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Ascorbate-Dependent Protection of Human Erythrocytes against Oxidant Stress Generated by Extracellular Diazobenzene Sulfonate

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ABSTRACT. Diazobenzene sulfonic acid (DABS) has been used to label thiols and amino groups on cell-surface proteins. However, we found that in addition to inhibiting an ascorbate-dependent trans-plasma membrane oxidoreductase in human erythrocytes, it also depleted α-tocopherol severely in the cell membrane. When erythrocytes were loaded with ascorbate, DABS-dependent loss of α-tocopherol was decreased, despite little change in intracellular ascorbate content. Sparing of α-tocopherol also was seen in erythrocyte ghosts resealed to contain ascorbate, although this was accompanied by loss of intravesicular ascorbate, probably due to the inability of ghosts to recycle ascorbate. A transmembrane transfer of electrons from ascorbate was confirmed by electron paramagnetic resonance spectroscopy, in which extracellular DABS was found to generate the ascorbate free radical within cells. When the membrane content of α-tocopherol was decreased to 20% of the initial value by DABS treatment, lipid peroxidation ensued, manifest by generation of F_2-isoprostanes in the cell membranes. Intracellular ascorbate also strongly protected against F_2-isoprostane formation. These results show that DABS causes an oxidant stress at the membrane surface that is transmitted within the cell, in part by an α-tocopherol-dependent mechanism, and that ascorbate recycling of α-tocopherol can protect against loss of α-tocopherol and the ensuing lipid peroxidation. BIOCHEM PHARMACOL **60**;1:47–53, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. ascorbic acid; α-tocopherol; diazobenzene sulfonic acid; F_2-isoprostanes; ascorbate free radical; human erythrocytes

All cells appear to have a trans-plasma membrane oxidoreductase activity that can transfer electrons from intracellular substrates, such as ascorbate and NADH, to extracellular electron acceptors, such as ferricyanide [1]. The function of this oxidoreductase activity is unknown, but it has been linked to cell growth [2, 3], iron uptake [1, 4], and most recently to protection of the cell membrane against oxidant damage [5]. In erythrocytes, this activity is sensitive to inhibition by *p*-chloromercuribenzene sulfonic acid [6, 7] and by DABS§ [6]. Since both reagents are charged and, thus, are likely to penetrate cells only very slowly, their inhibition of the oxidoreductase activity suggests that it is mediated by a protein with reactive exofacial groups. Further, treatment of intact cells with DABS resulted in a 35% decrease in NADH-dependent ferricyanide reduction in ghost membranes prepared from the cells [6]. These results showed that the effect of DABS persists during ghost preparation, and that the activity involved spans the plasma membrane.

DABS has also been used previously to label the exofacial cell surface proteins and phospholipids in erythrocytes [8, 9]. In attempting to use DABS as a probe for the transmembrane oxidoreductase activity in these cells, we found that under conditions in which inhibition of ferricyanide reduction was observed, DABS also depleted erythrocyte α-tocopherol by over 90% [10]. Depletion of α-tocopherol by DABS in intact cells could indicate an oxidant stress induced by the agent, and could also contribute to its inhibition of transmembrane ferricyanide reduction [11]. Since DABS is considered a protein-labeling agent [8, 12], the present studies were performed to establish the mechanism of the DABS-induced decrease in erythrocyte α-tocopherol. We found that DABS does induce oxidative stress in the plasma membrane of intact erythrocytes, that this stress is transmitted across the cell membrane, and that intracellular ascorbate, probably by recycling α-tocopherol, can protect against α-tocopherol loss and formation of lipid hydroperoxides in response to DABS.

MATERIALS AND METHODS

Materials

DHA, 2,2,6,6-tetramethyl-4-hydroxy-piperidine-*N*-oxyl (Tempol), and tetrapentyl ammonium bromide were

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§ *Abbreviations*: AFR, ascorbate free radical; DABS, diazobenzene sulfonic acid; and DHA, dehydroascorbic acid.

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obtained from the Aldrich Chemical Co. Other analytical reagents, including sulfanilic acid, sodium nitrite, and sodium ascorbate were purchased from the Sigma Chemical Co. DABS was prepared as previously described [6], stored on ice, and typically used within 30 min. An emulsion of α-tocopherol in Tween-80 was prepared by warming 5 mg of α-tocopherol with 10 mg of Tween-80, diluting with 2.2 mL of deionized water, and sonicating for 2–3 min to obtain an opalescent solution.

Preparation of Erythrocytes and Leaky and Resealed Erythrocyte Ghosts

Human erythrocytes were prepared from blood that had been drawn from normal volunteers, using heparin as an anticoagulant. Erythrocytes were washed three times by centrifugation in 10 vol. of PBS. The latter consisted of 140 mM NaCl and 12.5 mM Na_2HPO_4 in deionized water, adjusted to pH 7.4. The buffy coat of white cells was removed carefully from the surface of the erythrocyte pellet with each wash. Leaky erythrocyte ghosts were prepared as described by Gorga and Lienhard [13]. Erythrocyte ghost protein was measured with the Bradford reagent using 0.2% Triton X-100 to dissolve the membranes. Resealed erythrocyte ghosts were prepared by the method of Steck and Kant [14] to contain either no additions or 400 μM ascorbate as previously described [15]. Erythrocyte hemolysis was determined as the increase in absorbance at 540 nm of erythrocyte wash supernatants compared with absorbance produced by hemolysis of a known volume of erythrocytes in water. The concentration of methemoglobin in cell lysates was determined according to Winterbourn [16] as the difference in absorbance of diluted lysate between 577 and 630 nm, adjusted for the respective extinction coefficients of oxyhemoglobin and methemoglobin. Because hemoglobin was not purified before spectrophotometric measurements, there was a background of absorption at 630 nm due to cell membranes and cytosolic components. This background present in control cells was subtracted from each determination of methemoglobin, and the results are expressed as the percent increase in methemoglobin relative to total hemoglobin (oxyhemoglobin plus methemoglobin).

Antioxidant and Lipid Peroxidation Assays

The ascorbate content of erythrocytes and resealed erythrocyte ghosts was determined as previously described [15]. Erythrocyte and ghost contents of α-tocopherol were measured as follows. Packed erythrocytes or erythrocyte ghosts (0.2 mL) were mixed with 50 μL of aqueous 0.5% pyrogallol (w/v). To this was added 0.2 mL of reagent alcohol (95% ethanol:5% isopropanol, v/v), followed by vigorous mixing. Heptane (0.4 mL) was added, the resulting lysate was vortexed for at least 1 min and microfuged, and an aliquot of the upper heptane layer was removed and dried under a stream of nitrogen. To this was added 0.2 to 0.4 mL of a 1:1 mixture of ethanol and methanol, and 100 μL was assayed by HPLC with electrochemical detection. Separation was carried out on a 150-mm Waters DeltaPak C_{18} column (300 Å pore size, 5 μm particle size) that was preceded by a 4-mm guard column of the same packing material. The mobile phase was 95% methanol and contained 20 mM sodium perchlorate. Detection of α-tocopherol was carried out by a modification of the reduction–oxidation method described by Takeda *et al.* [17]. Briefly, an ESA model 5020 guard cell was placed just after the analytical column and set in the reducing mode at −0.5 V. This was followed by an ESA model 5011 analytical cell that was used for detection, with the first electrode in the analytical cell set at −0.5 V, and the second detecting electrode set at +0.6 V. At a flow rate of 1 mL/min, α-tocopherolquinone, γ-tocopherol, and α-tocopherol eluted sequentially between 4.3 and 6.5 min. The sensitivity of the assay was 2–5 pmol/injection for α-tocopherol. Values for ascorbate and α-tocopherol are expressed per milliliter of erythrocyte cytoplasm, which was taken as 80% of the packed cell volume [4]. Assay of F_2-isoprostanes in erythrocyte ghost membranes was carried out employing a highly precise and accurate mass spectrometric assay, as previously described [11].

Reduction of Extracellular Ferricyanide by Erythrocytes

Cells at a 5% hematocrit were incubated in PBS containing 5 mM D-glucose and 1 mM ferricyanide for 30 min at 37°, pelleted in a microfuge, and then duplicate aliquots of the supernatant were taken for assay of ferrocyanide by the method of Avron and Shavit [18], using 2,5-*o*-phenanthroline as the chelating agent. A correction was made for absorbance of medium from cells incubated in the absence of ferricyanide. The concentrations of ascorbate, α-tocopherol, and ferrocyanide are expressed relative to the intracellular water space of the erythrocytes, which was taken as 80% of the packed cell volume [4].

EPR Spectroscopic Measurements of the AFR

EPR measurements were taken on a Bruker EMX 8/27 spectrometer that was equipped with a BVT300 variable temperature controller. X-band EPR spectra were acquired using a ER041XG-DHA microwave bridge and ER4103TM/9614 cavity. Sample incubations were carried out at 37° in a Wilmad WG 804 aqueous flow flat cell. Instrument settings were as follows: 6 G magnetic field scan range (the conversion time of the instrument was set to result in an ~80-sec scan), 0.5 G modulation amplitude, 100 kHz modulation frequency, and 10 mW microwave power. The flat cell was preloaded with a suspension of erythrocytes, and the spectrometer was tuned before acquisition of data. Data acquisition was started by rapidly mixing 0.3 mM DABS with cells at 37° in PBS that contained 5 mM D-glucose. Sample was injected into the flat cell, and the spectral scan was started. The concentra-

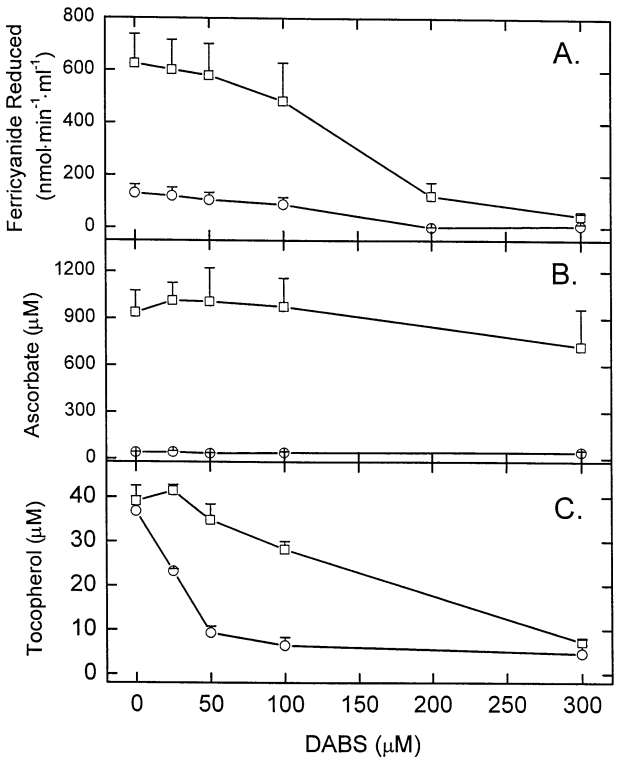

FIG. 1. Effects of DABS on erythrocyte ferricyanide reduction, ascorbate, and α-tocopherol. Cells at a 5 or 10% hematocrit were incubated for 15 min at 37° in the absence (circles) or presence (squares) of 200 μM DHA in PBS that contained 5 mM D-glucose. The cells were washed twice in PBS and suspended to the original concentration in PBS that contained 5 mM D-glucose and the indicated concentration of DABS. After 30 min of incubation at 37°, the cells were washed three times in PBS and taken for assay of ferricyanide reduction (panel A), ascorbate content (panel B), and α-tocopherol content (panel C). Data are means ± SEM from four experiments.

tion of the AFR was determined by calibration of the instrument using the signal of 10 μM Tempol.

Analytical and Statistical Methods

Except where indicated, data are expressed as means ± SEM from the indicated number of experiments. Differences between treatments were analyzed by two-way analysis of variance with post-hoc testing using Tukey's test or by the paired Student's *t*-test.

RESULTS

Incubation of erythrocytes with increasing concentrations of DABS for 30 min at 37° irreversibly decreased the ability of the cells to reduce ferricyanide. The DABS-induced inhibition was half-maximal at about 150 μM DABS, and almost complete at DABS concentrations of 200 μM and above (Fig. 1A). Cells that had been preloaded with ascorbic acid by incubation with DHA showed a 4-fold increase in the rate of ferricyanide reduction, but no change in the half-maximal inhibition induced by DABS (Fig. 1A). Thus, ascorbate-loading to concentrations 20-fold those in control cells (Fig. 1B) was without effect on DABS-induced inhibition of ferricyanide reduction. DABS treatment was also without a significant effect on intracellular ascorbate in either control or ascorbate-loaded cells (Fig. 1B). However, under these conditions, there was a concentration-dependent decrease in erythrocyte α-tocopherol (Fig. 1C) that was linear over the time of incubation (results not shown). The half-maximal decrease in α-tocopherol occurred at about 25 μM DABS, a concentration much less than that required for half-maximal inhibition of ferricyanide reduction (Fig. 1A). The DABS-induced decrease in α-tocopherol was retarded substantially by ascorbate-loading of the cells (Fig. 1C). The latter result suggests that ascorbate-loading of the cells helped to preserve their membrane content of α-tocopherol.

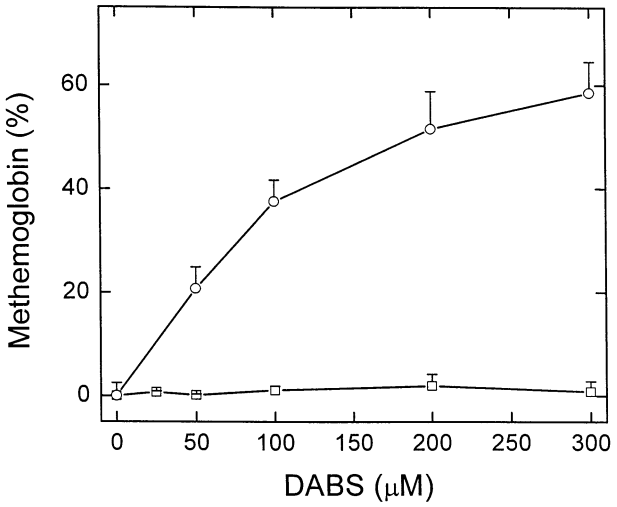

FIG. 2. DABS-induced oxidation of hemoglobin in cells and cell lysates. Squares: Erythrocytes at a 10% hematocrit were incubated for 20 min at 37° in the presence of the indicated concentration of DABS and 5 mM D-glucose. The cells were washed three times by centrifugation in PBS and hemolysed for determination of the percent methemoglobin in the hemolysates (means ± SEM, N = 3 experiments). Circles: Cells at a 10% hematocrit were treated with the indicated concentration of DABS in PBS containing 5 mM D-glucose, and immediately hemolysed in 100 vol. of deionized water, followed by determination of the percent methemoglobin (data are from two experiments ± range).

To determine whether the effects of DABS on intracellular ascorbate could be due to penetration of the agent into the cells, hemolysis and methemoglobin formation were measured. In the experiments of Fig. 1, the highest concentration of DABS (300 μM) had no effect on hemolysis (results not shown). Incubation of intact cells with DABS did not increase the cell content of methemoglobin (Fig. 2). On the other hand, incubation of fresh hemolysate with the same concentration range of DABS caused a marked increase in methemoglobin formation (Fig. 2). The failure of DABS to affect hemoglobin in intact cells shows that it did not penetrate the cell membrane.

The ability of ascorbate to spare erythrocyte α-tocoph-

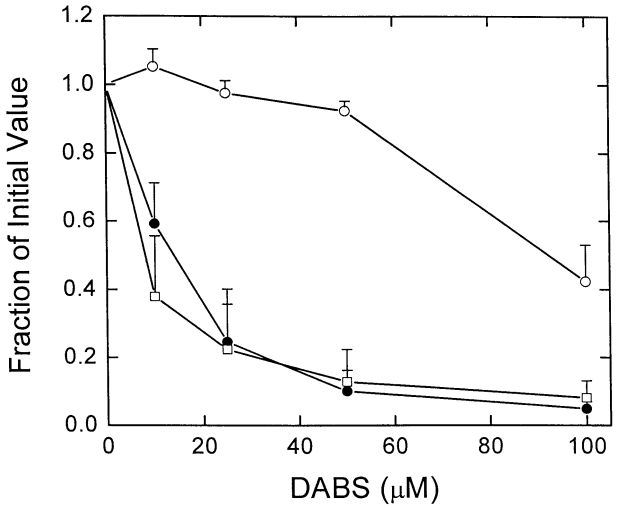

FIG. 3. Effects of DABS on the α-tocopherol content of resealed erythrocyte ghosts. Erythrocyte ghosts were resealed to contain either no ascorbate (solid symbols) or 1 mM ascorbate (open symbols). The ghosts were washed three times in ice-cold PBS to remove extravesicular ascorbate, and the packed ghosts were suspended in 9 vol. of PBS that contained the indicated concentration of DABS. Following incubation for 20 min at 37°, the ghosts were pelleted at 13,000 *g* for 5 min at 3°. The ghosts were washed twice more by centrifugation, and aliquots were taken for assay of α-tocopherol (circles) and ascorbate (squares). Results (means ± SEM) from three experiments are shown as a fraction of control for both α-tocopherol (initial values: control, 34 ± 6 μM; ascorbate-loaded, 27 ± 2 μM) and ascorbate (initial value: 1100 ± 270 μM).

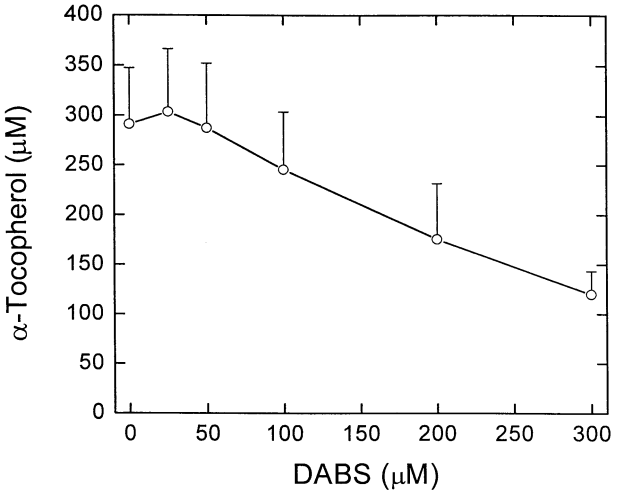

FIG. 4. Reaction of DABS with a Tween-80 emulsion of α-tocopherol. DABS at the indicated concentration was incubated in PBS for 15 min at 37°, followed by assay of α-tocopherol following dilution. Results are the means ± SEM from six experiments.

erol in response to treatment with DABS was also tested in resealed erythrocyte ghosts, which lack the ability to recycle oxidized ascorbate. DABS treatment of ghosts prepared without ascorbate-loading resulted in a sharp decrease in membrane α-tocopherol content (Fig. 3). A 50% fall in membrane α-tocopherol occurred at about 15 μM DABS, similar to that observed in intact cells. Ghosts that had been loaded with 400 μM ascorbate showed a markedly retarded loss of α-tocopherol compared with control ghosts. The ascorbate content of the ghosts also decreased with DABS treatment over the same concentration range as the decrease in α-tocopherol in control ghosts (Fig. 3). These results further indicate that ascorbate within ghosts spared α-tocopherol in response to DABS treatment.

The mechanism by which DABS lowered α-tocopherol in the erythrocyte membrane was investigated. Direct DABS treatment of α-tocopherol in Tween-80 emulsions caused a loss of α- tocopherol, as shown in Fig. 4. However, the decrease became significant ($P < 0.01$) only at a DABS concentration of 300 μM. In comparison to the more potent effect of DABS on membrane α-tocopherol in cells and resealed ghosts, this suggests that direct reaction fails to explain all of the effect of DABS to decrease membrane α-tocopherol. To determine whether DABS also induced an additional oxidant stress at the cell membrane, control and ascorbate-loaded cells were incubated with increasing concentrations of DABS, followed by measurement of F_2-isoprostanes in leaky erythrocyte ghost membranes prepared from the cells. Levels of F_2- isoprostanes varied considerably between different ghost preparations. However, as shown in Fig. 5A, treatment of cells with DABS concentrations above 100 μM caused increased F_2-isoprostane content of membranes prepared from such cells. Loading the cells with ascorbate substantially protected against formation of membrane-bound F_2-isoprostanes, as shown in the data from a single experiment in Fig. 5B.

To determine whether an oxidant stress induced by DABS was transmitted across the erythrocyte membrane to the cytoplasm, effects of DABS on intracellular AFR concentrations were measured by EPR spectroscopy. As shown by the top tracing of the inset in Fig. 6, cells containing endogenous ascorbate (typically 40–60 μM) had no detectable AFR when incubated in PBS. When 0.3 mM DABS was added, the spectral signature of the AFR appeared (inset of Fig. 6, lower tracing). The AFR signal was a linear function of the cell concentration at a constant DABS concentration of 0.3 mM (results not shown). As depicted in the main portion of Fig. 6, when cells were exposed to increasing concentrations of DABS, the AFR signal rose at a rate that was proportional to the DABS concentration when the latter was below 150 μM. The maximal amplitude of the signal was not a function of the DABS concentration, but was sustained longer at the higher DABS concentrations. Given the separation of extracellular DABS and intracellular ascorbate by the cell membrane over the short duration of this experiment, the appearance of the intracellular AFR indicates that the oxidant stress generated by DABS was transmitted across the cell membrane.

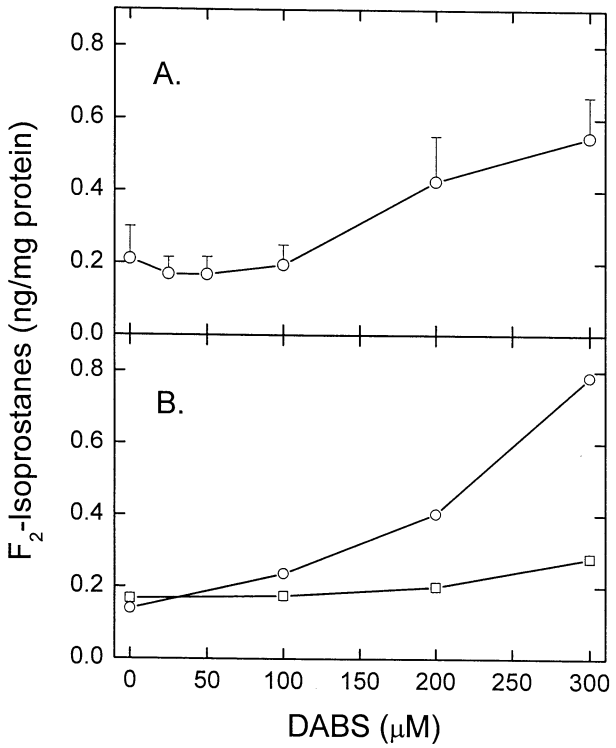

FIG. 5. DABS-induced formation of F_2-isoprostanes. (A) Erythrocytes at a 10% hematocrit were incubated with the indicated DABS concentration for 15 min at 37° in PBS that contained 5 mM D-glucose. The cells were washed three times by centrifugation, and taken for preparation of leaky ghosts and determination of F_2-isoprostane content. Data are from three experiments, and shown as means ± SEM. (B) Under the same conditions as in panel A, cells were incubated for 15 min without (circles) or with (squares) 200 μM DHA during DABS treatment before washing and preparation of leaky ghosts. Results in panel B are from one experiment of two that were performed with DHA loading.

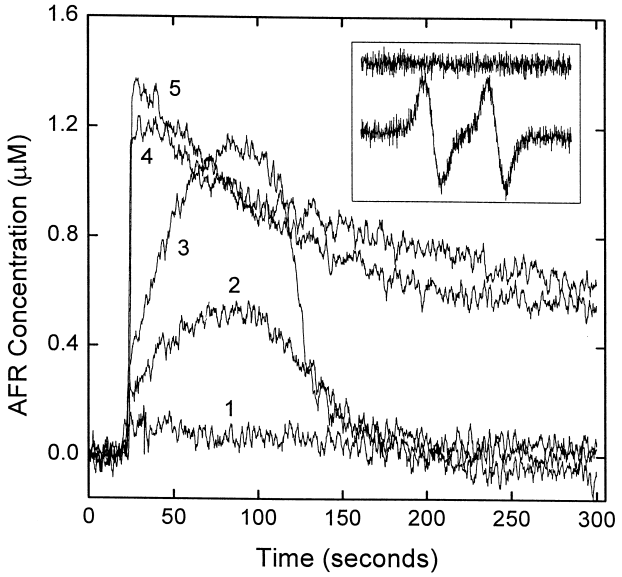

FIG. 6. Generation of the AFR by extracellular DABS. The inset shows the AFR signal in 40% cells alone (upper spectrum) and in 40% cells treated with 300 μM DABS (lower spectrum). In the main figure, the amplitude of the low-field line in the AFR signal at each concentration of DABS is plotted against time. DABS concentrations: 1 = 19 μM; 2 = 28 μM; 3 = 38 μM; 4 = 150 μM; and 5 = 300 μM. Data are from one of two experiments performed.

DISCUSSION

The major findings of this work are that the diazo compound DABS induces an oxidant stress that is transmitted into and across the erythrocyte membrane, and that in response to DABS treatment, intracellular ascorbate can both spare α-tocopherol in the membrane and prevent membrane lipid peroxidation. These conclusions are contingent on the inability of DABS to penetrate the cell membrane. Because of its zwitterionic nature at physiologic pH, DABS is highly unlikely to penetrate the plasma membrane at appreciable rates. Direct evidence for lack of penetrance is our finding that although DABS readily generated methemoglobin in cell lysates, this was not observed in erythrocytes (Fig. 2). Similar conclusions were reached previously by Berg [8], who found that [^{35}S]DABS did not label appreciable amounts of hemoglobin after incubation with intact erythrocytes.

The mechanism by which DABS lowered α-tocopherol in the erythrocyte membrane could involve direct reaction or be due to an oxidant stress generated at the cell surface. DABS is known to react with and couple to thiol and amino groups of proteins and lipids [8], but it also oxidizes primary and secondary alcohols to ketones [19]. Oxidation of the phenolic hydroxyl in α-tocopherol to form a semiquinone could have been responsible for our finding that DABS lowered the α-tocopherol concentration in emulsions (Fig. 4). On the other hand, the DABS-induced decrease in α-tocopherol in the membrane of the intact erythrocyte occurred at lower DABS concentrations (Fig. 1C) than required to decrease α-tocopherol directly. This and the finding that intracellular ascorbate protected α-tocopherol in the erythrocyte membrane (Figs. 1C and 3) argue for a one-electron oxidation of α-tocopherol to the α-tocopheroxyl radical rather than a two-electron oxidation to the semiquinone (which would not be reversed by ascorbate). The ability of ascorbate to preserve and possibly recycle α-tocopherol also does not favor a direct coupling between the reagent and α-tocopherol. The data do not rule out the possibility that the DABS-induced decrease in α-tocopherol was not direct, but secondary to oxidation by other reaction products in the membrane. However, based on the discussion in the following paragraph contrasting different sensitivities of α-tocopherol and lipid peroxidation to DABS, we favor a mechanism in which DABS directly oxidized α-tocopherol.

In these studies, we measured F_2-isoprostanes in erythrocyte ghosts as an indicator for lipid peroxidation. Isoprostanes of the F_2 class are derivatives of membrane arachidonic acid that have been shown to reflect non-enzymatic lipid peroxidation [20]. Over the same concentration range of DABS, membrane α-tocopherol was more sensitive to

depletion than was the appearance of F_2-isoprostanes (compare Fig. 1C with Fig. 5A). This suggests that lipid peroxidation proceeded only after α-tocopherol was depleted by 80% or more. This is also supported by the ability of increased intracellular ascorbate to protect against the loss of α-tocopherol (Figs. 1C and 3) and to delay the appearance of F_2-isoprostanes until higher concentrations of DABS are reached (Fig. 5B). We had shown similar results previously with the cell-impermeant oxidant ferricyanide [15], which is well known to oxidize α-tocopherol directly [21]. Together, these results suggest that intracellular ascorbate recycles the α-tocopheroxyl radical in the cell membrane. If so, one might expect ascorbate levels inside the cells to fall with or before depletion of α-tocopherol in the membrane. This was the observed effect in ghosts, which cannot recycle ascorbate. The failure of ascorbate to fall in the experiments with cells is very likely due to the ability of the cells to recycle and maintain intracellular ascorbate. In a previous study [10], we did find that 300 μM DABS induced a significant 20% decrease in intracellular ascorbate under similar conditions. Direct observation of the AFR in response to acute reaction of the cell surface with DABS (Fig. 6) also supports the notion that ascorbate is recycling α-tocopherol.

Regeneration of α-tocopherol from its free radical could occur by one of at least two mechanisms. The α-tocopheroxyl free radical could "flip-flop" from the exofacial surface of the outer membrane bilayer to the endofacial surface of the inner bilayer, and there be reduced directly by ascorbate. This mechanism has been demonstrated for simple liposomal systems in which α-tocopherol mediates electron transfer from intravesicular ascorbate to extravesicular ferricyanide [22, 23]. Assuming an initial equal distribution of α-tocopherol in each membrane face, the ability of extracellular DABS to decrease α-tocopherol by as much as 90% is compatible with a "flip-flop" mechanism. However, these results can also be explained by a mechanism in which electrons are transferred across the membrane by an oxidoreductase activity, such as that known to facilitate ferricyanide reduction by intracellular ascorbate in erythrocytes [4, 24].

In agreement with previous results [6, 10], we found that DABS treatment of erythrocytes irreversibly inhibited ferricyanide reduction by the cells (Fig. 1A), which suggests that DABS may react with the putative transmembrane oxidoreductase. In their initial use of DABS as a cell-impermeant reagent, Grebing *et al.* [6] found that ghosts prepared from cells treated with DABS had a 35% decrease in NADH-dependent ferricyanide reductase activity. This finding and the results of kinetic experiments prompted them to conclude that the activity spans the membrane and is probably mediated by a protein [6]. Although we found a larger decrease in ferricyanide reduction over the same concentration range of DABS in intact cells (Fig. 1A), our results also support the transmembrane nature of this activity. However, it is possible that the transmembrane activity involves redox cycling of α-tocopherol as a component. As noted previously, α-tocopherol can mediate transmembrane electron transfer, and we have shown that the addition of exogenous α-tocopherol to resealed human erythrocyte ghosts increases their ability to carry out ascorbate-dependent reduction of extravesicular ferricyanide [11]. A coupled protein–antioxidant transmembrane redox system is not without precedent, since it has been proposed that a ubiquinol-dependent cytochrome b_5 reductase mediates transmembrane AFR reduction in plasma membranes of the liver [25, 26].

In summary, we have found that the cell-impermeant diazo reagent DABS induces an oxidant stress when applied to the external surface of intact erythrocytes, and that this stress is manifest by loss of α-tocopherol, followed by formation of F_2-isoprostanes when α-tocopherol is depleted. The ability of intracellular ascorbate to protect against both loss of α-tocopherol and lipid peroxidation suggests that α-tocopherol is involved in the transfer of oxidant stress across the cell membrane, and that by recycling α-tocopherol, ascorbate can spare membrane lipids from peroxidative damage. This may have physiologic relevance in that ascorbate could spare α- tocopherol and membrane lipids from oxidation due to oxidized low density lipoprotein or to reactive oxygen species release from activated neutrophils [27]. Whereas DABS is a useful reagent for labeling external cell proteins or lipids, the present results suggest the need for caution, since this reagent also causes oxidant stress at the cell surface.

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