface; membrane blebs appeared devoid of sialoglycoproteins.

4.1, which anchors the skeletal network to the cell membrane [12,13] by binding to a major integral protein glycophorin on one hand and to spectrin on the other hand, one should expect menadione to be a potent modulator of the physical state of glycophorin and cytoskeletal proteins.

the peripheral cytoskeletal proteins. A major determinant of erythrocyte membrane shape and stability is the cytoskeleton, a protein lattice located on the inner surface

actin; 2 linking proteins, ankyrin and protein 4.1; and their respective attachment sites on the cytoplasmic poles of Band 3 and glycophorin. Protein 4.1 stabilizes the spectrin-actin lattice suggesting that protein 4.1 may be intimately involved in maintaining membrane integrity [16].

In this paper we report the molecular effects of oxidation by menadione on erythrocyte membranes using electron paramagnetic resonance (EPR) techniques of

tal protein [17,18], lipid bilayer [18,19] and cell-surface carbohydrate [19,20] domains of human erythrocyte membranes. This technique is sensitive to protein structural changes occurring in the spin labeled substrate. Changes in the relevant parameters of the EPR spectra reflect changes induced in protein components of erythrocyte membranes after oxidation.

2. Materials and methods2.

2.1. Chemicals

Menadione (2-methyl-1,4-naphtoquinone), the lipid-specific spin label 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (5-NS), the protein-specific spin label 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6), the spin

The rest of the chemicals that were used in this study were of reagent grade.

2.2. Preparation of ghosts

Fresh blood was obtained from healthy donors by venipuncture into heparinized

versity of Kentucky Human Subjects Committee to participate in this research study. Intact red cells were resuspended in PBS buffer (5 mM sodium phosphate/150 mM

phate buffer, pH 8.0). We employed 1 volume of cells to 20 volumes of SPB at 4°C for at least 30 min incubation time, and subsequent centrifugation at 4°C and 27 000 × *g*. This process was repeated until the membranes were free of residual hemoglobin. Protein content was estimated by the method of Lowry et al. [21].

For the selective spin labeling [20] of sialic acid residues of glycoproteins and glycolipids, the C-7, C-8, and C-9 vicinal hydroxyl groups of sialic acid residues of isolated membranes were oxidized to a terminal aldehyde by treatment with 2 mM $NaIO_4$ at 0°C for 10 min by reductive amination procedures developed in our labo-

tose and *N*-acetylgalactosamine residues, procedures also developed in our laboratory [22], intact cells were exposed to galactose oxidase as previously described. Isolated ghost membranes were then obtained as above, and the C-6 aldehyde on galactose and *N*-acetylgalactosamine were reacted with tempamine by reductive ami-

the major transmembrane protein (Band 3), Band 4.5, and glycophorin [22].

The cytoskeletal proteins are selectively and covalently spin labeled with MAL-6. Immunological studies of MAL-6-labeled ghost membranes suggest that nearly all the spin label is bound to the major erythrocyte cytoskeletal membrane, spectrin [reviewed in 17 and 18]. Lipid bilayers are labeled with a lipid-specific spin label 5-NS as previously described [18,19].

2.5. Spectra

perature and humidity on a Bruker ESP-300 EPR spectrometer with computerized data acquisition and analysis capabilities. Typical spectrometer conditions are given in the legends to the figures.

2.6. Menadione treatment

menadione in DMSO (dimethyl sulfoxide). In all experiments, the final concentration of protein content was 2.5 mg/ml. Prior to data acquisition, ghosts were incubated with 100 μM to 1 mM menadione (final concentrations) for 1 h at room temperature. For the control, 2% DMSO was added to the labeled membrane ghosts instead of the menadione solution.

2.7. Statistics

Data were analyzed by a two-tailed Student's *t*-test of paired data. A value of

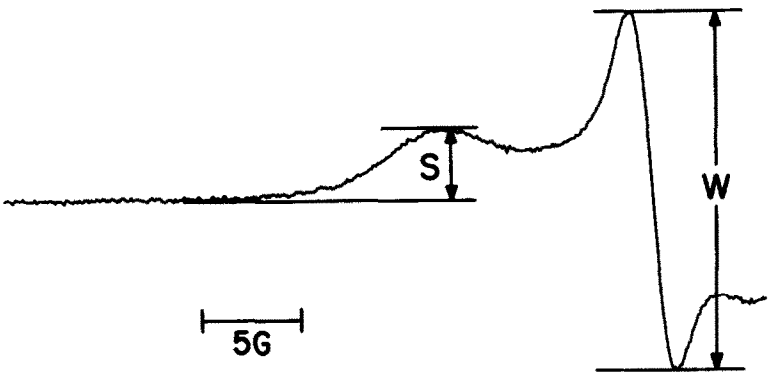

Fig. 1. A typical EPR spectrum of the M$_{+1}$ low field lines of MAL-6 labeled erythrocyte membranes

microwave power.

Effect of menadione[a] on the physical state of erythrocyte membrane skeletal proteins as monitored by the W/S ratio of MAL-6[b]

$(W/S)_{Control} - (W/S)_{Menadione}$	N	P^c
0.75 ± 0.29	7	< 0.001

[a]200 μM final concentration.
[b]Mean difference ± S.D. is presented.

3. Results

3.1. MAL-6 labeling (W/S ratio)

spin label signal intensity [17,18]. A typical EPR spectrum of MAL-6 covalently attached to erythrocyte membrane proteins is shown in Fig. 1. The relevant EPR parameter is the ratio of the weakly immobilized line (W) to that of the $M_I = +1$ low-field strongly immobilized

tal motion of spin-labeled proteins, thereby increasing the W/S ratio [17,18,23,24].

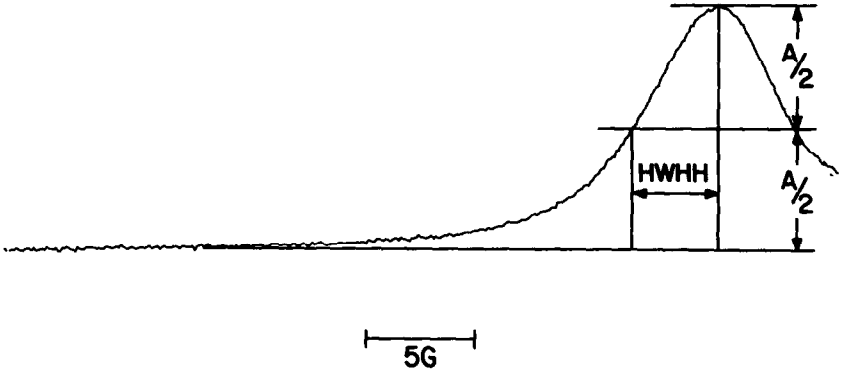

Fig. 1. A typical EPR spectrum of 5-NS labeled erythrocyte membranes. The $M_I = +1$ low field line is

tion amplitude and 30 G scan width.

In contrast, spermine, which crosslinks spectrin to the major transmembrane protein Band 3, i.e., increases membrane protein-protein interactions, reduces the segmental motion of spin-labeled proteins and causes a decrease in the W/S ratio [25].

Each sample was incubated with menadione for 1 h at room temperature before acquiring spectra. Different concentrations of menadione all decreased the W/S ratio relative to controls, suggesting that this oxidant increases protein-protein interac- [redacted] menadione concentrations for most of our experiments, the same concentration used by others [11]. Table 1 shows that 200 μM menadione decreased the W/S ratio relative to controls with high significance ($P < 0.001$), suggesting that this oxidant increases protein-protein interactions in erythrocyte membranes and affects spectrin, [redacted]

3.2. 5-NS labeling

We investigated whether menadione induces alterations of lipid bilayers. To test this idea, we employed the lipid-specific spin label 5-NS to assess the effect of menadione on the physical state of the lipid bilayer of human erythrocyte membranes. This lipid-specific probe undergoes rapid anisotropic motion about the long axis of the probe and orientational flipping of the principal axis of the nitroxide between parallel and perpendicular orientations relative to the membrane normal [17,18]. The half-width at half-height (HWHH) of the $M_I = +1$ low field line (Fig. 2) is sensitive to small variations in membrane motion [18,19]. The smaller the HWHH, the less motion and greater order in the local microenvironment reported by the nitroxide group of 5-NS [18]. The results shown in Table 2 suggest that under the condi- [redacted] cal state of cytoskeletal proteins (Table 1) are not due to secondary effects of alterations in the physical state of membrane lipids.

Table 2
Effect of menadione on the motion of 5-NS in the lipid bilayer of human erythrocyte membranes as [redacted]

[redacted]			
(μM)	(G)		
0	3.2 ± 0.2	6	ns**
100	3.3 ± 0.1	5	ns
200	3.2 ± 0.1	8	ns
500	3.2 ± 0.3	5	ns
1000	3.0 ± 0.1	4	ns

*The HWHH values are presented as means ± S.D. *P* values are calculated by a two-tailed Student's *t*-test with the null hypothesis that menadione treatment does not affect the HWHH value of 5-NS at each respective concentration relative to the control value.
**ns, not significant.

The the0.0672 Tthe30.0144 Tw3(of) Tj Tj 0 Tr

3.3. Tempamine labeling

Protein 4.1 anchors the cytoskeletal network to the cell membrane by binding to the major integral sialoglycoprotein (glycophorin) from one side and to spectrin on the other. If the oxidant menadione affects the 2 binding sites then the membrane

sialic acid. To test this possibility, sialic acid specific spin-labeling procedures were

characterized by an apparent rotational correlation time, τ_a, calculated by Eqn. 1

$$\tau_a = 6.5 \times 10^{-10} W_0 [(h_0/h_{-1})^{1/2} + (h_0/h_{+1})^{1/2} - 2] \quad (1)$$

where W_0, h_0, h_{-1} and h_{+1} are respectively the peak-to-peak linewidth of the $M_I = 0$ line in Gauss and peak-to-peak amplitude of the $M_I = 0$, $+1$, -1 lines. τ_a can be considered as the time required for the spin label to rotate through an angle of 1 radian [20]. An increase in τ_a implies reduced motion of the spin-labeled sialic

of glycophorin may be affected by the menadione-induced changes in the conformation of protein 4.1.

To gain insight into the specificity of menadione-induced changes in the physical state of transmembrane proteins, terminal galactose and *N*-acetylgalactosamine resi-

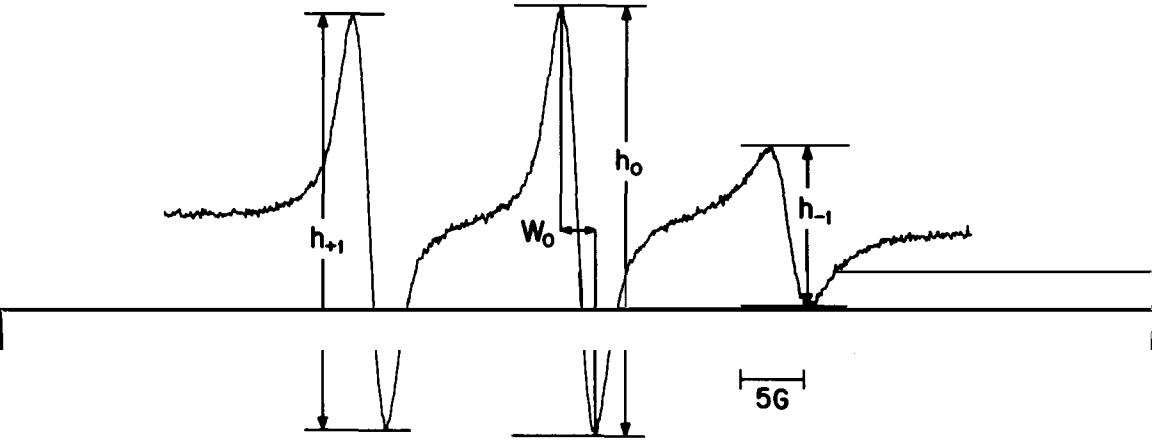

Fig. 3. A typical EPR spectrum of tempamine covalently bound to sialic acid residues of membrane glycoproteins in human erythrocyte membranes. The spectral measurements required for the determination of τ_a are indicated. Instrument settings: 75 G scan width, 19.1 mW microwave power and 0.32 G modulation amplitude.

Table 3
Effect of menadione[a] on the apparent rotation correlation time[b] of spin-labeled sialic acid relative to untreated controls

$(\tau_a)_{Menadione} - (\tau_a)_{Control}$	N	P^c
[redacted]	[redacted]	[redacted]

[redacted] 200 μM final concentration.
[b]Mean difference ± S.D. is presented.
[c]*P* values are calculated by a two-tailed Student's *t*-test of paired data with a null hypothesis that this [redacted]

dues, found mostly on Band 3 and Band 4.5, were also labeled with tempamine [22] in separate experiments. Again, the apparent rotational correlation time, τ_a, is used to analyze the spectrum. Contrary to our findings in glycophorin, Table 4 suggests that menadione has no statistically significant effect on the conformation of Band 3 and/or Band 4.5, also consistent with the notion of Band 4.1 oxidative damage by menadione.

4. Discussion

Greater than 90% of the protein 4.1 remains associated with the membrane under the low ionic strength conditions that remove spectrin [12,13]. Therefore, protein 4.1 [redacted]

The effect of menadione on glycophorin is less than that on the cytoskeletal pro- [redacted] μM final concentration). Since glycophorin is coupled to the major cytoskeletal protein by protein 4.1, this result is consistent with the suggestion that menadione may interact with protein 4.1, causing both increased cytoskeletal protein-protein interac-

Table 4
Effect of menadione[a] on the apparent rotation correlation time[b] of spin-labeled terminal galactose and [redacted]

$(\tau_a)_{Menadione} - (\tau_a)_{Control}$	N	P
$(0.08 \pm 0.3) \times 10^{-10}$ s	8	ns[d]

[a]200 μM final concentration.
[b]Mean difference ± S.D. is presented.
[c]*P* values are calculated by a two-tailed Student's *t*-test of paired data with the null hypothesis that this difference is zero, i.e., menadione has no effect on the motion of spin labeled galactose residues.
[d]ns, not significant.

spin label techniques have suggested that menadione leads to the oxidation of the

sharp contrast to that of the sialic acid.

A number of physiological or pathological processes might cause oxidant damage to red cells, and therefore it is important to determine the mechanism for membrane failure in an oxidative environment. Such damage may result in hemolysis, and therefore cytoskeletal protein damage may be a common pathway by which certain unstable hemoglobinopathies or antioxidant-compromised erythrocytes acquire membrane abnormalities [26]. Repka et al. [27] have shown that most of the

Schwartz et al. [28] strongly suggested that sickle protein 4.1 has sustained oxidative damage in vivo which is responsible for the membrane abnormalities reported in sickle erythrocytes. Parola et al. [29] reported that biliary epithelial cells and parenchymal cells were highly sensitive to 100 μM menadione. This oxidant induced cell a injury including a strong depletion of protein thiols and an increase in the extent of cell death.

Under the conditions used in this study, menadione did not alter the motion of the lipid-specific spin label 5-NS; however, others report that under prolonged expo

we assert only that under the specific conditions used in the current studies was menadione unable to alter lipid motion and order. Under different conditions lipid

reportedly did not alter lipid fluidity [8]. Finally, while lipid fluidity changes could

likely not secondary to alterations in the physical state of the lipid bilayer.

Although the precise molecular mechanisms to account for the alterations in the physical state of erythrocyte membrane cytoskeletal and transmembrane proteins

cytes might explain the cytotoxic nature of menadione. Studies designed to test this hypothesis are in progress.

Acknowledgements

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