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Comparative Biochemistry and Physiology Part C 134 (2003) 329–339

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Effect of oxidants on small intestinal brush border membranes and colonic apical membranes—a comparative study

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Received 16 September 2002; received in revised form 27 November 2002; accepted 28 November 2002

Abstract

This study compares composition of the rat small intestinal brush border membranes (BBM) and colonic apical membranes (CAM) and their susceptibility to in vitro exposure to various oxidants. Differences were observed between BBM and CAM in their lipid composition, sugar content, alkaline phosphatase (ALP) activity and cholesterol/phospholipid ratio. BBM and CAM were exposed to superoxide generated by xanthine + xanthine oxidase (X–XO) or peroxides such as tertiary butyl hydroperoxide (tBuOOH) and hydrogen peroxide (H_2O_2) and alterations in ALP activity, peroxidation parameters and membrane lipids were analyzed. Exposure of BBM and CAM to superoxide resulted in decrease in ALP activity and increase in peroxidation parameters such as protein carbonyl content, malondialdehyde and conjugated diene. Superoxide exposure also resulted in lipid alterations specifically in certain phospholipids. These alterations were prevented either by superoxide dismutase or by allopurinol. Peroxides did not have any significant effect. These results suggest that both BBM and CAM are susceptible to superoxide, which can bring about peroxidation and degradation of membrane lipids specifically, certain phospholipids.

Keywords: Brush border membrane; Colonic apical membrane; Phospholipids; Superoxide; Lipid peroxidation

1. Introduction

Gastrointestinal mucosa is likely to be exposed to various toxic compounds originating from food, bacterial metabolism, ingested drugs and oxidants formed during metabolism. These compounds can generate free radicals on their own or during reaction with other compounds (Mezes et al., 1997; Pons et al., 1991; Ribeiro, 1987). Presence of bacteria in the lumen attracts a large number of phagocytes under certain pathological conditions, which on activation release free radicals (Huycke et al., 2002; MacDonald et al., 1993), which can spill over to the surrounding mucosa.

Reactive oxygen species (ROS) play an important role in the pathogenesis of various tissue injuries including gastrointestinal mucosa. Intestinal epithelial cells are likely to be exposed to these active species generated both in the mucosa and in the lumen (Parks, 1989). Sources of ROS in the mucosa include activated neutrophills, high activity of xanthine oxidase and mitochondrial respiration (Otamiri, 1989; Yoshikowa and Kondo, 1990; Anup et al., 1999). Membrane lipids and proteins are the targets for ROS and one of the mechanisms by which these active species damage cells is through lipid peroxidation (Dipolk et al.,

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PII: S1532-0456(02)00250-8

1988). There are evidences showing peroxidation-induced alterations in the lipid organisation and function of biological membranes, i.e. a decrease in lipid fluidity (Courjault-Gautier et al., 1995), facilitation of ion permeability (Lambert et al., 2002) and inhibition of membrane-bound enzyme activities (Plaa and Witschi, 1976; Ohyashiki et al., 1994). Recent evidence suggests that mucosal lipid peroxidation may play an important role in the pathogenesis of inflammation-induced intestinal injury such as ulcerative colitis and inflammatory bowel disease (IBD) (Cuzzocrea et al., 2001; Koch et al., 2000). In addition, ROS may activate membrane-associated phospholipases resulting in degradation of phospholipids and generation of membrane lytic lysophospholipids and free fatty acids (Goldman et al., 1992). It was shown earlier that liver mitochondria contain a phospholipase A_2 that can be activated by superoxide (Madesh and Balasubramanian, 1997).

Small intestinal brush border membranes (BBM) and colonic apical membranes (CAM) are involved in different functions and the contents of the lumen in which they are closely associated are also different. BBM is mainly involved in the digestion and absorption of nutrients and is in contact with numerous digestive enzymes (Wolfgang, 1992). On the other hand, CAM are involved in absorption of different type of molecules including short chain fatty acids generated by the bacterial metabolism and are in close contact with bacteria and their metabolites. Although enterocytes and colonocyte are exposed to oxygen free radicals, their susceptibility may vary and distal ileal enterocytes and colonocytes are more prone to inflammation and oxidative stress associated with it (Nalini and Balasubramanian, 1993; Baskar and Balasubramanian, 1995). Hence, it is of interest to look at the susceptibility of these membranes to oxidative stress. The present study compares the susceptibility of BBM and CAM to in vitro exposure of ROS and the resultant alterations associated with it.

2. Materials and methods

Tris (hydroxy methyl) aminomethane (Tris), bovine serum albumin (BSA), *p*-nitrophenyl phosphate, thiobarbutaric acid (TBA), 2,4-dinitro phenyl hydrazine (DNPH), 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), xanthine, xanthine oxidase, allopurinol, superoxide dismutase (SOD), tertiary butyl hydroperoxide (tBuOOH) and lipid standards were all obtained from Sigma chemical Co., St Louis, USA. Polyethylene glycol (PEG) 4000 was obtained from Fluka AG, Switzerland. All other chemicals used were of analytical grade.

2.1. Isolation of brush border membranes

BBM were prepared from the rat small intestine using PEG precipitation method (Prabhu, and Balasubramanian, 2001). Briefly 3% homogenate of the scraped mucosa obtained from overnight fasted rats was prepared in 2 mM Tris–HCl containing 50 mM mannitol pH 7.1 using a Porter–Elvelhem homogenizer at full speed. This was allowed to remain at 4 °C for 15 min and filtered using nylon cloth. To this, a 50% PEG solution was added to make a final concentration of 10% PEG, stirred for 15 min and centrifuged at $7500 \times g$ for 15 min. The pellet was discarded and the supernatant was spun at $27\,000 \times g$ for 40 min. To the pellet, 30 ml suspension buffer (10 mM Tris–HCl and 300 mM mannitol, pH 7.1) was added, centrifuged at $27\,000 \times g$ for 40 min. The pellet was washed twice with the same suspension buffer and finally suspended in 1 ml of the same buffer using a syringe fitted with a 26-gauge needle. Enrichment of the marker enzyme alkaline phosphatase (ALP), sucrase and maltase were checked for purity of the isolated BBM. Protein was estimated using BSA as standard (Lowry et al. (1970). Membranes were prepared from three different animals with duplicate estimations ($n=3$). This study was approved by the Animal Experimentation Ethics Committee of the Institution.

2.2. Isolation of colonocytes and colonic apical membranes

Rat colonocytes were isolated as described (Roediger and True love, 1979). Briefly, overnight fasted rats were killed by decapitation, colon removed and washed with calcium free Krebs–Henseleit (K–H) buffer. The colon was filled with calcium free K–H buffer containing 5 mM EDTA, clamped at both ends and incubated in oxygenated calcium free K–H buffer at 37 °C for 20 min. Following incubation, contents of the colon were discarded and the colon was filled with calcium free K–H buffer without EDTA. Colonocytes were obtained by gently rubbing the colon along its

Table 1
Comparative data on the small intestinal BBM and CAM

	Small intestinal BBM	CAM
	(μmole/min/mg protein)	
ALP	$0.87 \pm 0.036^{\#\#\#}$	0.053 ± 0.004
	(nmole/mg protein)	
Thiol	26.4 ± 2	27.11 ± 1.26
Carbohydrates		
Hexose	$95.5 \pm 6.6^{\#\#\#}$	31.6 ± 3.6
Fucose	87.0 ± 2.8	$274.1 \pm 16.0^{***}$
Sailic acid	$92.2 \pm 11^{\#}$	70.2 ± 4.6
Lipids		
Cholesterol	61.4 ± 1.1	$102.3 \pm 4.5^{**}$
Cholesteryl ester	5 ± 0.4	$30.4 \pm 2.1^{**}$
TAG	110 ± 10	$128.0 \pm 3.4^{*}$
DAG	$65 \pm 4.5^{\#\#\#}$	14.1 ± 1.9
Total phospholipids	181.5 ± 10	159.7 ± 7.2
PC	$72.4 \pm 4.9^{\#\#\#}$	54.9 ± 1.2
PE	23.3 ± 0.9	$37.1 \pm 2.7^{***}$
Lyso PE	$17.4 \pm 1.2^{\#\#\#}$	8.6 ± 0.3
Lyso PC	$14.6 \pm 1.3^{\#\#\#}$	5.0 ± 0.4
Phosphotidyl serine and inositol	23.1 ± 1.1	26.9 ± 1.5
Sphingomylein	$25.9 \pm 0.6^{\#\#\#}$	16.1 ± 1.2
Phosphatidic acid	4.8 ± 0.3	$13.8 \pm 1.25^{***}$
	(mole/mole)	
C/P ratio	0.34	0.64

Each value represents mean ± S.D. of three ($n=3$) separate animal experiments with duplicate estimations.
* $P<0.05$ when compared to BBM.
** $P<0.005$ when compared to BBM.
*** $P<0.0005$ when compared to BBM.
$^{\#}$ $P<0.05$ when compared to CAM.
$^{\#\#\#}$ $P<0.005$ compared with CAM.

length. Isolated colonocytes were centrifuged at $600\times g$ for 5 min and washed twice using calcium free K–H buffer. The wet weight of the colonocytes was noted and 5% homogenate was made using a buffer containing 2 mM Tris–HCl containing 50 mM mannitol pH 7.1 and sonicated in a Soniprep150 (MSE) for 20 s at 14 amplitude micron. This was allowed to remain at 4 °C for 15 min and filtered using nylon mesh cloth. To this, a 50% PEG solution was added to make a final concentration of 7% PEG, stirred for 15 min and centrifuged at $7500\times g$ for 15 min. The pellet was discarded and the supernatant was spun at $12\,000\times g$ for 15 min. This pellet was also discarded and the supernatant was centrifuged at $27\,000\times g$ for 40 min. To this pellet, 15 ml suspension buffer (10 mM Tris–HCl and 300 mM mannitol, pH 7.1) was added and centrifuged at $27\,000\times g$ for 40 min. The pellet obtained was washed twice with the same suspension buffer and finally suspended in 1 ml of the same buffer using a syringe fitted with a 26-gauge needle. Enrichment of the marker enzyme ALP, leucine amino peptidase and γ-glutamyl transpeptidase were checked for purity of the isolated CAM.

2.3. Determination of membrane bound sugars and thiol contents

Membrane bound-hexoses and fucose were determined as described with slight modification (Djurdjic and Mandic, 1990). Two hundred microlitre of membrane corresponding to 10–20 μg 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 nmole. Membrane associated hexoses and fucose were expressed as nmole/mg protein. Sialic acid content of the isolated membranes was estimated as described (Serifer and Gerstenfeld, 1962).

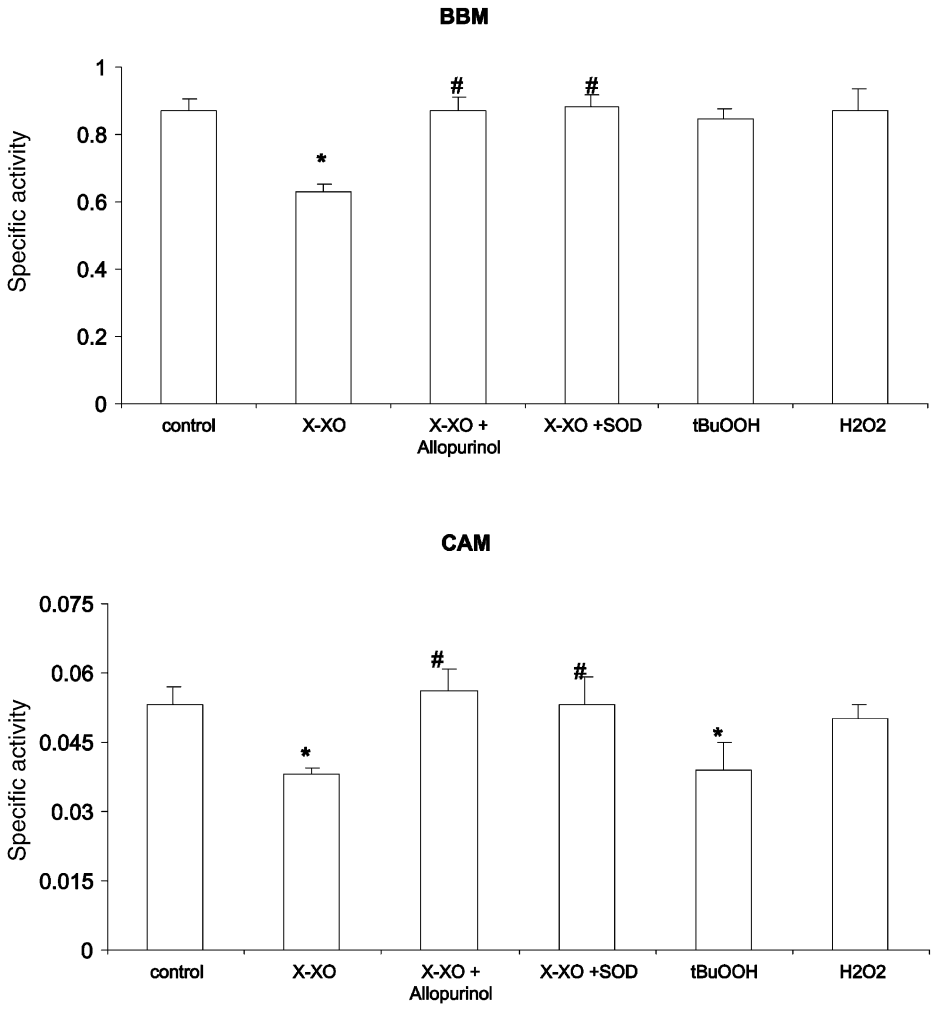

Fig. 1. ALP activity of BBM and CAM after various oxidant exposure. (Each value represents mean ± S.D. of three ($n = 3$) separate animal experiments with triplicate estimations. *$P < 0.05$ compared to control. #$P < 0.05$ compared to X–XO). The final concentration of compounds used and incubation conditions are described in the text.

Membrane corresponding to 25–50 μg protein was used. Standard curve was made using sailic acid in the range of 5–50 nmole and membrane bound sailic acid was expressed as nmole/mg protein. Thiol content of the membranes was measured using DTNB as described (Habeeb, 1972).

2.4. Oxidant exposure of isolated membranes

BBM and CAM corresponding to 1–2 mg protein were incubated in a total volume of 1 ml suspension buffer for 30 min at 37 °C with each of the following free radical generating systems separately: (a) 1 mM xanthine + 100 munits xanthine oxidase, with and without allopurinol (1 mM) or SOD (1000 units), (b) 300 μM of tBuOOH and (c) 10 μM H202 (all final concentrations). As a control, corresponding membranes were incubated at 37 °C for 30 min but without the addition of oxidants. Controls containing only xanthine or XO were also tested. Following exposure to various oxidants, membranes were tested for ALP, peroxidation parameters and lipid composition. Activity of ALP (Thambidurai and Bachawat, 1977) was measured using *p*-nitrophenyl

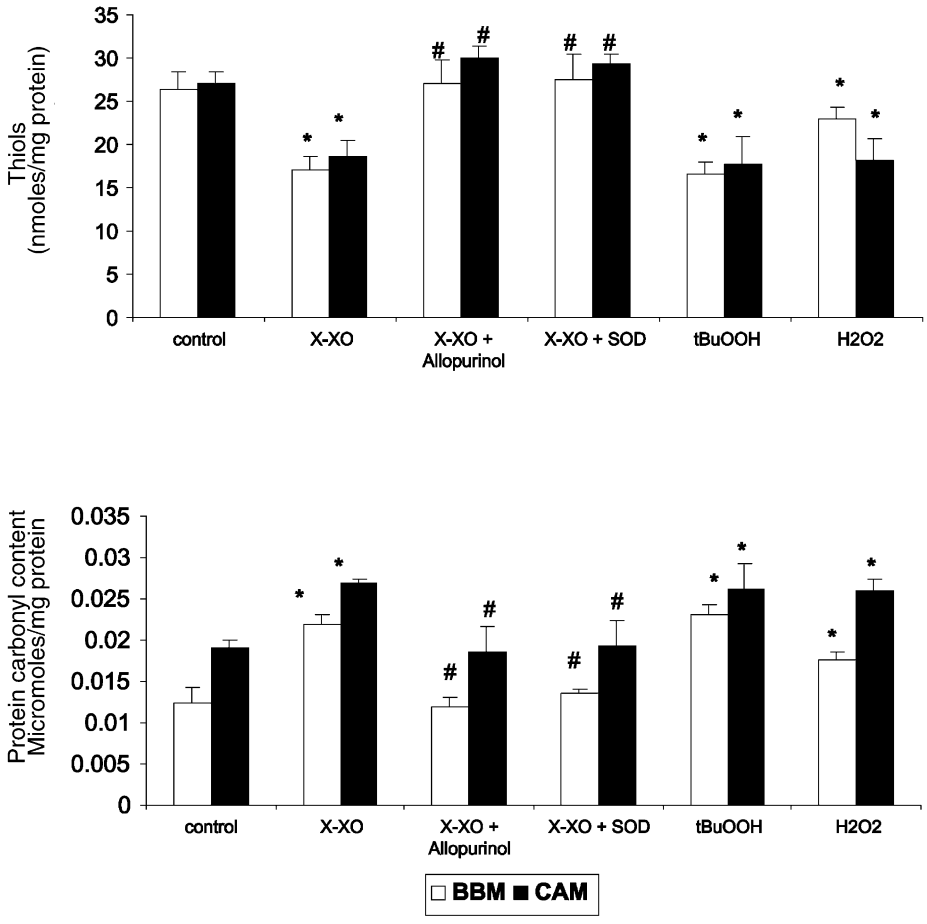

Fig. 2. Thiol and protein carbonyl content of BBM and CAM after exposure to various oxidants. (Each value represents mean ± S.D. of three ($n=3$) separate animal experiments with triplicate estimations. *$P<0.05$ compared to control. #$P<0.05$ compared to X–XO). The final concentration of compounds used and incubation conditions are described in the text.

phosphate as substrate and specific activity expressed as μmole/min/mg protein.

2.5. Peroxidation parameters

Malondialdehyde (MDA) was measured using TBA method (Ohkawn et al., 1979). The amount of MDA formed was calculated from the standard curve prepared using 1,1′,3,3′ tetramethoxy propane and expressed as nmole/mg protein. For conjugated diene measurement, total lipids from the membrane were extracted as described (Bligh and Dyer, 1959), dissolved in 1 ml heptane and read at 233 nm using Shimadzu spectrophotometer. The amount of conjugated diene formed was calculated using molar absorption co-efficient of 2.52×10^4 and expressed as μmole/mg protein (Chan and Levett, 1972). Protein carbonyl content was measured using 2,4-DNPH (Sohal et al., 1993) and calculated using extinction co-efficient of 22/mM cm.

2.6. Lipid analysis

Following incubation, membrane lipids were extracted by the Bligh and Dyer (1959) method. The lower organic phase was concentrated using nitrogen, 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: diethyl ether: acetic acid (80:20:1, v/v). Spots corresponding to standard were identified by iodine exposure and eluted. Cholesterol and cholesteryl ester (Zaltkis

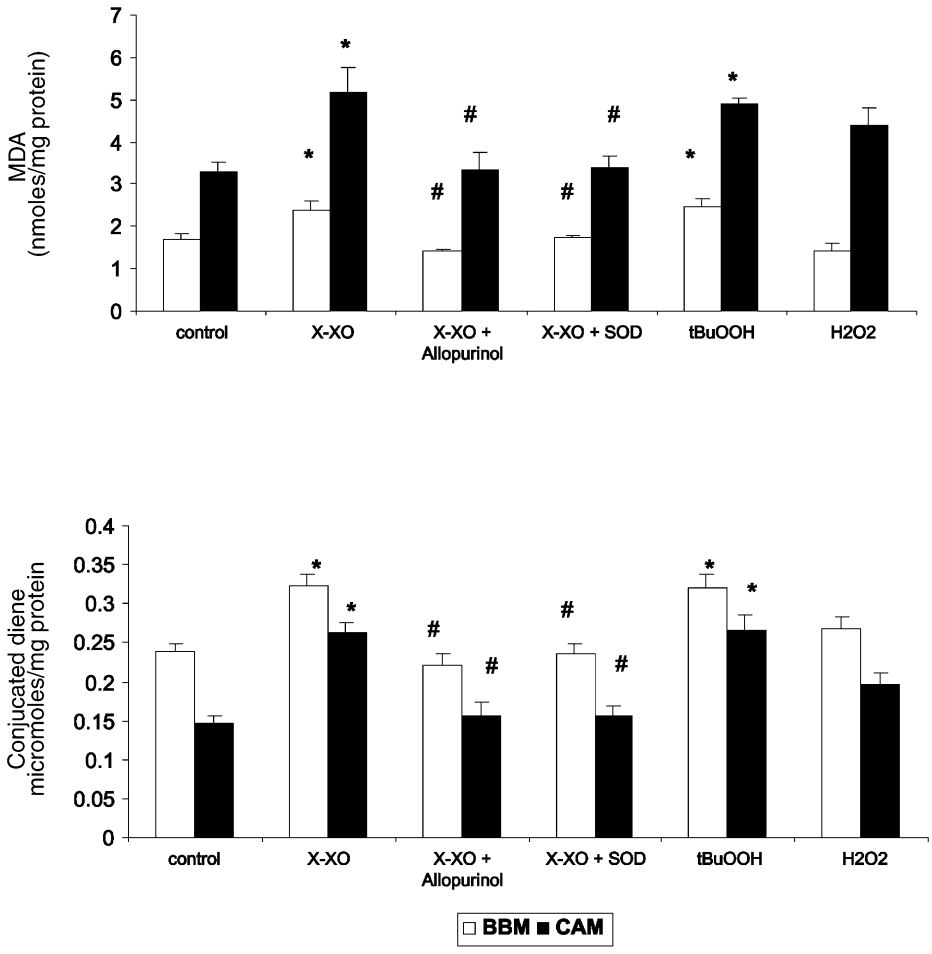

Fig. 3. Lipid peroxidation parameters after exposure of BBM and CAM to various oxidants invitro. (Each value represents mean ± S.D. of three ($n=3$) separate animal experiments with triplicate estimations. *$P<0.05$ compared to control. #$P<0.05$ compared to X–XO). The final concentration of compounds used and incubation conditions are described in the text.

et al., 1953), diacyl glycerol (DAG) and triacyl glycerol (TAG) (Synder and Stephen, 1959) were quantitated as described. Individual phospholipids were separated on silica gel H plate using the solvent system chloroform: methanol: acetic acid: water (25:14:4:2, v/v) and quantitated by phosphate estimation after acid hydrolysis (Bartlett, 1959).

2.7. Statistical analyses

Data are expressed as mean ± S.D. from a minimum of three animals with duplicate estimation. Statistical analyses were performed with Student's '*t*'-test and Mann–Whitney test to compare the changes.

3. Results

Purity of the isolated membranes was assessed by marker enzymes. An enrichment of ALP (12-fold), sucrase (10-fold) and maltase (10-fold) for BBM and ALP (11-fold), γ-glutamyl transpeptidase (10-fold) and leucine amino peptidase (4-fold) for CAM were obtained as compared to the respective homogenates (data not shown). Table 1 shows the comparative data on certain components of the BBM and CAM. As can be seen, ALP activity is high in BBM as compared to CAM and thiol content is similar in both the membranes. Among the sugars, decrease in hexose and sialic acid and a considerable increase in fucose were seen in CAM as compared to BBM. Among the

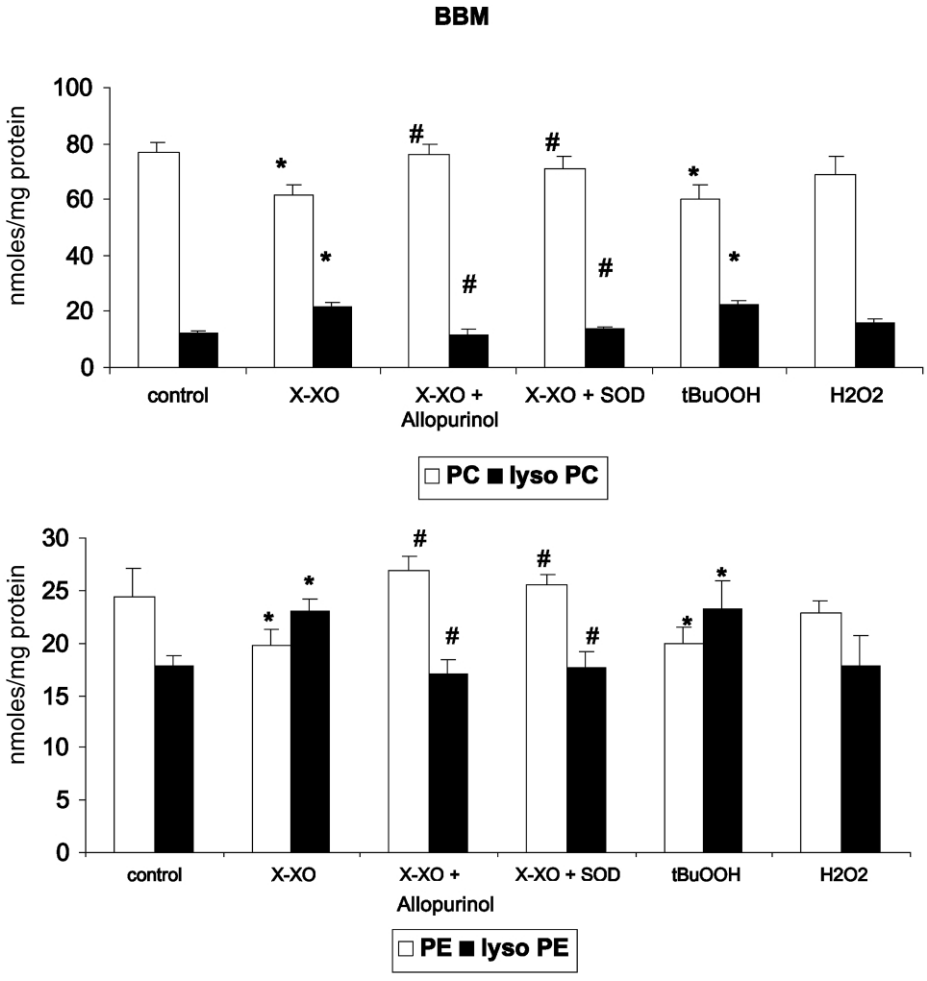

Fig. 4. Phospholipid alterations in BBM following exposure to oxidants. (a) PC and lyso PC. (b) PE and lyso PE. (Each value represents mean ± S.D. of three ($n=3$) separate animal experiments with duplicate estimations. *$P<0.05$ compared to control. #$P<0.05$ compared to X–XO). The final concentration of compounds used and incubation conditions are described in the text.

lipids, higher content of cholesterol and TAG and decrease in DAG were seen in CAM as compared to BBM. A slight decrease in total phospholipid was seen in CAM as compared to BBM, and among the individual phospholipids, a decrease in phosphotidylcholine (PC), sphingomylein, lyso phospholipids and phosphatidic acid and an increase in phosphotidylethanolamine (PE) were seen in CAM as compared to BBM. Lipid analysis also showed a higher cholesterol/phospholipid (C/P) ratio in CAM as compared to BBM.

Membranes are susceptible to free radicals since they contain lipids and proteins, which can undergo oxidation. Membranes were exposed in vitro to oxidants and different parameters were studied. Fig. 1a and b shows ALP activity in BBM and CAM on exposure to various oxidants. A decrease in activity on exposure to superoxide generated by XO was seen which was prevented by the simultaneous presence of an inhibitor of XO, allopurinol or SOD. In addition tBuOOH exposure also decreased the ALP activity of CAM, which was not seen in BBM. Incubation with H_2O_2 did not alter this activity. A decrease in thiol content and an increase in protein carbonyl content were seen in these membranes when exposed to superoxide or tBuOOH and inhibition of superoxide formation by allopurinol or scavenging by SOD prevented these changes (Fig. 2a and b). These changes were similar in both BBM and CAM. A similar observation was made with regard to lipid peroxidation parameters, MDA and conjugated diene (Fig. 3a

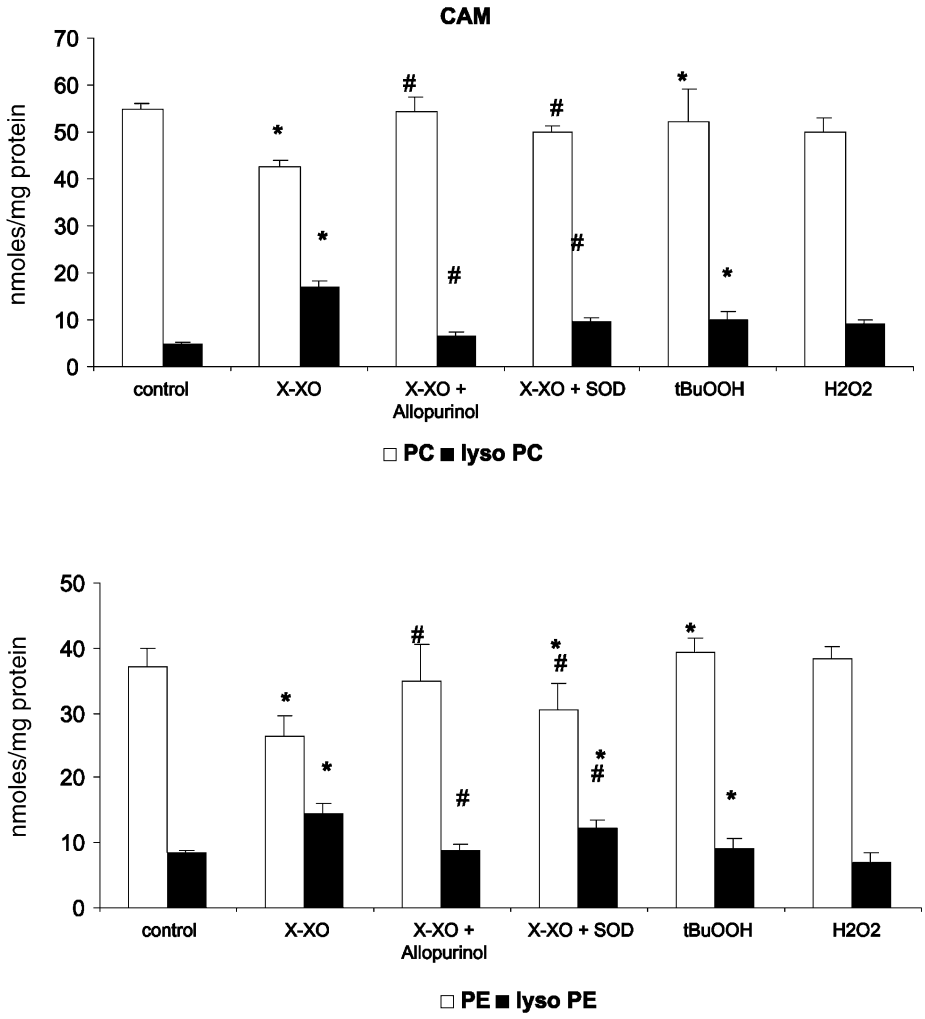

Fig. 5. Phospholipid changes in CAM after exposure to various oxidants. (a) PC and lyso PC. (b) PE and lyso PE. (Each value represents mean ± S.D. of three ($n=3$) separate animal experiments with duplicate estimations. *$P<0.05$ compared to control. #$P<0.05$ compared to X–XO). The final concentration of compounds used and incubation conditions are described in the text.

and b). Among the phospholipids, a decrease in PC and PE with a concomitant increase in lyso PC and lyso PE were seen on exposure of BBM to superoxide or tBuOOH (Fig. 4a and b), which was prevented by allopurinol or SOD. In the case of CAM, superoxide and not tBuOOH was able to alter the phospholipid composition. Degradation of PC and PE to lyso PC and lyso PE were seen on exposure to superoxide (Fig. 5a and b). H_2O_2 exposure did not alter these phospholipids either in the BBM or CAM. There was no alteration in other phospholipids or neutral lipids either in BBM or CAM on exposure to oxidants. Membranes either incubated with xanthine or xanthine oxidase alone were similar to incubated control membrane (data not shown).

4. Discussion

In the present study a comparison was made on the susceptibility of small intestinal BBM and CAM to oxygen free radicals. These membranes seem to differ in their enzyme activities, sugar content and lipid composition. The luminal contents with which they are in contact are also different. Small intestinal BBM is in contact with dietary materials, secretions from bile and pancreas, which contain variety of lytic enzymes, deter-

gents and intestinal secretions. On the other hand, CAM is in contact with a variety of bacteria along with unabsorbed dietary materials and their degradation products. Hence it is likely that they may differ in their composition, which is seen in this study, specifically, differences in the sugar content and lipid composition and an altered C/P ratio.

ROS have been implicated in the pathophysiology of certain gastrointestinal diseases (Parks, 1989), including IBDs such as Crohn's disease, ulcerative colitis (Pavlick et al., 2002; Babbs, 1992) and colorectal cancer (Babbs, 1990; Blakeborough et al., 1989). In these conditions inflammation is normally seen in the distal ileal and colonic mucosa and ROS formed by activated neutrophils play a role in damage to the mucosa (Otamiri, 1989). In addition, inflammatory cells from patients with Crohn's disease or IBD generate significantly higher levels of ROS than inflammatory cells isolated from normal humans (Williams, 1990a,b). Small intestine is susceptible to ROS generated during hypoxia and these ROS are generated mainly by XO, which is present abundantly in this tissue and by infiltered phagocytes. These effects of ROS might lead to membrane damage which may facilitate entry of luminal contents including bacteria and bacterial products in to systemic circulation. We have earlier shown that during surgical stress to the small intestine, ROS are generated by activation of XO resulting in structural and functional alterations to the BBM (Prabhu et al., 2000). Very little information is available on the susceptibility of CAM to oxidants and in the present study, a comparison was made between CAM and BBM on their susceptibility to oxidants. These membranes were found to be susceptible to superoxide generated by X–XO, which was confirmed by inhibiting XO by allopurinol or scavenging superoxide by SOD. These alterations in the membrane include decrease in ALP activity, lipid and protein oxidation and altered lipid composition. It has been shown earlier that activity of ALP is decreased on exposure to superoxide (Dudeja and Brasitus, 1993). Lipid peroxidation was seen both in BBM and CAM although to a lesser extent as compared to other membranes and this is similar to an earlier report on the effect of oxidants on monkey intestinal BBM (Nalini et al., 1993). Lipids in the membrane are known to be susceptible to ROS attack and in addition to lipid peroxidation, they can also undergo phospholipid degradation by the phospholipases (Nigam and Schewe, 2000; Goldman et al., 1992). Alterations in phospholipid composition suggest activation of PLA_2 by superoxide whereas other oxidants did not have any effect. It is known that PLA_2 is activated by superoxide (Madesh and Balasubramanian, 1997) and peroxides like t-BuOOH (Borowitz and Montgonem, 1989) and H_2O_2 (Birbes et al., 2000). In the present study, although superoxide exposure brought about phospholipid degradation, no alteration was seen on exposure to H_2O_2. PLA_2 activation during intestinal ischemia/reperfusion has been shown (Otamiri et al., 1988, 1987) and ROS has been implicated in the PLA_2 activation. To our knowledge, there is no report on the susceptibility of CAM to oxidants although these membranes are known to be exposed to ROS during clinical situations such as inflammation of the colon which can lead to increased permeability, altered secretion and cellular toxicity. In conclusion, this study has compared the susceptibility of BBM and CAM to oxidants and although they differ in their composition, they are similarly affected by oxidants.

Acknowledgments

The Wellcome Trust Research Laboratory is supported by the Wellcome Trust, London. The financial assistance from the Council of Scientific and Industrial Research, Government of India, is gratefully acknowledged. R. Prabhu is a Senior Research Fellow of the Council of Scientific and Industrial Research, Government of India.

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