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# Isolation of Human Small Intestinal Brush Border Membranes Using Polyethylene Glycol and Effect of Exposure to Various Oxidants *In Vitro*

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This study presents a method of brush border membrane (BBM) preparation from the human small intestine using polyethylene glycol (PEG) precipitation and also looks at the effect of *in vitro* oxidant exposure on structural and functional alterations in the membrane. Isolated BBM were relatively pure as judged by 10- to 14-fold enrichment of marker enzymes with less than 1% contamination by other subcellular organelles. These membranes showed uphill transport of glucose and lipid analysis showed a cholesterol–phospholipid (C/P) ratio of 1.19. Isolated BBM were found to be susceptible to superoxide generated by xanthine oxidase (XO), resulting in lipid and protein oxidation along with altered glucose uptake. Superoxide exposure also resulted in phospholipid alterations, especially generation of lyso phospholipids. These changes were prevented by inhibiting XO by allopurinol or scavenging superoxide by superoxide dismutase (SOD). Other oxidants studied did not have significant affect on these membranes. These studies suggest that PEG can be used for preparation of BBM from the human small intestine and these membranes undergo structural and functional alterations on exposure to superoxide.

**KEY WORDS:** human small intestine; brush border membrane; polyethylene glycol; oxidants; lipids.

The brush border is an important and interesting feature of intestinal epithelial cells, since they constitute a barrier between the cell and lumen of the intestine, contain a number of important digestive enzymes, and form a digestive–absorptive surface (1, 2). This appears to be true for all mammals including man. Brush border membranes (BBM) have been isolated by various methods including fractionation by density gradient and divalent cation precipitation. Welsh et al were the first to isolate human brush border membranes using discontinous gradient in presence of EDTA (3). Schimtz et al and Hauser et al reported a method for isolation of brush border membranes from rat small intestine using divalent cation ($Ca^{2+}$ or $Mg^{2+}$) precipitation (4, 5). Cation precipitation is a widely used method that is also rapid, and subsequent treatment of the isolated BBM with potassium thiocyanate (KSCN) selectively removes cytoskeletal material, which improves the purity.

BBM prepared from rodent intestine by $Ca^{2+}$ or $Mg^{2+}$ precipitation were found to differ in their lipid composition (6), size and shape (7), intramembrane particle distribution, and protein composition. Our earlier work has shown that BBM prepared by $Ca^{2+}$ or $Mg^{2+}$ precipitation

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from rat intestine differ in their purity and lipid composition, whereas BBM prepared from monkey small intestine using these cations were similar (6). One of the problems associated with the use of cations, especially $Ca^{2+}$ for BBM preparation is that $Ca^{2+}$ can activate membrane-associated lipase, phospholipase, and possibly proteases, which can alter the structural components of the membrane. Since structural and functional aspects of BBM are influenced by their lipid and protein composition (8), it is important to isolate membranes in the purest form with intact structural features. Earlier we have developed a method for isolation of BBM from rat small intestine using polyethylene glycol precipitation (9), which avoids the use of divalent cations.

Oxygen-derived free radical-mediated damage has been implicated in the pathophysiology of certain gastrointestinal disease including small intestinal ischemia–reperfusion injury, inflammatory bowel diseases, pancreatitis, and gastric ulcer. The source of free radicals in the intestinal mucosa includes activated neutrophils, high activity of xanthine oxidase, and altered mitochondrial respiration (10, 11). Membrane lipids and proteins are the target of free radicals and one of the mechanisms by which these active species damage cells is through lipid peroxidation. Our earlier work has shown that *in vitro* exposure of monkey BBM to oxidants results in altered enzyme activity and transport (12).

In the present study we have developed a procedure for the isolation of human intestinal brush border membranes using polyethylene glycol precipitation that avoids the use of divalent cations. We have further studied the *in vitro* exposure of these membranes to oxidants and their effect on structural and functional alterations.

## MATERIALS AND METHODS

Tris(hydroxymethyl)aminomethane (Tris), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), bovine serum albumin (BSA), *p*-nitrophenyl phosphate, peroxidase, *O*-dianisidine, glucose oxidase, gamma-glutamyl *p*-nitroanilide, glycyl glycine, L-leucine *p*-nitroanilide, ATP, glucose-6-phosphate, *p*-nitrocatechol sulfate, thiobarbituric acid (TBA), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), allopurinol, superoxide dismutase (SOD), and lipid standards were obtained from Sigma Chemical Co. (St.) Louis, Missouri, USA). Polyethylene glycol (PEG) 4000 was obtained from Fluka AG, Switzerland. $^{14}$C-labeled glucose was obtained from Bhaba Atomic Research Center, Bombay, India. Millipore membranes (pore size 0.45 $\mu$m) were obtained from Millipore, India. All other chemicals used were of analytical grade.

**Isolation of Human Brush Border Membranes.** Pieces of normal human small intestine 5 to 10 cm long and obtained in the course of surgery for tumors involving the gastrointestinal tract were used for BBM preparation. This study was approved by the Institutional Research Committee and informed consent was obtained from patients. Tissues were washed thoroughly with ice-cold saline, and the mucosa was scraped with a metal spatula and stored at $-20^{\circ}$C. A 3% homogenate of the mucosa was prepared in buffer containing 2 mM Tris HCl and 50 mM mannitol, pH 7.1, using a Potter-Elvehjem homogenizer for 2–3 min at full speed. This was allowed to stand at 4°C for 15 min and filtered using nylon mesh cloth. To this, a 50% solution of PEG was added to make final concentration of 10% PEG, stirred for 15 min continuously at 4°C and centrifuged at 7500*g* for 15 min. The pellet was discarded and the supernatant was spun at 12,000*g* for 15 min. This pellet was also discarded, and the supernatant was centrifuged at 27,000*g* for 40 min. The pellet was washed twice with the suspension buffer containing 10 mM Tris HCl and 300 mM mannitol, pH 7.1, and finally suspended in 1 ml of the same buffer using a syringe fitted with a 26-gauge needle. Protein was estimated using bovine serum albumin as standard (13).

**Enzyme Assays.** Activity of alkaline phosphatase (14), sucrase (15), leucine amino peptidase (16), $\gamma$-glutamyltranspeptidase (17), $Na^+$,$K^+$-ATPase (18), glucose-6-phosphatase (19), and arylsulfatase (20) were assayed as described. The enzyme activities are expressed as units per milligram of protein. (Units are expressed as micromoles per minute per milligram of protein for alkaline phosphatase, leucine amino peptidase, $\gamma$-glutamyltranspeptidase, and glucose-6-phosphatase, as nanomoles per hour per milligram of protein for sucrase, and arylsulfatase, and as nanomoles per minute per milligram of protein for $Na^+$,$K^+$-ATPase.)

**Electron Microscopy.** BBM vesicles were fixed in suspension at 4°C with 1.5 % glutaraldehyde in 50 mM sodium cacodylate buffer, pH 7.4, for 30 min and centrifuged at 10,000*g* for 10 min. Pellets were gently rinsed with the same buffer and post-fixed for 2 hr on ice with 1% osmium tetraoxide in 50 mM sodium cacodylate buffer, pH 7.6. Pellets were then rinsed with same buffer. After dehydration in an ethanol series, pellets were embedded in polybed resins, thin sectioned, and stained with uranyl acetate and lead citrate. This material was then photographed in a Phillips 201 electron microscope .

**Determination of Membrane-Bound Sugars and Thiol Content.** Membrane-bound hexoses and fucose were determined as described with slight modification (21). Two hundred microliters of membrane corresponding to 10–20 $\mu$g protein was used. Calibration curves were made using a solution of D-galactose for hexoses or L-fucose, in the concentration range of 5–50 nmol. Membrane-associated hexoses and fucose were expressed as nanomoles per milligram of protein. Sialic acid content of the isolated membranes was estimated as described (22). Membrane corresponding to 25–50 $\mu$g protein was used. A standard curve was made using sialic acid in the range of 5–50 nmol, and membrane-bound sialic acid was expressed as nanomoles per milligram of protein. Thiol content of the membranes was measured using DTNB as described (23).

**Oxidant Exposure of BBM.** Isolated BBM corresponding to 1–2 mg protein were incubated in a total volume of 1 ml of suspension buffer (10 mM Tris HCl and 300 mM mannitol, pH 7.1) for 30 min at 37°C with each of the following free radical generating systems separately: (1) 1 mM xanthine + 100 munits XO, with and without allopurinol (1 mM) or SOD (1000 units), (2) 300 $\mu$M of tBuOOH and (3) 10 $\mu$M $H_2O_2$ (all final concentrations). We also incubated membranes with 50 $\mu$M of calcium separately. As a control, corresponding membranes were incubated at 37°C for 30 min without the addition of oxidants.

TABLE 1. ACTIVITY OF MARKER ENZYMES IN ISOLATED BRUSH BORDER MEMBRANES

| | *Specific activity** | | | |
|---|---|---|---|---|
| | *Homogenate* | *Brush border membrane* | *(fold) enrichment* | *Recovery (%)* |
| Alkaline phosphatase | 0.055 ± 0.003 | 0.574 ± 0.038 | 10–12 | 34 |
| Sucrase | 1.19 ± 0.09 | 14.46 ± 1.34 | 12–14 | 36 |
| $\gamma$-Glutamyl transpeptidase | 0.0025 ± 0.0003 | 0.024 ± 00.002 | 10 | 30 |
| Leucine aminopeptidase | 0.0135 ± 0.001 | 0.145 ± 0.01 | 10 | 27 |

*Each value represents mean ± SD of four separate experiments with triplicate estimations.

Controls containing only xanthine or XO were also tested. Following incubation, BBM lipids, enzymes, lipid peroxidation parameters, and glucose uptake were analyzed as described below.

**Peroxidation Parameters.** Malonaldehyde (MDA) was measured using the TBA method (24). The amount of malondialdehyde (MDA) formed was calculated from the standard curve prepared using 1,1′,3,3′-tetramethoxypropane and value expressed as nanomoles per milligram of protein. For conjugated diene measurement, total lipids from BBM were extracted as described (25), dissolved in 1 ml heptane, and read at 233 nm using a Shimadzu spectrophotometer. The amount of conjugated diene formed was calculated using a molar absorption coefficient of $2.52 \times 10^4$ and expressed as micromoles per milligram of protein (26). Protein carbonyl content was measured using 2,4-dinitrophenylhydrazine (DNPH) (27) and calculated using an extinction coefficient of 22 $mM^{-1}cm^{-1}$.

**Lipid Analysis.** BBM lipids were extracted by the method of Bligh and Dyer (25). The lower organic phase was evaporated to dryness, resuspended in a small volume of chloroform–methanol (2:1), and used for lipid analysis. Neutral lipids were separated on silica gel G plates using the solvent system hexane–diethylether–acetic acid (80:20:1, v/v). Spots corresponding to standard were identified by iodine exposure and eluted. Cholesterol and cholesteryl ester (28), and diacylglycerol and triacylglycerol (29) were quantitated as described. Individual phospholipids were separated on silica gel H plates using the solvent system chloroform–methanol–acetic acid–water (25:14:4:2, v/v) and quantitated by phosphate estimation after acid hydrolysis (30).

**Measurement of D-Glucose Uptake.** Isolated BBM vesicles were assessed for their ability to transport glucose by uptake measurements carried out by a rapid filtration technique at room temperature as described (31). Briefly 50 $\mu$l of BBM corresponding to 100 $\mu$g protein was incubated with 150 $\mu$l of uptake buffer containing 150 mM NaSCN, 50 $\mu$M D-glucose, 0.8 $\mu$Ci D-[$^{14}$C]glucose, and 10 mM HEPES (pH 7.5) at varying time intervals. At the end of incubation, the mixture was diluted with 2 ml of ice cold stop buffer (150 mM NaCl, 10 mM HEPES, pH 7.5) and immediately filtered using a 0.45-$\mu$m Millipore filter under constant vacuum. The filter was washed three times with 5 ml of stop buffer and transferred to counting vials. The radioactivity retained in the filter was counted using LKB Rack-Beta scintillation counter.

**Statistics.** Data are expressed as mean ± SD from a minimum of four experiments. Statistical analyses were performed with Student's *t* test and Mann-Whitney test to compare the changes.

## RESULTS

Purity of the isolated BBM was assessed by enrichment of marker enzymes, which showed a 10- to-14-fold enrichment in the activity of alkaline phosphatase, sucrase, $\gamma$-glutamyltranspeptidase, and leucine aminopeptidase (Table 1). Contamination by other subcellular fractions was checked by assaying $Na^+$,$K^+$-ATPase for basolateral membrane, arylsulfatase for lysosomes, and glucose-6-phosphatase for microsomes. As shown in Table 2, there is very little contamination by these fractions in the final preparation. Figure 1 shows an electron micrograph of the final BBM preparation, which appears predominantly in vesicular form. The thiol and carbohydrate content of the isolated BBM were measured and showed a total thiol of $10.33 \pm 0.72$, sialic acid of $166 \pm 6.62$, fucose of $254 \pm 14$, and hexose of $66.37 \pm 6.6$ nmol/mg protein. Amounts of fucose and sialic acid are found to be higher in these membranes as compared to hexoses. Analysis of the lipid composition of BBM showed the presence of various lipids with a cholesterol–phospholipid ratio of 1.19 (Table 3). The ability of the membrane vesicles to transport glucose was checked using $^{14}$C-labeled glucose, which showed an uphill transport with maximum at 20 sec (Figure 2).

Human BBM were exposed to different oxidants *in vitro*, and we looked for structural and functional

TABLE 2. ACTIVITY OF SUBCELLULAR MARKER ENZYMES IN ISOLATED BBM

| | *Specific activity** | | |
|---|---|---|---|
| | *Homogenate* | *BBM membrane* | *Contamination (%)* |
| $Na^+$,$K^+$-ATPase | 0.666 ± 0.09 | 0.1 ± 0.01 | 0.48 |
| Arylsulfatase | 0.2 ± 0.013 | 0.058 ± 0.006 | 0.8 |
| Glucose-6-phosphatase | 0.405 ± 0.018 | 0.123 ± 0.01 | 0.78 |

*Each value represents mean ± SD of four separate experiments with triplicate estimations.

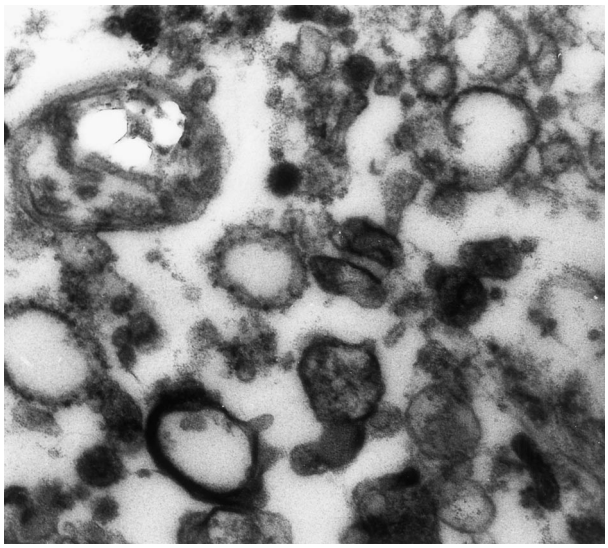

**Fig 1.** Electron micrograph of brush border membrane vesicles isolated from human small intestine (82,000× magnification).

alterations. Exposure to superoxide generated by X-XO resulted in a decrease in alkaline phosphatase activity and glucose uptake, accompanied by an increase in lipid peroxidation parameters (Figures 3 and 4). Oxidant exposure did not alter disaccharidase activity (data not shown). These alterations could be prevented by the simultaneous presence of xanthine oxidase (XO) inhibitor, allopurinol, or superoxide dismutase (SOD) in the incubation system (Figures 3 and 4). Exposure to other oxidants or calcium did not result in any alterations. Superoxide exposure resulted in changes in certain phospholipids and other oxidants did not have any effect (Figure 5). A decrease in phosphatidylcholine (PC) and phosphatidylethanolamine

TABLE 3. LIPID COMPOSITION OF HUMAN BBM

| | *Amount (nmol/mg protein)** |
|---|---|
| Neutral lipids | |
| Cholesterol | 172.6 ± 6.99 |
| Cholesteryl ester | 16.61 ± 1.1 |
| Triacylglycerol | 170.88 ± 6.48 |
| Diacylglycerol | 50.3 ± 5 |
| Phospholipids | |
| Phosphatidylcholine | 47.04 ± 11.61 |
| Lysophosphatidylcholine | 11.61 ± 0.63 |
| Phosphatidylethanolamine | 20.88 ± 1.11 |
| Lysophosphatidylethanolamine | 12.1 ± 0.91 |
| Phosphatidic acid and cardiolipin | 20.97 ± 1.56 |
| Sphingomyelin | 14.46 ± 1.14 |
| Phosphatidylserine and phosphatidylinositol | 17.93 ± 1.16 |
| Cholesterol/Phospholipid Ratio (mol/mol) | 1.19 |

*Each value represents mean ± SD of four separate experiments with duplicate estimations.

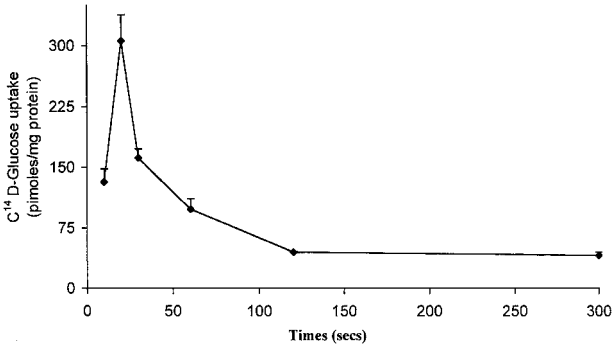


**Fig 2.** D-Glucose transport by BBM vesicles prepared from human small intestine. Each value represents mean ± SD from four separate intestines with triplicate estimation.

(PE) with a concomitant increase in lyso-PC and lyso-PE was seen. These changes were prevented either by inhibiting XO or by superoxide dismutase. Interestingly, calcium also brought about changes in these phospholipids when incubated with the membrane (Figure 5). There were no changes in other lipids (data not shown).

## DISCUSSION

In this study, brush border membranes from human small intestine were isolated using PEG precipitation,

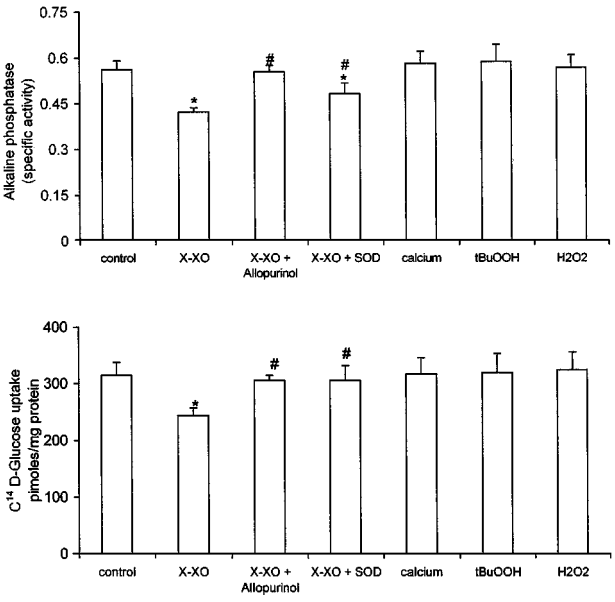


**Fig 3.** Effects of oxidants on human BBM alkaline phosphatase activity and D-glucose uptake (20 sec). Each value represents mean ± SD from four different intestines with duplicate estimation. (*$P < 0.05$ when compared to control, #$P < 0.05$ when compared to X-XO). The final concentration of compounds and incubation conditions are described in the text.

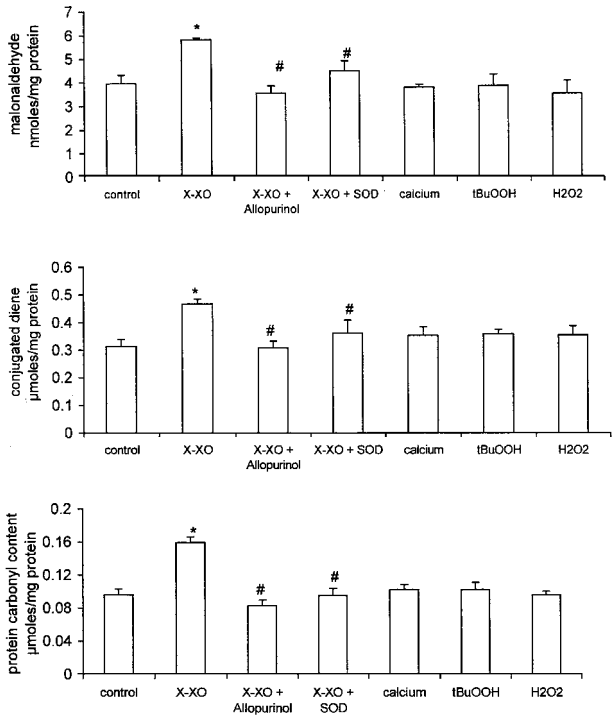


**Fig 4.** Effect of oxidants on human BBM lipid and protein parameters. Each value represents mean ± SD of four separate experiments with duplicate estimation. (*$P < 0.05$ when compared to control, #$P < 0.05$ when compared to X-XO). The final concentration of compounds and the incubation conditions are described in the text.

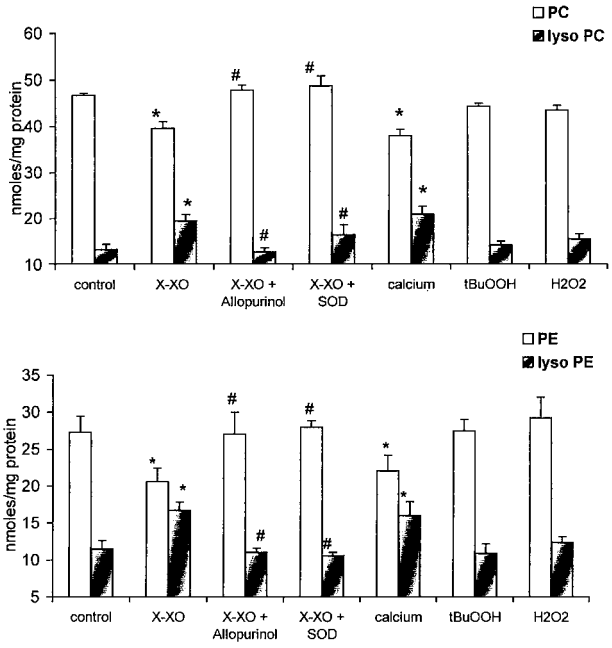


**Fig 5.** Phospholipid composition of BBM after exposure to various oxidants. (Top) phosphatidylcholine (PC) and lyso-PC and (bottom) phosphatidylethanolamine (PE) and lyso-PE. Each value represents mean ± SD of four separate experiments with duplicate estimation. (*$P < 0.05$ when compared to control, #$P < 0.05$ when compared to X-XO). The final concentration of compounds and the incubation conditions are described in the text.

which showed relatively pure preparation as judged by enrichment of marker enzymes and free from contamination by other subcellular organelles. The divalent precipitation is a commonly used method for BBM preparation, but one of the problems associated with this method is the possible activation of membrane-associated lipases, phospholipases, and possibly proteases, which is likely to alter lipid and protein composition. Hauser et al reported that membrane vesicles prepared from fresh or frozen tissue by the $Ca^{2+}$-precipitation technique (5) contained high levels of lyso-PC, lyso-PE, and free fatty acids. It is known that polyethylene glycol can selectively precipitate certain subcellular organelles (32) and our earlier work has shown isolation of brush border membrane vesicles from rat and rabbit small intestine using polyethylene glycol. This preparation was comparable to the divalent $Ca^{2+}$ precipitation method (9) in purity, and it avoided the alterations in lipid composition. Very little information is available on the isolation and composition of human brush border membranes, and in this study a relatively pure membrane preparation was obtained using polyethylene glycol precipitation. This method also avoided alteration in the lipid composition as an artifact of the isolation procedure. Intestinal epithelial cells are known to contain $Ca^{2+}$-activated phospholipase that may degrade membrane phospholipids (33). BBMs prepared from human small intestine using PEG precipitation showed an increased lipid content and C/P ratio as compared to the divalent cation-precipitated membranes reported earlier (34). The membrane lipids play an important role in modulating the function including enzyme activity and transport often by altering membrane fluidity (10, 35).

Cellular membranes are the main targets of oxygen-derived free radicals, which result in peroxidation of lipids. Reactive oxygen species have been implicated in the pathophysiology of certain gastrointestinal disorders, including small intestinal ischemia–reperfusion injury (36), acute pancreatitis (37), gastrointestinal cancer (38), necrotizing enterocolitis (39), and gastric ulcers (40). In the present work, susceptiblity of BBM to oxidants was studied using *in vitro* exposure of these membranes to different oxidants. It was seen that only superoxide generated by XO could bring about an alteration in membrane-associated enzyme activity, transport properties, and lipid composition, which were prevented by inhibiting XO by allopurinol or scavenging superoxide by SOD. Although $Ca^{2+}$ exposure did not alter membrane-associated enzyme

activity or glucose transport, it could bring about phospholipid degradation, suggesting activation of phospholipase $A_2$ ($PLA_2$).

Oxygen free radicals are known to activate phospholipases, and we have earlier shown activation of $PLA_2$ in liver mitochondria by superoxide, which brings about an alteration in the mitochondrial lipids (41). Increased lyso-PC and lyso-PE was evident following lipid peroxidation of $PLA_2$-containing liposomes and microsomes (42). It was also shown that rat renal mitochondrial phospholipids are altered by $Ca^{2+}$-activated $PLA_2$ during ischemia (43). It is known that the BBM contains calcium-dependent and calcium-independent $PLA_2$, which can be activated by free radicals and calcium (44, 45). A decrease in PC and PE along with an increase in lyso-PC and lyso-PE on exposure to superoxide suggests the presence of a $PLA_2$ in these membranes that is activated by superoxide. Similar changes in the lipid composition were also seen in presence of $Ca^{2+}$. Free radicals bring about lipid peroxidation of membrane lipids and human BBM also undergoes lipid peroxidation on exposure to superoxide. Our earlier report has shown that spontaneous free-radical generating 2,2′-azobis(2-amidinopropane)dihydrochloride (ABAP) is capable of inducing lipid peroxidation of monkey small intestinal BBM as judged by production of MDA, conjugated diene, and depletion of tocopherol (12).

In summary, this study has shown a novel method of BBM preparation from human small intestine using PEG precipitation that avoids degradation of membrane lipids as an isolation artifact. It was also seen that *in vitro* exposure of these membranes to superoxide results in structural and functional alterations, suggesting a possible role for free radicals in damaging these membranes, which is likely to occur in certain gastrointestinal disorders.

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