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Detection of oxidized lipid-modified erythrocyte membrane proteins by radiolabeling with tritiated borohydride

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Human erythrocyte ghosts treated with *tert*-butyl hydroperoxide or ADP-Fe^{3+} incorporated radioactivity on reduction with tritiated borohydride. The tritium incorporation closely correlated with membrane lipid oxidation as assessed by the formation of thiobarbituric acid-reactive substances and fluorescent substances. Treatment of ghosts with the inducers in the presence of butylated hydroxytoluene, thiourea, or desferrioxamine suppressed the tritium incorporation in the subsequent reduction. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the tritiated ghost proteins showed that the label was incorporated into the intermolecularly cross-linked and the uncross-linked proteins of bands 1, 2, 3, 4.1, 4.2, 5 and 6, and into the noncross-linked glycophorin A (PAS-1). Glycophorin A was hardly cross-linkable but modified during membrane lipid oxidation. Possible candidates for producing borohydride-reducible functions in the proteins are various mono- and bifunctional aldehydes, as well as those for producing fluorescence and cross-links. A part of thiobarbituric acid-reactive or fluorescent substances may be involved in borohydride reduction and tritium labeling.

Introduction

Lipid oxidation in biological systems has been implicated in oxidative damage of cells and tissues which may be relevant to some pathological processes and aging [1–3]. Membranes are considered to be the major sites of the deteriorative effects of lipid oxidation owing to their high content of polyunsaturated fatty acids [4]. Lipid hydroperoxides arising in the membrane from the polyunsaturated fatty acids decompose to produce reactive lipid radicals and secondary products like aldehydes [5,6]. The reactive species thus generated react with the adjacent membrane components, i.e., proteins and phospholipids, which may eventually lead to membrane damage [1,7]. It is important to identify the membrane proteins modified with the oxidized lipids, since they play diverse roles in supporting the structure and function of the membrane.

It has been suggested that proteins exposed to oxidized lipids undergo modifications such as protein-protein cross-linking, protein-lipid adducts, amino acid damage and fluorescence formation [1,2,7–10]. The modifications occurring in membrane proteins can be detected only by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis for intermolecular protein cross-linking [2,10–13], where the cross-linked proteins are identified by loss of the original protein bands and the appearance of high molecular weight bands. In view of detecting the membrane pro-

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teins modified with oxidized lipids sensitively, irrespective of intermolecular cross-link formation, we tried to label the modified proteins by the use of tritiated borohydride. In this paper, we demonstrate that human erythrocyte membrane proteins incorporated tritium by tritiated borohydride reduction when lipid oxidation was induced in the membrane, and that tritium was incorporated into not only intermolecularly cross-linked proteins but also into noncross-linked proteins.

Materials and Methods

Materials. *tert*-Butyl hydroperoxide (*t*-BuOOH) and ADP monopotassium salt were purchased from Nakarai Chemicals, Ltd., Kyoto and Oriental Yeast Company, Ltd., Tokyo, respectively. Butylated hydroxytoluene and desferrioxamine ('Desferal') were from Nikki Universal Company, Ltd., Tokyo and Ciba-Geigy, Basel, respectively. *trans,trans*-2,4-Decadienal was obtained from Aldrich Chemical Company, Milwaukee WI. Malonaldehyde was obtained as the sodium salt according to the method of Marnett and Tuttle [14] with slight modifications as previously described [15]. 13-Linoleic acid hydroperoxide (13-LOOH) was prepared according to the method of Gardner [16] by the use of linoleic acid (Nippon Oil and Fats Company, Ltd., Tokyo) and soy bean lipoxygenase (Lipoxidase Type I, Sigma Chemical Company, St. Louis). Bovine serum albumin (fatty acid free) was from Sigma. Hemoglobin (CO form) was purified from the human erythrocyte lysate by CM Sephadex C-50 ion exchange column chromatography as described previously [17]. NaB^3H_4 (solid, 10 Ci/mmol) from Amersham International Bucks. was dissolved in 0.01 M NaOH, and stored frozen at −80°C under nitrogen gas. All other chemicals were reagent grade products of Wako Pure Chemical Industries, Ltd., Osaka.

Human erythrocyte ghost preparations. Human venous blood was collected from a healthy donor using citrate/phosphate/dextrose as an anticoagulant, and stored at 4°C for use within a few days. The blood was centrifuged to remove plasma and buffy coats. Erythrocytes were washed four times with isotonic saline, and the ghosts were isolated according to the method of Dodge et al. [18] using 5 mM phosphate buffer (pH 8.0) for hypotonic hemolysis and wash medium. Ghosts were stored at −20°C in aliquots for use. Hemoglobin content of the ghosts was 1–3% of the total proteins as determined by the pyridine hemochromogen method [18]. Protein concentrations were determined by the method of Lowry et al. [19].

Induction of lipid oxidation in ghosts. A suspension of ghosts (2.5 mg protein/ml) was incubated with various concentrations of *t*-BuOOH in 0.1 M phosphate buffer (pH 7.0) at 37°C for 1 h in the presence of hemoglobin (2.5 μM, CO form) added as a catalyst. The ghosts were washed five times by centrifugation (11 000 × *g*, 20 min) at 4°C in 5 mM phosphate buffer (pH 7.0) and resuspended in 0.1 M phosphate buffer (pH 7.0) at 3–4 mg protein/ml. For the inhibition of lipid oxidation, the ghost suspensions (2.5 mg protein/ml) with the added hemoglobin was preincubated with 0.05 mM butylated hydroxytoluene, 30 mM thiourea, or 0.5 mM desferrioxamine at 4°C for 30 min, followed by incubation with 0.1 mM *t*-BuOOH at 37°C for 1 h, and processed as described above. For lipid oxidation induced with ADP-Fe^{3+}, the ghost suspension without additional hemoglobin was incubated with a mixture of ADP and $FeCl_3$ (molar ratio 17 : 1) in 0.1 M borate buffer (pH 7.2) at 37°C for 5 h.

Modification of ghosts with aldehydes. A suspension of ghosts (2.5 mg protein/ml) was incubated with various aldehydes in 0.1 M phosphate buffer (pH 7.0) at 37°C for 1 h. The modified ghosts were washed by centrifugation and resuspended in the same buffer.

Modification of bovine serum albumin with 13-LOOH. Bovine serum albumin (10 mg/ml) was incubated with 13-LOOH (0.001–1 mM) in0.1 M phosphate buffer (pH 7.0) at 37°C for 23 h. The modified protein was recovered by gel filtration through a Sephadex G-25 column equilibrated with the same buffer.

Separation of proteins and lipids of the modified ghosts. Proteins and lipids of the modified ghosts were separated by extracting the total lipid according to the method of Folch et al. [20]. A suspension of the ghosts (0.5 ml) was mixed with 20 vol. of chloroform/methanol (2 : 1, v/v), and centrifuged (1500 × *g*, 20 min) to separate into the clear supernatant and the insoluble residue (pro-

tein fraction). The protein residue was solubilized in 5% SDS/10 mM sodium phosphate buffer (pH 7.0). Sonication was sometimes required for the complete solubilization. The supernatant was washed with 0.2 vol. of water, centrifuged, and the lower phase was obtained (lipid fraction). In this procedure, protein recovery was usually more than 80%, and the protein composition in the ghosts was little affected as determined by SDS-polyacrylamide gel electrophoresis.

Measurement of lipid oxidation A. Formation of thiobarbituric acid-reactive substances. Lipid oxidation of the ghosts was assessed by measuring thiobarbituric acid-reactive substances according to the method of Buege and Aust [21]. Briefly, 1 ml of a washed ghost suspension was mixed with 2.0 ml of a solution of 0.375% thiobarbituric acid/15% thiochloroacetic acid/0.25 M HCl containing 0.01% butylated hydroxytoluene, and the mixture was heated at 100°C for 15 min. After cooling, the mixture was centrifuged and the absorbance at 532 nm of the supernatant was measured. The amount of thiobarbituric acid-reactive substances was expressed as the absorbance per mg of protein. Thiobarbituric acid-reactive substances were also released into the reaction medium during lipid oxidation, and their increase paralleled those of the washed ghosts.

B. Formation of fluorescence. Formation of fluorescent substances was assessed as another index of membrane lipid oxidation [22]. For measurement of the fluorescence of whole ghost and its protein fraction, they were solubilized in 5% SDS/10 mM sodium phosphate buffer (pH 7.0). Lipid fractions of the ghosts were clarified by the addition of 0.1 vol. of methanol before fluorescence measurement. Fluorescence intensities of the ghosts treated with *t*-BuOOH or ADP-Fe^{3+}, and their protein and lipid fractions were measured at an excitation maximum of 356 nm and an emission maximum of 434 nm. Excitation and emission maxima of the aldehyde-modified ghosts were 350–362 nm and 428–441 nm, respectively, except those of malonaldehyde-modified ghosts, which were 395 nm and 463 nm, respectively. The intensities of the aldehyde-modified ghosts were measured at the respective maximum wavelengths. The relative fluorescence intensities of the ghosts and the proteins were expressed against 0.1 μM quinine sulfate for the protein concentration of 10 mg/ml, and those of the ghost lipids were presented for their concentration contained in the solubilized ghosts at 10 mg protein/ml.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis of the ghost proteins was performed in the discontinuous buffer system of Laemmli [23] with a 4% stacking gel and 7.5% separating gel. Ghosts were solubilized in the Laemmli's sample buffer. Phenylmethylsulfonyl fluoride (1 mM) was included in the buffer to prevent the degradation of the proteins by proteinases. After electrophoresis, proteins and sialoglycoproteins were visualized by Coomassie brilliant blue R-250 staining and periodic acid-Schiff (PAS) staining [24], respectively. Samples containing 25 μg protein were applied for Coomassie blue staining, and 75 μg protein for PAS staining. Major protein and sialoglycoprotein bands are numbered according to Steck [25].

Tritium labeling by tritiated borohydride reduction. Immediately after the preparation of oxidized ghosts or aldehyde-modified ghosts, the ghost suspension (2–5 mg protein/ml) in 0.1 M phosphate buffer (pH 7.0) was mixed with an equal vol. of 2 mM NaB^3H_4 (diluted to 1.65 Ci/mmol with unlabeled $NaBH_4$ before use) in 0.01 M NaOH, and allowed to stand at room temperature for 90 min. The ghost suspension was dialyzed extensively against 5 mM phosphate buffer/0.15 M NaCl (pH 7.0) at 4°C until free radioactivity was removed to the background level, and was solubilized in 76 mM Tris-HCl containing 2.4% SDS (pH 6.8) Separation of proteins and lipids of the labeled ghosts were carried out by mixing an aliquot of the SDS-solubilized labeled ghosts with 10 vols. of an unlabeled carrier ghost suspension (5 mg protein/ml) followed by fractionation into protein and lipid fractions as described above. Radioactivities of the SDS-solubilized whole ghost and protein and lipid fractions were measured in a toluene-Triton X-100 scintillation cocktail. For measurement of radioactivities of the lipid fractions, the solvent was evaporated and the lipid residue was dissolved in the scintillation cocktail and counted. Approx. 80% of the radioactivity in whole ghost was recovered in the protein and lipid fractions. The data were presented for the radioactivities of whole ghost and its protein and lipid

fractions obtained from the same amount of ghosts. Reduction of the modified bovine serum albumin with 1 mM NaB^3H_4 (1.65 Ci/mmol) was performed similarly.

SDS-polyacrylamide gel electrophoresis of the labeled ghosts was carried out as described above. After electrophoresis, the gel was fixed in 10% trichloroacetic acid, rinsed in water and treated with ENHANCE (New England Nuclear, Boston, MA) for fluorographic detection of the radioactive protein bands according to the manufacturer's specifications. The treated gel was dried onto Whatman 3 MM paper at 60–70°C in vacuo, then exposed to Kodak X-OMAT AR film at −80°C.

Results

Tritium incorporation into oxidized ghosts by reduction with tritiated borohydride

Lipid oxidation of human erythrocyte ghosts was induced by incubating the ghosts with various concentrations of *t*-BuOOH in the presence of hemoglobin (2.5 μM) at 37°C for 1 h under aerobic conditions. Presence of hemoglobin was necessary to catalyze the reaction in the hydroperoxide-induced lipid oxidation of ghosts [13,26]. After washing, the extent of lipid oxidation of the ghosts was assessed by thiobarbituric acid reaction and fluorescence formation. Both thiobarbituric acid-reactive substances and fluorescent substances in the ghosts significantly increased at *t*-BuOOH concentrations above 0.1 mM (Fig. 1A and B). The protein and the lipid fractions of the ghosts showed similar increase in fluorescence (Fig. 1B). Lipid oxidation took place in the ghosts, and the membrane proteins and lipids were modified under the conditions described. The oxidized ghosts were treated with NaB^3H_4. The incorporation of radioactivity into the ghosts and the protein and the lipid fractions significantly increased at *t*-BuOOH concentrations above 0.1 mM (Fig. 1C), which was in good agreement with the extent of lipid oxidation. Fig. 2A shows time-dependent increase in lipid oxidation of the *t*-BuOOH-treated ghosts as assessed by fluorescence formation. ^3H incorporation into the ghosts by reduction with NaB^3H_4 also increased dependent on the time of exposure to *t*-BuOOH (Fig. 2B).

Formation of the thiobarbituric acid-reactive substances and fluorescence substances were suppressed by an antioxidant butylated hydroxytoluene, a hydroxyl radical scavenger thiourea, and a potent iron chelator desferrioxamine to the level of the unoxidized control ghosts (Table I). The ghosts treated with *t*-BuOOH in the presence of these compounds were subjected to NaB^3H_4 reduction, and the radioactivities incorporated into the ghosts were as low as that of the unoxidized control ghosts (Table I).

When lipid oxidation of ghosts was induced by ADP-Fe^{3+}, an effective catalyst of lipid oxidation [12,27], the oxidized ghosts incorporated radioactivity on reduction with NaB^3H_4, which is in accordance with the increase of lipid oxidation (Fig. 3). The presence of the inhibitors of lipid oxidation in the ADP-Fe^{3+} treatment suppressed ^3H incorporation (Table II).

The close correlation between the lipid oxida-

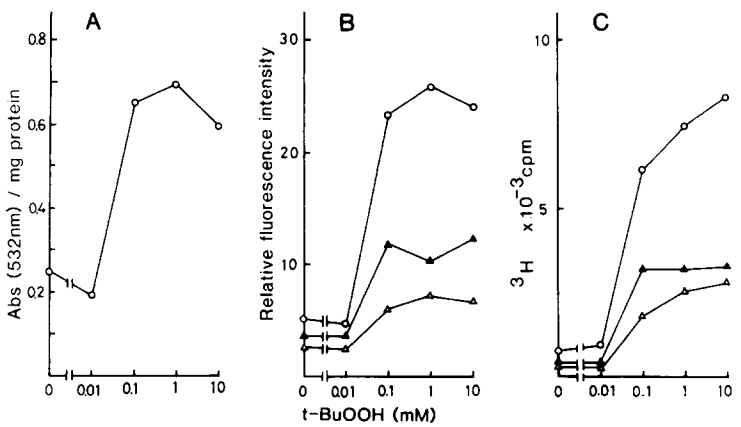

Fig. 1. Incorporation of tritium into the ghosts oxidized with *t*-BuOOH. Ghosts were incubated with the indicated concentrations of *t*-BuOOH in the presence of 2.5 μM hemoglobin at 37°C for 1 h, followed by reduction with NaB^3H_4. Thiobarbituric acid-reactive substances (A), fluorescence (B) and ^3H incorporation (C) were measured for the oxidized ghosts (○) and their protein (△) and lipid (▲) fractions. Radioactivities are expressed for the ghosts containing 1 μg protein.

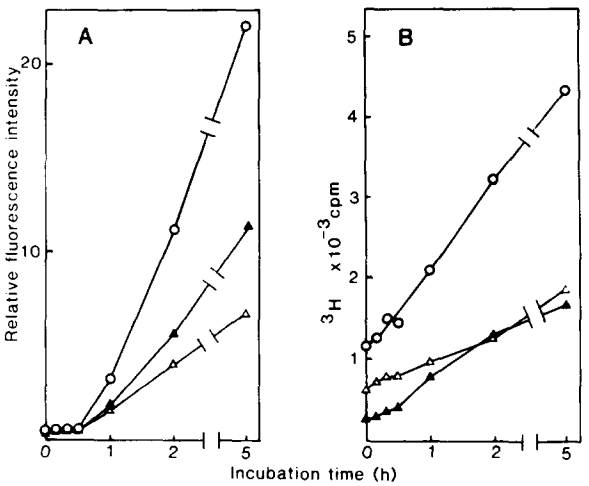

Fig. 2. Effect of incubation time of ghosts with *t*-BuOOH on tritium incorporation. Ghosts were incubated with 0.1 mM *t*-BuOOH in the presence of 2.5 μM hemoglobin at 37°C for the indicated periods. Butylated hydroxytoluene (0.5 mM) was added to terminate the reaction. The ghosts were then reduced with NaB^3H_4. Fluorescence (A), and ^3H incorporation (B) were measured for the oxidized ghosts (○) and their protein (△) and lipid (▲) fractions.

tion of ghosts treated with *t*-BuOOH and ADP-Fe^{3+} and the ^3H incorporation into the modified ghosts indicates that NaB^3H_4 reduced and labeled certain particular functions formed in the membrane protein and lipid fractions during lipid oxidation.

Identification of the tritium-labeled ghost proteins

We analyzed the ^3H-labeled ghost proteins by SDS-polyacrylamide gel electrophoresis in order to identify the proteins modified during lipid oxidation. Fig. 4A shows the Coomassie blue-stained proteins of the *t*-BuOOH-treated ghosts. There were no significant changes in the protein pattern of the ghosts treated with 0.01 mM *t*-BuOOH (lane b). At the *t*-BuOOH concentrations higher than 0.1 mM, the original proteins bands 1, 2, 3, 4.1, 4.2, 5 and 6 disappeared, and intermolecular protein cross-linking was observed as indicated by the formation of high molecular weight proteins at the top of the 4% stacking gel and near the top of the 7.5% separating gel (lanes c–e). The formation of intermolecularly cross-linked proteins depends on the degree of lipid oxidation of the ghosts. A similar effect of *t*-BuOOH on the cross-linking of erythrocyte membrane proteins has been observed previously [11].

Fig. 4B shows the PAS stained sialoglycoproteins of the *t*-BuOOH-treated ghosts. PAS-1 and PAS-2 sialoglycoproteins diminished, but a major part of them remained at the original position, and only small amounts of intermolecularly cross-linked products were observed at the top of the stacking gel (lanes c–e). It has been shown that the component observed in PAS-1 is the dimeric form of glycophorin A and the major component in PAS-2 is the monomeric form of

TABLE I

TRITIUM INCORPORATION INTO THE GHOSTS OXIDIZED WITH *t*-BuOOH IN THE PRESENCE OF THE INHIBITORS OF LIPID OXIDATION

Ghosts were incubated with 0.1 mM *t*-BuOOH and 2.5 μM hemoglobin at 37°C for 1 h in the presence or absence of the inhibitors of lipid oxidation, followed by reduction with NaB^3H_4. Thiobarbituric acid-reactive substances, fluorescence and ^3H incorporation were measured for the oxidized ghosts. Data are expressed as a percentage of the values of the *t*-BuOOH-treated ghosts in the absence of the inhibitors.

Ghosts incubated with:	Lipid oxidation		^3H incorporation
	thiobarbituric acid-reactive substances	fluorescence intensity	
t-BuOOH	100	100	100
t-BuOOH in the presence of			
butylated hydroxytoluene (0.05 mM)	9.9	9.1	14.4
thiourea (30 mM)	14.7	20.2	22.3
desferrioxamine (0.5 mM)	8.1	10.1	16.8
Control	5.5	9.1	19.0

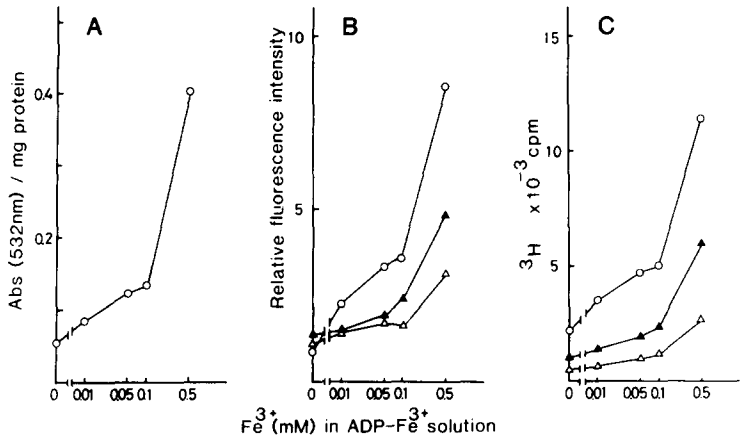

Fig. 3. Incorporation of tritium into the ghosts oxidized with ADP-Fe^{3+}. Ghosts were incubated with the indicated concentrations of Fe^{3+} chelated with ADP (ADP to Fe^{3+} molar ratio, 17:1) at 37°C for 5 h, followed by reduction with NaB^3H_4. Thiobarbituric acid-reactive substances (A), fluorescence (B) and ^3H incorporation (C) were measured for the oxidized ghosts (○) and their protein (△) and lipid (▲) fractions. Radioactivities are expressed for the ghosts containing 10 μg protein.

glycophorin A [28,29]. From PAS staining of the gel it appears that most of these sialoglycoproteins were much less cross-linked by lipid oxidation.

When the ghosts modified with *t*-BuOOH were reduced with $NaBH_4$, the electrophoretic patterns of the proteins were the same as those shown in Fig. 4A and B. The modified ghosts were reduced with NaB^3H_4 and the ^3H-labeled proteins were visualized by fluorography following SDS-polyacrylamide gel electrophoresis (Fig. 4C). The radioactivities in the control ghosts and the ghosts treated with 0.01 mM *t*-BuOOH were too weak to exhibit visual bands (lanes a and b, respectively). In contrast, the ghosts treated with *t*-BuOOH at concentrations above 0.1 mM exhibited highly radioactive protein bands (lanes c–e). The high molecular weight cross-linked protein bands at the top of the 4% stacking gel and the 7.5% separating gel were highly labeled with ^3H. The Coomassie blue-stained proteins corresponding to bands 1, 2, 3, 4.1, 4.2, 5 and 6 were labeled in significant intensity. The PAS-stained sialoglycoproteins corresponding to PAS-1 and PAS-2 were highly labeled. The unidentified low molecular weight proteins at the gel front, and lipids running faster than the gel front were also intensely labeled. A similar distribution of the radioactivity in the gel was obtained when the gel was sliced, and the radioactivity of each gel slice was counted. These results indicate that lipid oxidation of ghosts in-

TABLE II

TRITIUM INCORPORATION INTO THE GHOSTS OXIDIZED WITH ADP-Fe^{3+} IN THE PRESENCE OF THE INHIBITORS OF LIPID OXIDATION

Ghosts were incubated with ADP-Fe^{3+} (1.7 mM ADP, 0.1 mM $FeCl_3$) at 37°C for 5 h in the presence or absence of the inhibitors of lipid oxidation, followed by reduction with NaB^3H_4. Thiobarbituric acid-reactive substances, fluorescence and ^3H incorporation were measured for the oxidized ghosts. Data are expressed as a percentage of the values of the ADP-Fe^{3+}-treated ghosts in the absence of the inhibitors.

Ghosts incubated with:	Lipid oxidation		^3H incorporation
	thiobarbituric acid-reactive substances	fluorescence intensity	
ADP-Fe^{3+}	100	100	100
ADP-Fe^{3+} in the presence of			
butylated hydroxytoluene (0.05 mM)	36.1	15.9	37.2
thiourea (30 mM)	41.4	31.6	57.3
Control	39.8	26.5	43.3

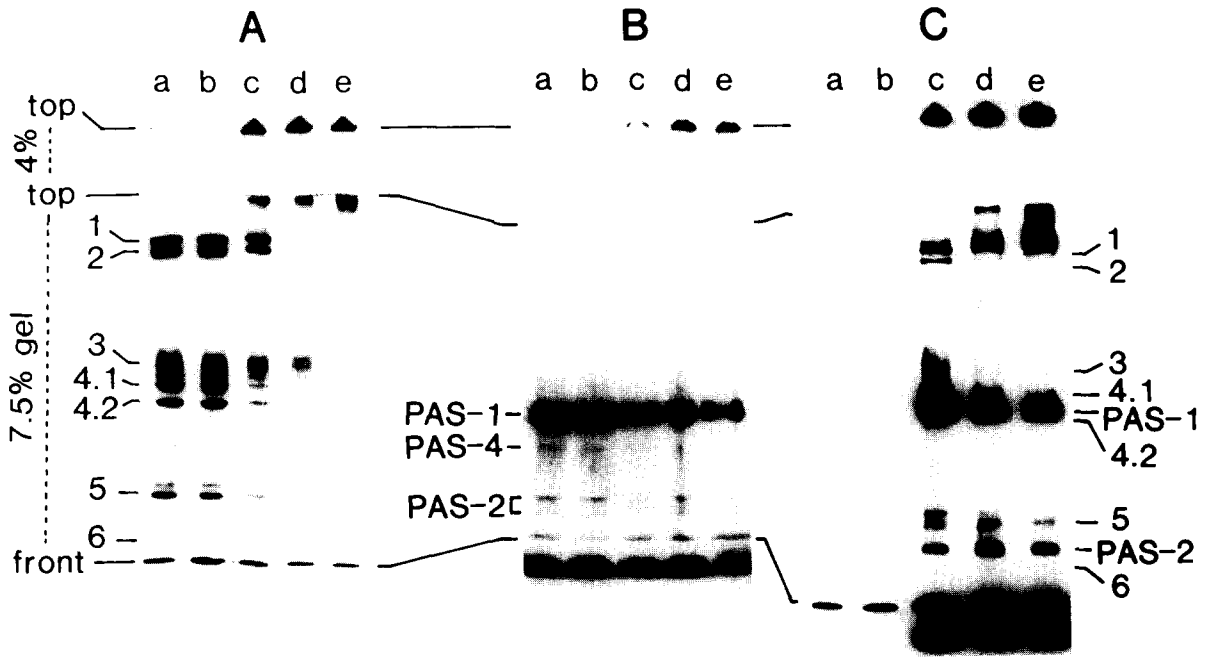

Fig. 4. SDS-polyacrylamide gel electrophoresis of the ghosts oxidized with *t*-BuOOH (A and B) and the oxidized ghosts reduced with tritiated borohydride (C). Ghosts were incubated with *t*-BuOOH, and then reduced with NaB^3H_4 as described in the legend to Fig. 1. (A) Coomassie blue staining of the *t*-BuOOH-treated ghosts. (B) PAS staining of the *t*-BuOOH-treated ghosts. (C) Fluorography of the *t*-BuOOH-treated ghosts reduced with NaB^3H_4. a, b, c, d and e represent the ghosts incubated with 0, 0.01, 0.1, 1 and 10 mM *t*-BuOOH, respectively. Positions corresponding to the major protein and sialoglycoprotein bands are indicated. The band running faster than the gel front corresponds to lipids [25].

duced by *t*-BuOOH resulted in the formation of not only the intermolecularly cross-linked proteins but the modified proteins which were not cross-linked. It is likely that most of the membrane proteins were intermolecularly cross-linked and labeled, and glycophorin A was not cross-linked but was modified and labeled.

Fig. 5 shows SDS-polyacrylamide gel electrophoretic patterns of the ADP Fe^{3+}-treated ghosts. At the ADP-Fe^{3+} concentrations of 8.3 mM-0.5 mM, where significant increases in lipid oxidation and ^3H incorporation were observed (see Fig. 3), formation of the intermolecularly cross-linked proteins was very small (Figs. 5A and B, lanes b and c). While the radioactivity was incorporated into the intermolecularly cross-linked bands, the noncross-linked bands 1, 2, 3, 4.1, 4.2, 5, PAS-1 and PAS-2 were labeled similarly or more intensely (Fig. 5C lane c). The radioactivity of PAS-1 was most prominent. It was found that most of the proteins in the ghosts treated with ADP-Fe^{3+} were modified without being crosslinked.

These results demonstrate that incorporation of ^3H by reduction with NaB^3H_4 is a good measure for the modification of ghost proteins with oxidized lipids.

Possible candidates producing borohydride-reducible functions in the ghost proteins

In order to discover whether lipid hydroperoxides can modify proteins to produce borohydride-reducible functions, bovine serum albumin was treated with 13-LOOH (0.001–1 mM) at 37°C for 23 h and subjected to NaB^3H_4 reduction. No intermolecularly cross-linked proteins were formed in the modification. A concentration-dependent increase of ^3H incorporation was observed, and SDS-polyacrylamide gel electrophoresis of the labeled protein followed by fluorography gave a single radioactive band of the protein. It is evident that the functions that can be reduced and labeled with NaB^3H_4 are formed in proteins

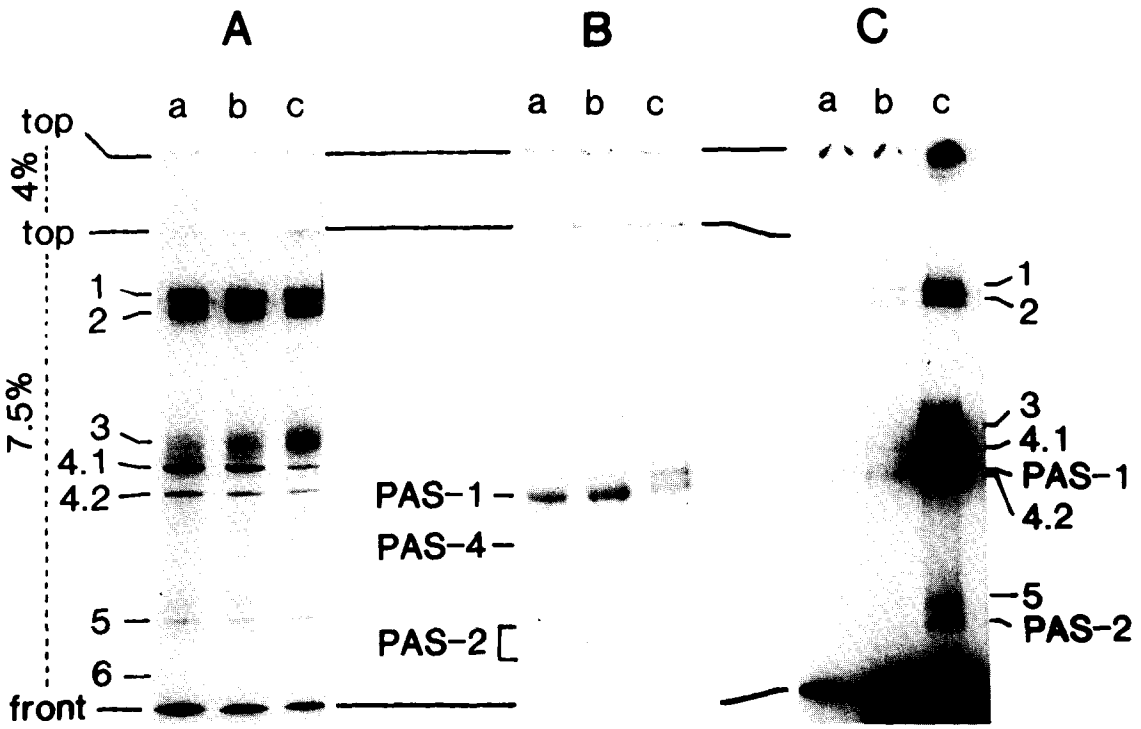

Fig. 5. SDS-polyacrylamide gel electrophoresis of the ghosts oxidized with ADP-Fe^{3+} (A and B) and the oxidized ghosts reduced with tritiated borohydride (C). Ghosts were incubated with ADP-Fe^{3+}, and then reduced with NaB^3H_4 as described in the legend to Fig. 3. (A) Coomassie blue staining of the ADP-Fe^{3+}-treated ghosts. (B) PAS staining of the ADP-Fe^{3+}-treated ghosts. (C) Fluorography of the ADP-Fe^{3+}-treated ghosts reduced with NaB^3H_4. a, b and c represent the ghosts incubated with 0, 0.1 and 0.5 mM Fe^{3+} chelated with 0, 1.7 and 8.3 mM ADP, respectively. Positions corresponding to the major protein and sialoglycoprotein bands are indicated.

treated with lipid hydroperoxides.

Lipid hydroperoxides readily degrade into the secondary products including aldehydes [5,6]. We investigated the reaction of the aldehydes with ghosts as possible candidates for the reactive species producing the borohydride-reducible functions in the ghost proteins during lipid oxidation. The aldehydes used were saturated monofunctional aldehydes including acetaldehyde, hexanal and heptanal, an unsaturated monofunctional aldehyde 2,4-decadienal, and bifunctional aldehydes, malonaldehyde and glutaraldehyde. Ghosts were incubated with 0.5–50 mM concentrations of each of the aldehydes at 37°C for 1 h, and the modified ghosts were analyzed for intermolecular protein cross-linking and fluorescence formation. The concentrations of the aldehydes were extremely different for significant or extensive formation of cross-links. These aldehydes at the concentrations indicated in Fig. 6A produced intermolecularly cross-linked proteins. Marked fluorescence was observed in the ghosts treated with any of these aldehydes at the indicated concentrations (Table III). There was no correlation of the abilities of these aldehydes in cross-linking the ghost proteins and fluorescence formation. Malonaldehyde produced much more fluorescence than did the other aldehydes, but its cross-linking ability was not more than that of the others. Reduction of the modified ghosts with NaB^3H_4 resulted in ^3H incorporation into the whole ghost, and its protein and lipid fractions (Table III). Monofunctional aldehydes such as hexanal, heptanal and 2,4-decadienal were effective in ^3H incorporation. Incorporation of the radioactivity into the malonaldehyde-modified

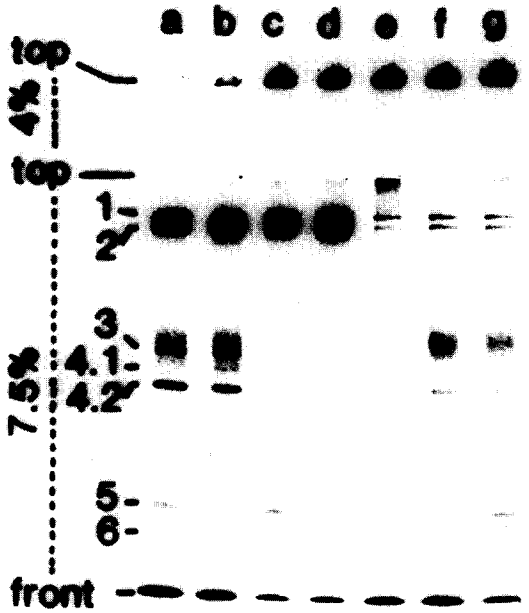

Fig. 6. SDS-polyacrylamide gel electrophoresis of the aldehyde-modified ghosts. Ghosts were incubated with various aldehydes at 37°c for 1 h, electrophoresed and proteins were stained with Coomassie blue. a, control; b, 50 mM acetaldehyde; c, 50 mM hexanal; d, 25 mM heptanal; e, 0.5 mM 2,4-decadienal; f, 25 mM malonaldehyde; g, 0.5 mM glutaraldehyde.

ghosts was much less in spite of their extensive protein cross-linking and prominent fluorescence.

It is possible that the mixture of various aldehydes produced in lipid oxidation of ghosts would form borohydride-reducible functions by reaction with the membrane proteins. The borohydride-reducible functions may be comprised of many compounds with different structures. It seems likely that the structures for the borohydride-reducible functions are not always identical with those for the cross-links and fluorophores produced by the same mixture of the aldehydes.

Effects of borohydride reduction of the oxidized ghosts on their thiobarbituric acid reactivity and fluorescence intensity were examined. Thiobarbituric acid-reactive substances in the *t*-BuOOH-treated ghosts were destroyed by 17% on reduction with 1 mM $NaBH_4$. Fluorescent substances in the ghosts, and their lipid and protein fractions were also destroyed by 24%, 22% and 26%, respectively. These results suggest that a part of the functions susceptible to NaB^3H_4 reduction and radiolabeling involves thiobarbituric acid-reactive substances and fluorescent substances.

Discussion

The present study has shown a close correlation between the erythrocyte membrane lipid oxidation and the formation of borohydride-reducible functions in the membrane proteins and has allowed us to detect the proteins modified with oxidized lipids by radiolabeling with ^3H by the use of NaB^3H_4.

TABLE III

INCORPORATION OF TRITIUM INTO THE ALDEHYDE-MODIFIED GHOSTS

Ghosts were incubated with various aldehydes at 37°C for 1 h, followed by reduction with NaB^3H_4. Fluorescence and ^3H incorporation were measured for the modified ghosts.

Aldehydes	(mM)	Relative fluorescence intensity of ghosts [a]	Incorporation of ^3H (cpm) ($\times 10^{-3}$) into:		
			ghosts [b]	lipid fraction [b]	protein fraction [b]
Control		2.8	88	65	27
Acetaldehyde	(50)	9.7	104	56	29
Hexanal	(50)	14.4	325	201	90
Heptanal	(25)	45.6	255	142	55
2,4-Decadienal	(0.5)	22.3	270	112	80
Malonaldehyde	(25)	233.7	98	56	41
Glutaraldehyde	(0.5)	10.0	281	90	71

[a] Ghost concentration of 10 mg protein/ml.

[b] Radioactivities in the ghosts containing 1 mg protein.

When ghosts were treated with 0.1–10 mM *t*-BuOOH for 1 h and reduced with 1 mM NaB^3H_4 (1.65 Ci/mmol), the radioactivity incorporated into the proteins of the ghosts was approx. 5–10-fold that of the control ghosts. Use of 10 mM concentration of NaB^3H_4 with same specific activity resulted in an increase of about 2-fold in the ^3H incorporation into the proteins, but this incorporation was unspecific. The borohydride-reducible functions in the *t*-BuOOH, ADP-Fe^{3+} and aldehyde-modified ghosts appeared to be labile since the ^3H incorporation remarkably decreased after a 24-h storage of the modified ghosts at 4 or −80°C. It is likely that the ^3H-labeled sites represent a part of the modified sites.

Bailey et al. [30] reported that human erythrocyte membrane proteins contained borohydride-reducible components, and the major components were hexosyl lysines, which formed by nonenzymatic glucosylation of the lysil residues of the proteins. Miller et al. [31] demonstrated that all of the major proteins of erythrocyte membranes were equally labeled with NaB^3H_4 and suggested the dependence of nonenzymatic glucosylation on blood glucose levels and erythrocyte age. Thus, part of the radioactivity incorporation into the proteins in our control ghosts may be due to the ketoamine linkage [31] of the glycosylated proteins.

SDS-polyacrylamide gel electrophoresis analysis was effective for detection of the labeled proteins. It was shown that most membrane proteins of the *t*-BuOOH-treated ghosts, bands 1, 2, 3, 4.1, 4.2, 5 and 6 were readily cross-linked and ^3H was incorporated not only into the cross-linked proteins but also into the noncross-linked proteins. In contrast, glycophorin A (PAS-1) could hardly be cross-linked but could be intensely labeled. ADP-Fe^{3+} treatment of the ghosts did not cause extensive cross-linking, but the noncross-linked proteins and glycophorin A were labeled with NaB^3H_4. Therefore, reduction with NaB^3H_4 followed by SDS-polyacrylamide gel electrophoresis analysis may be useful in detecting and identifying the membrane proteins modified with oxidized lipids.

A mixture of various aldehydes produced in lipid oxidation may form borohydride-reducible functions by reaction with the membrane proteins, but their structures are not known. Yet, the most probable candidates for them are Schiff bases formed by the reaction of aldehydes with amino groups of the proteins. They are susceptible to borohydride reduction, and the bases are stabilized [32]. Of a variety of aldehydes produced through lipid oxidation, malonaldehyde reacts with amino groups to form various compounds [1,8,15, 33–35] which are susceptible to borohydride reduction [15]. Thus, these compounds would be reduced and labeled with NaB^3H_4 if they are produced in the membrane. Allen et al. [36] reported that radioactivity was incorporated into the protein aggregates of the malonaldehyde-modified ghosts upon reduction with NaB^3H_4. Similarly, radiolabeling with NaB^3H_4 was applied for the measurement of malonaldehyde-DNA cross-links [37]. However, the characteristics of the fluorescence spectra of the reaction products of proteins with oxidized unsaturated fatty acids were different from those of the reaction products with malonaldehyde, and indicated that malonaldehyde plays only a minor role in the protein modification in oxidized membranes [10,15,33,35,38]. In our present experiments, incorporation of the radioactivity into the malonaldehyde-modified ghosts was much less than that incorporated into the monofunctional aldehyde-modified ghosts, in spite of their extensive protein cross-linking and prominent fluorescence.

Modification of ghosts with monofunctional aldehydes resulted in the formation of borohydride-reducible functions in the membrane proteins. These aldehydes were capable of cross-linking the membrane proteins and forming fluorescence with similar spectral characteristics to those formed by reaction of lipid peroxides with proteins [10,35]. Monofunctional aldehydes may play an important role in the formation of borohydride-reducible functions. Their Schiff bases are one of the probable candidates, but other unidentified functions may be involved in the NaB^3H_4 labeling since monofunctional aldehydes react with proteins yielding complexed products, i.e., cross-links and fluorophores. A part of thiobarbituric acid-reactive substances and fluorescent substances may be responsible for NaB^3H_4 reduction.

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