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

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Abstract

Menadione (2-methyl-1,4-naphtoquinone) is cytotoxic to hepatocytes. In order to begin to investigate the changes in the physical state of membranes induced by this cytotoxic substance, electron paramagnetic resonance (EPR) spin-labeling techniques were used in conjunction with spin labels specific for cytoskeletal proteins, bilayer lipids, or cell-surface sialic acid or galactose to investigate erythrocyte membranes. We studied the molecular effects of oxidation of 200 μM menadione on the different membrane domains. The major findings are: (1) 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 interactions of the cytoskeletal network and its anchoring domains in the membrane. These results are discussed with reference to possible mechanisms involved in the cytotoxic action of menadione.

Keywords: Menadione; Erythrocyte membranes; Cytoskeletal proteins; Band 4.1; Oxidation; Spin labeling; Sialic acid

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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 cells is partially or completely due to their capacity to stimulate superoxide production by generating considerable amounts of oxygen reactive species or by arylation of cellular thiols [2].

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 oxidation of thiol groups in proteins [7]. Menadione reportedly does not cause lipid peroxidation in hepatocytes [8].

Menadione is a model compound employed to investigate cell injury and oxidant-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 membrane proteins as well as the dissociation of the cytoskeleton network. Studies performed on the cytoskeletal fraction extracted from menadione-treated cultured mammalian cells have revealed a series of oxidative changes affecting the 3 major classes of cytoskeletal proteins: spectrin, actin and protein 4.1. Furthermore, immunocytochemical 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.

The simplest hypothesis to explain this phenomena is that the direct or indirect breaking of connections anchoring transmembrane proteins may induce a rearrangement of such proteins, one manifestation of which could be the dissociation of the cytoskeleton network. If this hypothesis is true, focusing our attention on protein 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.

To test this hypothesis, we used erythrocytes to provide a simple model system [14,15] for the study of interaction between cell membrane integral components and the peripheral cytoskeletal proteins. A major determinant of erythrocyte membrane shape and stability is the cytoskeleton, a protein lattice located on the inner surface of the cell membrane. It is composed of 2 major supporting proteins, spectrin and 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 spin labeling [17,18]. The results of investigations are reported using EPR in conjunction with spin labels specific to different membrane components: the cytoskeletal 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 methods

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 label used to label cell-surface carbohydrates, 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl (tempamine), and galactose oxidase type IV were purchased from Sigma. 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 tubes that were put immediately in ice, and processed within 30 min of collection. These donors signed informed consent forms under a protocol approval by the University 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 NaCl, pH 8.0) and washed 3 times by centrifugation at 600 × *g* for 5 min at 4 °C. After each wash, the buffy coats were carefully removed by aspiration. Erythrocyte ghost membranes were obtained by hypotonic lysis with 5P8 (5 mM sodium phosphate buffer, pH 8.0). We employed 1 volume of cells to 20 volumes of 5P8 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].

2.3. Labeling of sialic acid and galactose residues

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 laboratory as previously described [20]. Seventy percent of erythrocyte membrane sialic acid is found on glycophorin [14]. For the selective spin labeling of terminal galactose 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-

nation as described [22]. Most of the spin label is bound to the glycoconjugates of the major transmembrane protein (Band 3), Band 4.5, and glycophorin [22].

2.4. Labeling of proteins and lipids

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

All EPR spectra were recorded at room temperature in a room with constant temperature 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

A 10-mM stock solution of menadione was prepared (always fresh) by dissolving 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 $P < 0.05$ was considered statistically significant.

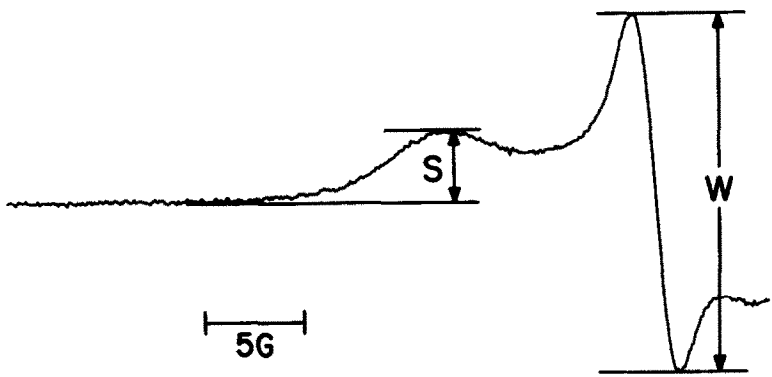

Fig. 1. A typical EPR spectrum of the $M_I = +1$ low field lines of MAL-6 labeled erythrocyte membranes. The spectral amplitudes of the spin label covalently bound to strongly and weakly immobilized sites are indicated by S and W. EPR parameters: 75 G sweep width, 0.32 G modulation amplitude and 19.1 mW microwave power.

Table 1
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.
[c]*P* value calculated by a two-tailed Student's *t*-test of paired data with the null hypothesis that this difference is zero, i.e., that menadione had no effect on the W/S ratio of MAL-6 relative to the control.

3. Results

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

The protein-specific MAL-6 spin label binds covalently to SH groups of cytoskeletal proteins, with the spectrin-actin complex accounting for 70–90% of the 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 measured is the ratio of the spectral amplitude of the $M_I = +1$ low-field weakly immobilized line (W) to that of the $M_I = +1$ low-field strongly immobilized line (S), known as the W/S ratio. Changes in the W/S ratio are known to be strong indicators of alterations in the conformation and interactions of cytoskeletal proteins [17,18]. For example, agents such as hemin, polyphosphates and selective digestion of ankyrin, the protein which bridges a fraction of Band 3 molecules to spectrin, decrease cytoskeletal protein-protein interactions and consequently increase segmental motion of spin-labeled proteins, thereby increasing the W/S ratio [17,18,23,24].

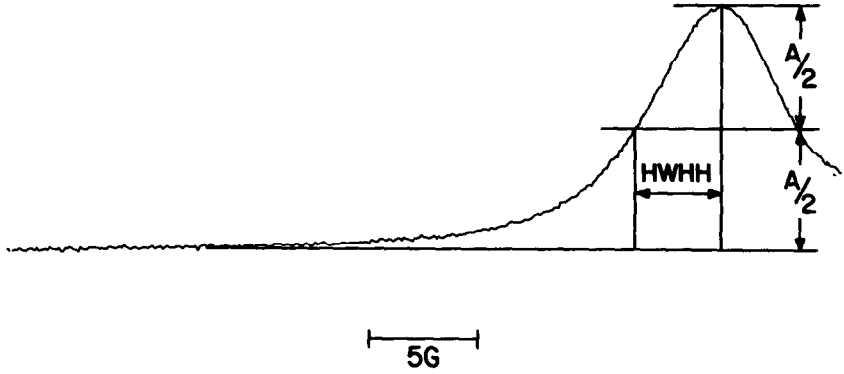

Fig. 2. A typical EPR spectrum of 5-NS labeled erythrocyte membranes. The $M_I = +1$ low field line is enlarged showing the HWHH parameter. EPR parameters: 19.1 mW microwave power, 0.32 G modulation amplitude and 30 G scan width.

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 interactions in erythrocyte membranes. Based on the dose-response data, we chose 200-μM 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, where most of the MAL-6 is found.

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 conditions employed in this study menadione has no effect on the fluidity of the lipid bilayer, consistent with the notion that this agent reportedly does not cause lipid peroxidation in hepatocytes [8]. These results also suggest that the changes in the physical 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 monitored by HWHH of the low-field line*

Menadione (μM)	HWHH (G)	N	*P*
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.

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 integrity will be affected. Therefore, the menadione-induced increase of cytoskeletal-protein interactions, protein 4.1 in particular, might be expected to alter the motion of extracellular sialic acid residues; glycophorin contains up to 70% of the membrane sialic acid. To test this possibility, sialic acid specific spin-labeling procedures were employed [20]. An EPR spectrum of the tempamine spin label covalently attached to terminal sialic acid is presented in Fig. 3. The motion of the spin label is 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 acid [17,20]. Table 3 shows that menadione has the slightly statistically significant ($P < 0.05$) effect of slowing the rotational motion of tempamine on terminal sialic acid residues. This result is consistent with our expectations that the conformation 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-

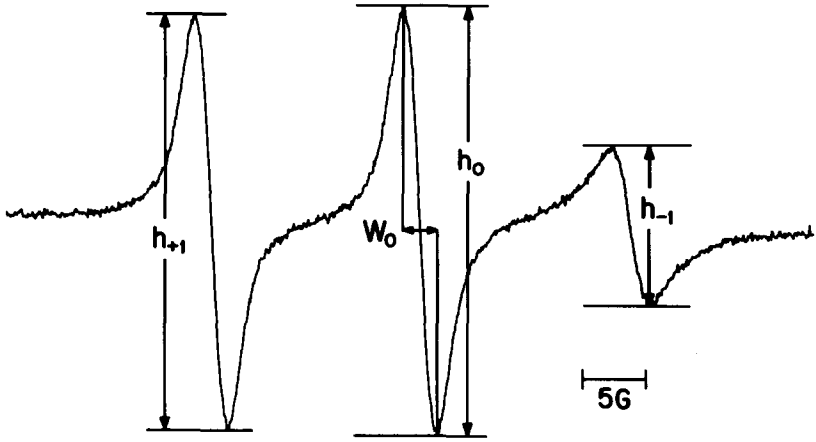

Fig. 3. A typical EPR spectrum of tempamine covalently bound to sialic acid residues of membrane glycoproteins in human erythrocyte membranes. The spectral measurements required for the determination of τ_a are indicated. Instrument settings: 75 G scan width, 19.1 mW microwave power and 0.32 G modulation 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
$(0.53 \pm 0.65) \times 10^{-10}$ s	9	< 0.05

[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 a null hypothesis that this difference is zero, i.e., that menadione had no effect on the motion of spin labeled sialic acid.

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 would appear to have a membrane association site distinct from its known interaction with spectrin and actin. It has been reported that protein 4.1 binds preferentially to glycophorin [12]. Both of these observations suggest that protein 4.1 may be intimately involved in maintaining membrane integrity.

The effect of menadione on glycophorin is less than that on the cytoskeletal proteins (a 10% increase vs. a 17% decrease of the respective control parameter at 200 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 *N*-acetylgalactosamine residues relative to untreated controls

$(\tau_a)_{Menadione} - (\tau_a)_{Control}$	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.

tions and decreased molecular motion of sialic acid on glycophorin. These parallel spin label techniques have suggested that menadione leads to the oxidation of the erythrocyte membrane by interacting with certain, perhaps specific, protein components. The physical state of galactose residues was unaffected by menadione, in 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]. Rank et al. [27] have shown that most of the cytoskeletal proteins in sickle cell membranes contained oxidized or blocked thiols. 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 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 exposure of peroxidation products alteration of lipid fluidity can occur [30]. Therefore, 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 alterations might well be important. However, in hepatocytes 200 μM menadione reportedly did not alter lipid fluidity [8]. Finally, while lipid fluidity changes could be important at higher levels or longer exposure of menadione, the current results suggest that changes in the physical state of cytoskeletal proteins and sialic acid are 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 upon treatment with menadione remain yet unclear, the current results are consistent with a major involvement of the cytoskeletal protein 4.1. Similar changes in hepatocytes might explain the cytotoxic nature of menadione. Studies designed to test this hypothesis are in progress.

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