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EFFECT OF LIPID PEROXIDATION ON THE ACCESSIBILITY OF DANSYL CHLORIDE LABELING TO LIPIDS AND PROTEINS OF BOVINE MYELIN

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In our previous studies we have demonstrated that bovine myelin appears highly susceptible to oxidative damage to both its lipid and protein composition. In order to determine whether these alterations would affect the accessibility of myelin components to a fluorescent probe, we have performed various labeling experiments using dansyl chloride. Results from labeling of purified bovine myelin treated with or without cumene hydroperoxide show that basic protein from treated myelin incorporated more dansyl chloride than basic protein from untreated myelin. This increase of labeling could be prevented by the addition of the antioxdant agent, butylated hydroxytoluene. This evidence suggests that lipid peroxidation may play an important role in the pathogenesis of inflammatory demyelinating diseases.

Key words: Lipid Peroxidation; Myelin Proteins; Phospholipids; Fluorescent Probe; Demielinating Disease

INTRODUCTION

Lipid peroxidation in biological systems has long been considered to be the basis for a variety of pathological processes. Recently, the central nervous system has been the object of several studies which have focused on oxidative-induced damage. These reports conclude that, pathological experimental conditions such as brain edema, ischemia and epileptiform discharges, seem to share peroxidative reactions which mediate the development of such conditions[1,2,3]. Despite the large body of evidence on the involvement of lipid peroxidation in brain injury, little is known about the molecular mechanisms involved.

In our laboratory we have investigated the effects of chemically-induced lipid peroxidation in purified bovine myelin and found that both lipid and protein composition of myelin appears to be highly susceptible to oxidative damage[4]. We have

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also reported that erythrocytes from patients with multiple sclerosis contain higher levels of superoxide dismutase and appear more susceptible to oxidative stress than normal subjects[5]. Our results indicate that lipid peroxidation may play an important role in inflammatory demyelinating diseases. In addition, lipid-protein and lipid-lipid interactions in myelin, believed to be an important factor in its stability[6], may be altered when an oxidative challenge takes place.

The present study focuses on the accessibility of fluorescent probes in both lipids and proteins of purified bovine myelin altered by chemically induced peroxidation.

MATERIALS AND METHODS

Myelin Purification

Fresh bovine brains were obtained from a local slaughter house. The cortex was removed and the white matter was separated from the gray matter. Myelin was separated and purified by an earlier method described by Uyemura *et al.*[7]. Myelin was lyophilized and dessicated at −20 C.

Lipid Peroxidation and Dansyl-Chloride Labeling

Myelin (15 mg) was suspended in 3 ml of phosphate buffer (1 mM, pH 7.4) and aliquoted into three equal volume samples. Two of these samples were treated with cumene hydroperoxide at final concentrations of 0.5 mM and 2 mM, while the third sample was treated with an equal volume of phosphate buffer. After an incubation of 20 minutes at 37 C, the samples were washed twice with phosphate buffer and finally resuspended in 1 ml of 10 mM bicarbonate buffer (pH 8.3). The myelin suspensions were then treated with 15 ul of a stock solution of dansyl-chloride in acetone (0.5 M) and allowed to react for 1 hour at 25 C. The reaction was terminated by centrifugation of the samples. The resulting pellets were resuspended in phosphate buffer and then recentrifuged, this cycle was repeated five times. From time to time some occasional determination of lipid peroxidation, at the end of the incubation with the oxidative compound, was carried out as described earlier[2].

Analysis of Labeled and Unlabeled Myelin Proteins

Samples to be analyzed by gel electrophoresis were partially delipidated by the treatment with ether-ethanol (3:2, v:v) as described previously[8]. SDS-polyacrylamide gel electrophoresis in a discontinuous buffer system were executed with 12 percent and 7 percent gels according to the method of Laemmli[9].

Samples to be analyzed by gel exclusion chromotography were solubilized in a 7-fold weight excess of SDS and applied to a 60 cm × 2 cm column that had been packed with Sephadex G-200 and equilibrated with 0.2 percent SDS in 10 mM Tris-HCl buffer (pH 7.2) and 40 mM NaCl. The proteins were eluted with the equilibrating buffer. For spectrofluorometric measurements were carried out using a Perkin-Elmer, model 650-10 S, with emission and excitation wavelengths set at 515 nm and 340 nm, respectively. Electrophoresis gels containing dansylated proteins were excited with UV light (360 nm) and the fluorescence emission of the protein bands were photographed using a Polaroid camera equipped with a Kodak 2E filter. The polaroid negatives were finally scanned in a Hoefer scanner (GS 300) set in transmittance operational mode.

RESULTS AND DISCUSSION

Treatment of myelin with cumene hydroperoxide resulted in partial loss of the proteolipid band (Figure 1). Since lipid peroxidation could alter lipid-protein interactions, we have performed various labeling experiments using dansyl chloride in order to investigate the effect of oxdative treatment on the accessibility of this fluorescent probe to intrinsic and/or extrinsic proteins. The results of these experiments are shown in the inset of Figure 2, indicating that there is an increase in the extent of labeling of basic protein. Such increase is found to be directly correlated with the extent of treatment of myelin with oxidant since there is higher incorporation of dansyl chloride in the basic protein component of myelin treated with 2 mM oxidant than myelin treated with only 0.5 mM oxidant. The fluorescence intensity of the bands corresponding to the dansylated components of treated and untreated myelin were quantified by scanning densitometry of the negatives of gel electrophoresis photographs (Figure 2). In comparison to the basic protein, the proteolipid protein showed only a slight increase in dansyl chloride labeling upon cumene hydroperoxide

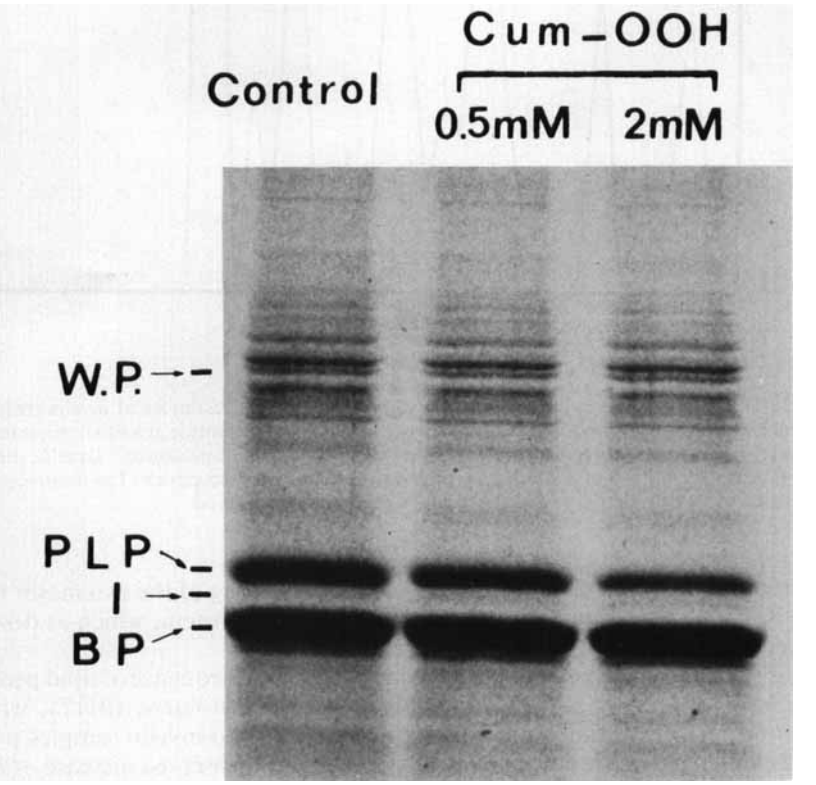

FIGURE 1 Effect of cumene hydroperoxide on myelin. Samples of myelin (1 mg/ml) were treated with or without cument hydroperoxide and subjected, prior delipidation, to electrophoresis using 12 percent polyacrylamide gels. Lane 1 corresponds to untreated myelin. Lanes 2 and 3 correspond to myelin treated with 0.5 mM and 2 mM cumene hydroperoxide. PLP, proteolipid protein; I, intermediate protein; BP, basic protein.

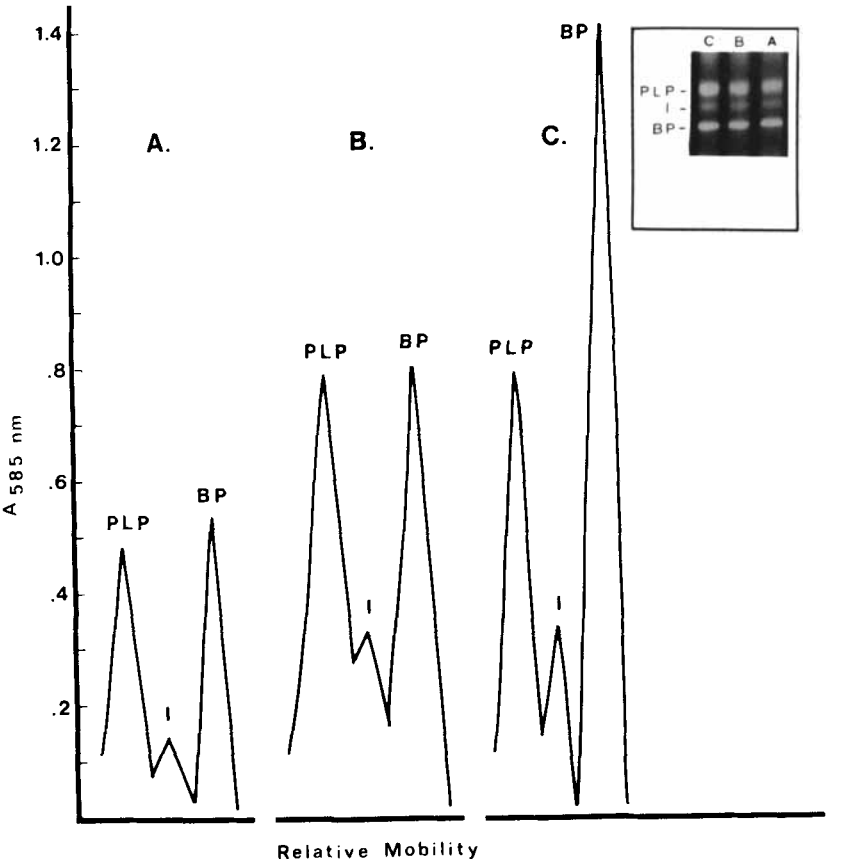

FIGURE 2 Dansyl chloride labeling of treated and untreated myelin. Samples of myelin treated with 0.5 mM and 2 mM of cumene hydroperoxide were reacted with dansyl chloride at a final concentration of 7.5 mM and then subjected to electrophoresis in 7 percent polyacrylamide gels (inset). Lane A, untreated. Lanes B and C treated with 0.5 mM and 2 mM cumene hydroperoxide, respectively. The fluorescent bands were quantified by densitometric scan as described in materials and methods.

treatment of myelin. The difference in the extent of labeling of the two major myelin proteins is most likely due to the loss of the proteolipid protein, which as described above, occurs in the course of oxidative treatment.

To see if the observed increase in protein labeling is a consequence of lipid peroxidation, we have added the anti-oxidant butylated hydroxytoluene (BHT), which is known to protect membrane lipids from peroxidation[10], to myelin samples prior to oxidative challenge. The presence of BHT prevented the observed increase of dansyl chloride incorporation (Figure 3). Addition of 12 mM of BHT to untreated myelin did not have any effect on the extent of dansyl chloride labeling (data not shown).

In order to verify whether lipid peroxidation induced any changes in the accessibility of dansyl chloride to phospholipids, extracts of the ether-ethanol delipidation system were spotted on silica gel TLC plate and developed in chloroform/methanol/isopropanol (8.5:0.5:1, v:v). Two fluorescent spots were

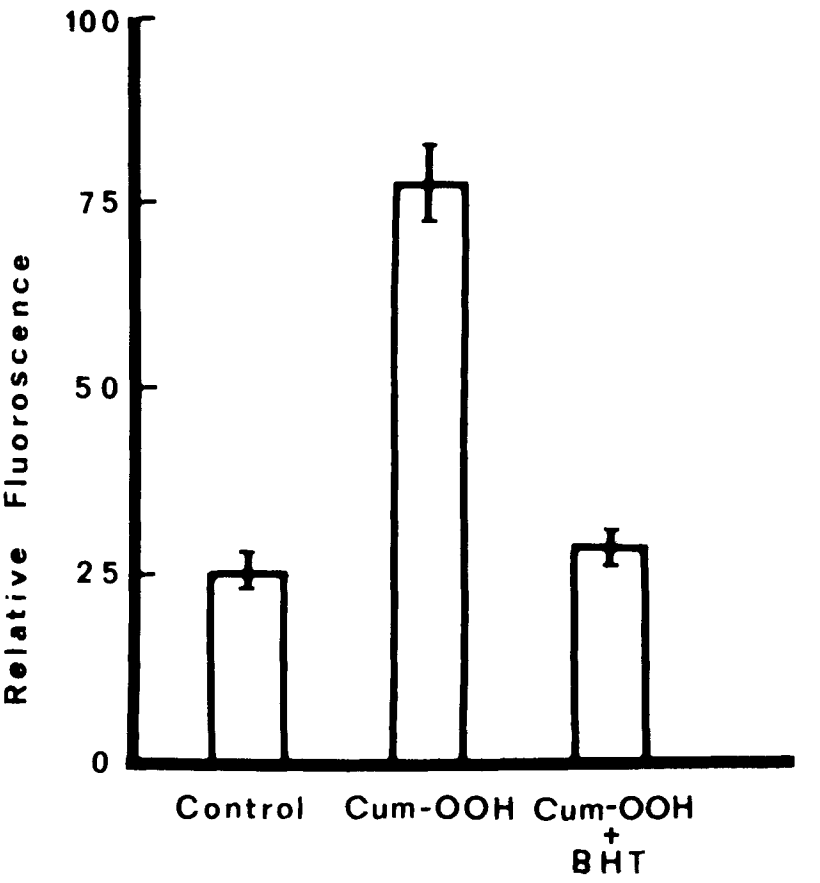

FIGURE 3 Effect of BHT on dansyl chloride labeling of cumene hydroperoxide treated myelin. Untreated myelin and treated myelin in the presence and absence of BHT were analyzed in 7 percent polyacrylamide gel electrophoresis. The fluorescent bands corresponding to the basic protein, as detected by UV excitation, were excised and transferred to tubes containing 4 ml of buffer. After an incubation period of 24 hours, the buffer was quantitatively transferred from each tube to a cuvette and its fluorescence measured. Bar 1, untreated myelin. Bar 2, 2 mM cumene hydroperoxide treated myelin. Bar 3, 2 mM cumene hydroperoxide treated myelin in the presence of 12 mM BHT.

observed for each treatment condition: one spot, which we have indentified as dansyl-phosphotidylethanolamine, migrated close to the solvent front and was intensely fluorescent, while the other spot, corresponding to dansyl-phosphotidylserine, was less mobil and less fluorescent. To quantitate the fluorescence from each spot corresponding to treated and untreated myelin, the silica gel corresponding to the fluorescent spots was scraped from the plate, transfered to test tubes and extreacted with chloroform. The obtained fluorescence intensity measurements revealed no change in phospholipid labeling upon oxidative challenge.

In all of our labeling experiments on myelin we have observed the presence of fluorescence in both basic protein and proteolipid protein; these results are in disagreement with the work of Crang and Rumsby[11], in which they show that only the

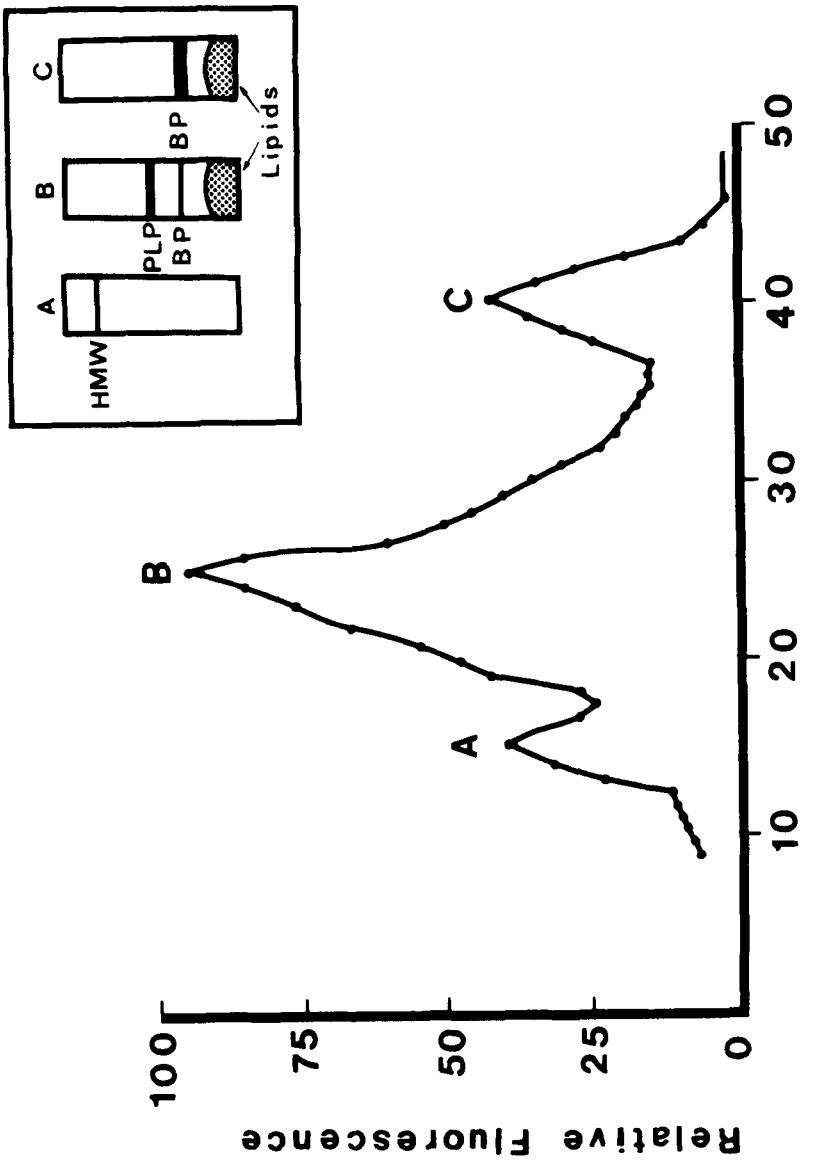

FIGURE 4 Elution pattern of dansyl chloride labeled myelin. Non-delipidated myelin, labeled with dansyl chloride, was separated on Sephadex G-200 column. The fluorescently labeled myelin components eluted into three peaks. Analysis of each peak was carried out by 7 percent polyacrylamide gel electrophoresis (inset).

proteolipid protein is labeled by dansyl chloride. In an attempt to reconcile our data with those of the afore mentioned group, we have separated untreated myelin, which had been labeled with dansyl chloride, by exclusion column chromatography as described by Crang *et al.*[11]. The fluorescence elution pattern which is composed of three separate peaks is shown in Figure 3. After pooling the fractions into the respective peaks, gel electrophoresis analysis of each peak was carried out (inset Figure 3). The first peak to elute off the column was found to be mostly high molecular weight material; the second peak was a mixture of proteolipid protein, some basic protein and phospholipids; the third peak was mostly basic protein and phospholipids. The relative ratio of dansyl incorporation into the myelin components was found to remain approximately the same, even when smaller concentration of dansyl chloride were used in the labeling procedure. These results indicate that basic protein is one of the components to be labeled by dansyl chloride. In addition, it has been reported that salicylaldehyde, a membrane-permeant probe, can label all the myelin proteins in intact myelin as well as in isolated myelin[12].

Thus, lipid peroxidation, in inducing the well known alterations in the lipid bilayer, also would facilitate the discovery of bouried potential reacting sites of basic protein to be labeled. A similar finding was observed in myelin from patients with acute multiple sclerosis, in which basic protein was more accessible to a non-penetrating agent than in myelin from normal subjects[13]. It is tempting now to speculate that lipid peroxidation can be one of the most likely candidate in the pathogenesis of the acute demyelinating disease.

Acknowledgment

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