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Effect of oxidant exposure on monkey intestinal brush-border membrane

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This study looks at the effect of oxidant exposure on changes in structural components and functional properties of monkey intestinal brush-border membrane vesicles (BBMV). These membranes were found resistant to iron-dependent lipid peroxidation as judged by measurement of various parameters such as formation of malonaldehyde (MDA) and conjugated diene and depletion of total arachidonic acid, tocopherol and membrane-associated protein thiol groups. Free radicals generated by thermal decomposition of 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP) which does not require iron, were capable of inducing lipid peroxidation in this membrane. Fluorescence polarisation studies used to assess the physical state of the membrane lipids after exposure to various free radical generating systems showed that ABAP could decrease the fluidity of BBMV whereas other systems had no effect. Exposure of BBMV to ABAP or cumene hydroperoxide decreased the glucose and amino acid transport. BBMV had a high content of nonesterified fatty acids as part of the total lipids and removal of these free fatty acids by treatment with fatty acid free albumin made the membranes susceptible to iron-dependent peroxidation. These studies suggest that intestinal epithelial cell membranes are resistant to iron-dependent lipid peroxidation due to the presence of membrane-associated free fatty acids. Possibly lipid peroxidation may play a less significant role in damage to these cells.

Introduction

Nutrient absorption is the major function of intestinal mucosa and any damage to the epithelial cell membrane might lead to malabsorption. Oxygen-derived free radical mediated damage has been implicated in the pathophysiology of certain gastrointestinal diseases. Gastrointestinal epithelial cells are likely to be exposed to these active species generated both in the mucosa and in the lumen [1,2]. Sources of free radicals in the mucosa include activated neutrophils, high activity of the enzyme xanthine oxidase and the normal mitochondrial oxidation. In the lumen, dietary materials such as ascorbic acid, transition metals, peroxidized lipids and bacterial metabolites form the source of free radicals.

Membrane lipids and proteins are the targets of free radicals and one of the mechanisms by which these active species damage cells is through lipid peroxidation. Biological membranes are readily susceptible to peroxidation damage since they are rich in polyunsaturated fatty acids as constituents of lipids. Our earlier work has shown that rat intestinal epithelial cell membranes are resistant to lipid peroxidation as judged by malonaldehyde production [3–5].

Intestinal microvillus membrane is highly specialized to perform transport and enzymatic functions essential for normal digestion and absorption. Since it is believed that lipid–lipid and lipid–protein interactions in the membrane play a major role in their function, it is important to study the effect of free radicals on these dynamic features in membrane components in order to understand the molecular mechanism mediating the modifications of the membrane functions induced by free radical damage. Earlier studies using fluorescent probes have shown that lipid peroxidation alter the fluidity of rat liver plasma membrane and certain tumour cell membranes [6]. Studies on porcine intestinal brush-border membrane vesicles have shown an increase in molecular rigidity of the protein conformation by lipid peroxidation [7]. The present study looks at the effect of in vitro exposure of monkey intestinal brush-border membranes to variety of oxidants by quantitating products of lipid peroxidation, assessing physical changes in the membrane by fluidity measurements and functional changes by studying transport of glucose and amino acids.

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Materials and Methods

Hepes, 2-thiobarbituric acid, bovine serum albumin (BSA), ascorbic acid, Tris, α-tocopherol, standard fatty acids, xanthine, xanthine oxidase, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), pyrene and 1,6-diphenyl-1,3,5-hexatriene (DPH) were all purchased from Sigma. 2,2′-Azobis(2-amidinopropane) dihydrochloride (ABAP) was obtained from Polysciences, USA, D-[^{14}C]glucose and L-[^{3}H]leucine were obtained from Bhabha Atomic Research Centre, Bombay. All other chemicals used were of analytical grade.

Isolation of brush-border membrane vesicles

A modified Kessler's method [8] was used to prepare BBMV from monkey intestinal mucosa. Overnight fasted monkeys (*Macacca radiata*) were killed by Nembutal injection and contents of the small intestine were washed thoroughly with ice-cold saline. Mucosa was scraped using a glass slide and stored at −20°C in aliquots of 5 g. Approximately 2–3% homogenate of mucosa was prepared in 6 mM Tris buffer (pH 7.5) containing 150 mM mannitol by homogenising in a Waring blender for 2 min at full speed. This was allowed to stand for 10–15 min and filtered through nylon mesh. 1 M $MgCl_2$ was added to a final concentration of 10 mM to the above homogenate, stirred for 1 min and allowed to stand for 15 min. This was centrifuged at $3000 \times g$ for 15 min, the pellet discarded and the supernatant was spun at $27000 \times g$ for 30 min. To the pellet, 0.5 ml preloading buffer (10 mM Hepes-Tris, 300 mM mannitol (pH 7.5)) was added and suspended gently using 1 ml syringe fitted with 26 gauge needle, five to six times. The suspension was diluted to 30 ml with the same buffer and spun down at $27000 \times g$ for 30 min. The above step was repeated once more and the final pellet was suspended in preloading buffer and used for further studies. Purity of isolated BBMV was checked by enrichment of the marker enzyme alkaline phosphatase [9] and vesicle formation using electron microscopy. Protein was measured using BSA as standard [10].

Removal of free fatty acids from BBMV

Isolated BBMV were divided into two portions and one portion was treated with fatty acid free albumin corresponding to 12 times the protein concentration of BBMV protein. The other untreated portion was taken as control. They were incubated at 4°C for 30 min followed by centrifugation at $27000 \times g$ for 30 min. The pellet was washed twice with the buffer, the final BBMV was suspended in the pre-loading buffer.

Peroxidation of BBMV

Peroxidation was induced by exposing BBMV to various free radical generating systems. BBMV corresponding to 1 mg protein in a total volume of 1 ml pre-loading buffer were incubated at 37°C for 30 min separately with the following radical generating systems. (a) Ascorbate 500 μM + ferrous sulfate 5 μM, (b) cumene hydroperoxide 100 μM, (c) H_2O_2 10 μM + ferrous sulfate 10 μM, (d) xanthine 1 mM + xanthine oxidase 10 milliunits, (e) ABAP 50 mM (all final concentrations). Control incubation had only membrane and buffer. Osmolality of the incubation buffer after addition of various components was measured using Wescor osmometer and maintained around 300 mosmol/kg by varying the mannitol content. Concentration of mannitol used in the incubation buffer did not inhibit peroxidation as confirmed by measurement of MDA production from liver microsomes in presence of mannitol.

Peroxidation parameters studied

(a) MDA production. After incubation of BBMV with the above systems, reaction was stopped with trichloroacetic acid and MDA in the protein free supernatant was measured using thiobarbituric acid [11]. Amount of MDA formed was calculated from standard curve prepared using 1,1′,3,3′-tetramethoxypropane and values expressed as nmol per mg protein.

(b) Conjugated diene. Total lipids from incubated BBMV were extracted as described [12], dissolved in 1 ml heptane and read at 233 nm. The amount of conjugated diene formed was calculated using a molar absorption coefficient of $2.52 \cdot 10^4$ and expressed as nmol/mg protein.

(c) Total arachidonic acid. Total lipids obtained from control and various peroxidized BBMV preparations were hydrolysed and methylated using methanolic HCl. Fatty acid methyl esters were quantitated after separation using Pye Unicam gas chromatograph fitted with 5% EGSS-X column and Spectraphysics integrator. Heptadecanoic acid was used as internal standard.

(d) Protein thiol group estimation. Thiol groups of the membrane protein in control and peroxidized BBMV were measured using DTNB after precipitation with trichloroacetic acid as described [13] and expressed as nmol/mg protein.

(e) Tocopherol estimation. α-Tocopherol content of control and peroxidized BBMV was measured using HPLC. Membrane tocopherol was extracted as described for liver microsomes [14] and quantitated using Shimadzu 6A HPLC as described [15]. Separation was achieved with silica column using the solvent system hexane/methanol 98:2 with a flow rate of 1 ml/min and the tocopherol was detected at 294 nm using UV detector. Extracted total lipids from control and peroxidized BBMV were used to measure total cholesterol [16] and total phospholipids were quantitated after acid digestion and phosphate estimation [17].

Measurement of sugar and amino acid transport

Control and oxidant exposed BBMV were tested for their ability to transport sugars and amino acids. Transport studies were carried out using labelled glucose and leucine. Uptake measurements were carried out by rapid filtration technique using Millipore filters (pore size 0.45 μm) [18]. Uptake was initiated using 50 μl of control and peroxidized BBMV suspension corresponding to 100 μg protein. This was incubated with 150 μl uptake buffer (150 mM NaSCN, 10 mM Hepes-Tris (pH 7.5) containing either 50 μM [^{14}C]glucose or 0.5 μM [^{3}H]leucine). At 20 s uptake was stopped by adding 3 ml ice-cold stop buffer (10 mM Hepes-Tris, 150 mM NaCl (pH 7.5)) and filtered rapidly through membrane filter. The filter was washed three times with 5 ml of stop buffer and counted using LKB Rackbeta scintillation counter. Experiments were also done by inclusion of 1000 units of superoxide dismutase in the incubation mixture prior to transport measurements. Equilibrium uptake was performed after 30 min incubation with uptake buffer. Specificity of sodium-dependent transport was confirmed by inclusion of 1 mM phlorizin, a specific inhibitor of sodium dependent transport in the incubation mixture.

Fluorescence polarization studies

Fluorescence measurements were performed on both control and peroxidized BBMV using Shimadzu RF-5000 spectrofluorometer. Both steady-state fluorescence polarization using DPH and excimer fluorescence intensity using pyrene were used to estimate relative membrane fluIdity [19]. For steady state fluorescence a 2 mM stock solution of DPH in tetrahydrofuran was prepared and stored protected from light at -20°C. Just prior to use, an aqueous suspension of the probe was prepared by diluting DPH stock solution in 2000 volume of 0.02 M phosphate buffered saline (pH 7.4) and stirred vigorously for 2–3 h at 25°C until no odour of tetrahydrofuran could be detected. The resulting dispersion of 2 μM DPH was clear and devoid of fluorescence. Control and peroxidized membranes, equivalent to 100 μg protein were incubated in 3 ml of DPH suspension at 37°C for 1 h. Fluorescence emission intensities (excitation wavelength 360 nm, emission wavelength 430 nm) were recorded parallel and perpendicular to excitation plane. Fluorescence polarization was expressed as fluorescence anisotropy, r, determined from the equation $r = (I_2 - I_1)/(I_2 + 2I_1)$, where I_2 and I_1 are fluorescence intensities parallel and perpendicular to the excitation plane. The anisotropy parameter $(r_0/r - 1)^{-1}$ was calculated using the value of $r_0 = 0.362$ for DPH [20]. According to the Perrin equation, a form of which can be written as $r_0/r = 1 + 3t/p$ where t is the excited state life time and p is the rotational relaxation time of the probe. These values vary directly with p under conditions of constant t and are therefore considered to be inversely related to fluidity [21–23].

Excimer fluorescence intensity was studied using pyrene as the probe. Pyrene 100 μm (final concentration) was incubated with BBMV corresponding to 2 mg protein (control and peroxidized) in 0.1 M Tris-HCl buffer pH 7.1 for 2 h at 37°C. After incubation, it was centrifuged at 25 000 $\times g$ for 20 min and the pellet was washed twice with 10 mM Tris-HCl buffer (pH 7.1) and resuspended in the same buffer. An aliquot corresponding to 100 μg protein in a total volume of 2 ml of 0.1 M Tris-HCl (pH 7.1) was used for fluorescence measurements. Fluorescence intensity of the incorporated pyrene was measured at an excitation wavelength

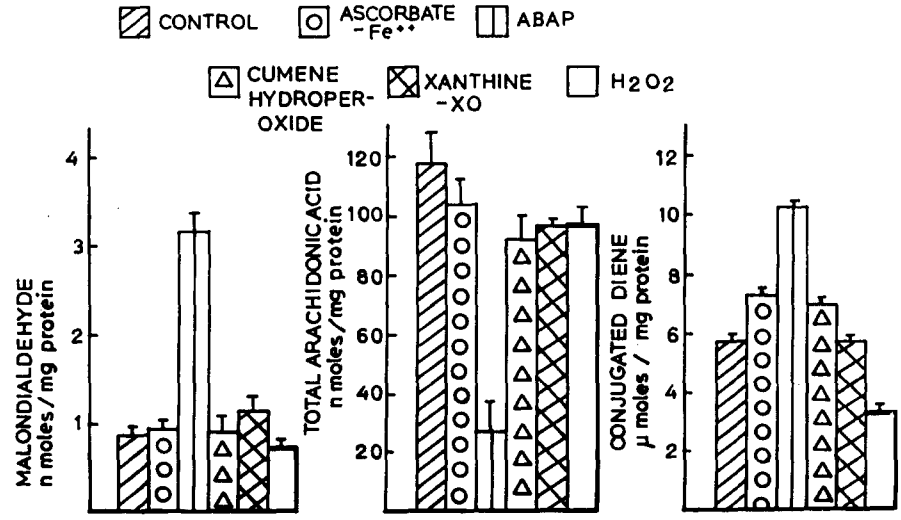

Fig. 1. Effect of oxidants on lipid peroxidation of monkey intestinal brush-border membranes. Each value represents mean ± S.D of six separate experiments. Experimental details are given in the text.

of 340 nm, the monomer fluorescence was measured at 390 nm and the excited dimer or excimer fluorescence at 465 nm. Estimation of excimer/monomer fluorescence intensity ratio provides a convenient index of fluidity.

Results

BBMV isolation

Nearly 15-fold purification was achieved on the brush-border membrane vesicles prepared from monkey small intestinal mucosa as judged by marker enzyme, alkaline phosphatase (specific activity 0.061 ± 0.011 and 0.922 ± 0.071 μmol/min per mg protein in homogenate and BBMV, respectively). Representative samples were found to be free of contamination with basolateral membranes as assessed by the absence of Na^+/K^+-ATPase. Examination under electron microscope showed that the membrane preparation was homogeneous and vesicular.

Peroxidation parameters

The extent of lipid peroxidation in BBMV which were exposed to different free radical generating systems was assessed by measuring parameters such as MDA, conjugated diene and arachidonic acid content and are shown in Fig. 1. Exposure to free radicals generated from ABAP showed significant increase in the formation of MDA and conjugated diene and decrease in total arachidonic acid. All other systems used did not show significant alteration in any of the peroxidation products as compared to control BBMV. Similar observation was made regarding the loss of protein thiol groups and α-tocopherol in the BBMV exposed to various oxidants (Fig. 2). Only ABAP was able to decrease protein thiol groups and it completely eliminated α-tocopherol content of the membrane. None of the other systems were capable of significantly altering these constituents of the membrane. Lipid composition of BBMV was analysed in order to assess the fatty acid composition and alteration in lipid constituents. This membrane had considerable amount of polyunsaturated fatty acids including arachidonic acid and in addition it had a high content of nonesterified fatty acids, about 120 nmoles of free fatty acids per mg protein. Composition of various fatty acids in total and nonesterified fatty acid fraction are shown in Fig. 3. It was observed that oxidant exposure did not alter the content of total cholesterol or phospholipids of the membrane (data not shown).

Membrane transport studies

Transport of glucose and amino acid were compared with control and oxidant exposed BBMV. Uptake of D-glucose and L-leucine measured at 20 s was decreased in ABAP and cumene hydroperoxide exposed

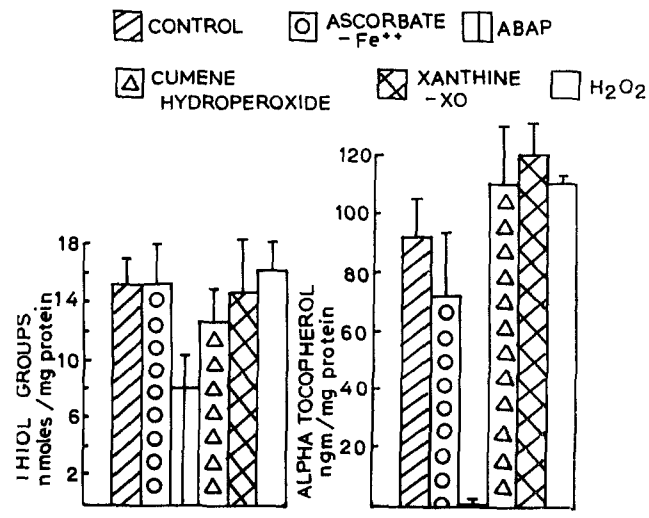

Fig. 2. Effect of oxidants on the protein thiol levels and tocopherol content of monkey intestinal brush-border membranes. Each value represents mean ± S.D of four separate experiments. Experimental details are given in the text.

BBMV as compared to control and inclusion of superoxide dismutase in the incubation system did not prevent the loss of glucose and amino acid uptake (Fig. 4a and b). The equilibrium uptake after exposure to cumene hydroperoxide was 0.24 ± 0.07 nmol/mg protein and 1.87 ± 0.11 pmol/mg protein compared to the control values of 0.21 ± 0.05 nmol/mg protein and 2.98 pmol/mg protein for [^{14}C]glucose and [^{3}H]leucine, respectively. But the same values after exposure to ABAP was 0.023 ± 0.004 nmol/mg protein and 0.099 ± 0.017 pmol/mg protein, suggesting a complete damage to the membrane vesicle. Comparison of kinetic constants of D-glucose transport by control and cumene hydroperoxide exposed BBMV showed a similar K_m of 67.9

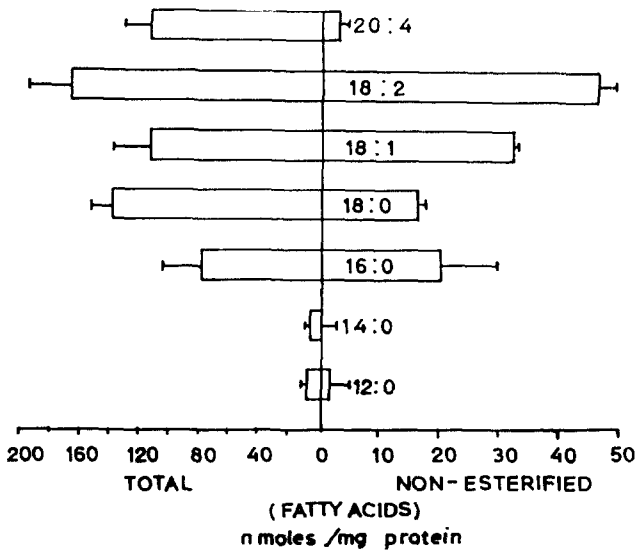

Fig. 3. Total and non-esterified fatty acid composition of the brush border membrane vesicles. Each value represents mean ± S.D of four separate experiments.

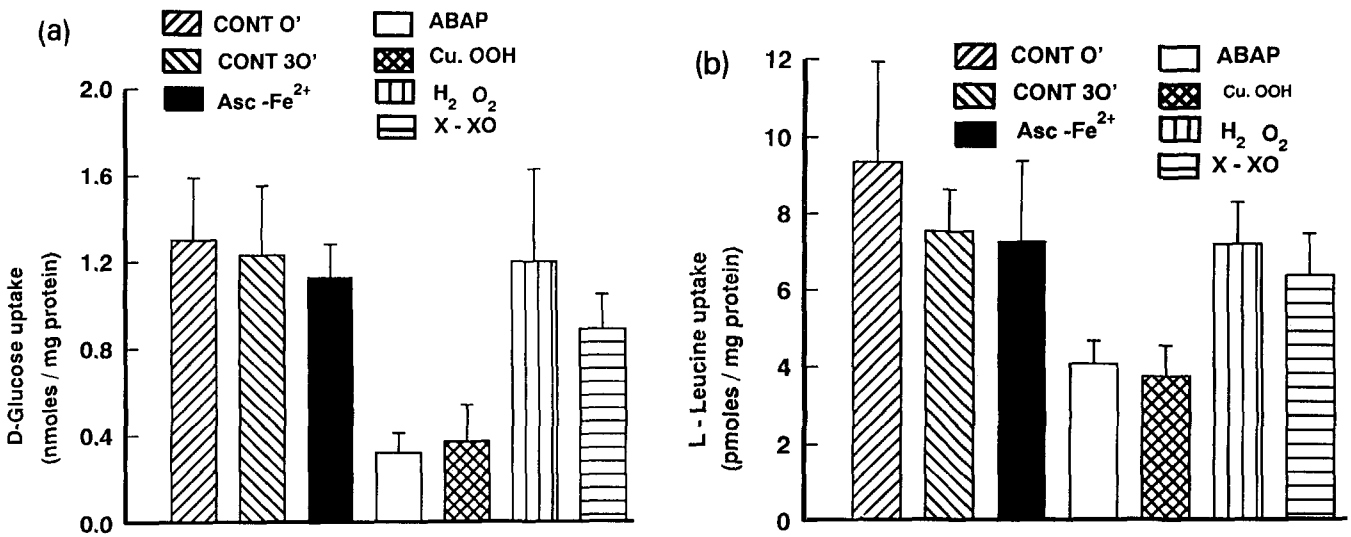

Fig. 4. D-Glucose (a) and L-leucine (b) uptake by control and oxidant exposed intestinal BBMV. Experimental details are given in the text. Each value represents mean ± S.D of three separate experiments. Equilibrium values of D-glucose uptake at 30 min range from 210 to 230 pmoles D-glucose/mg protein and for L-leucine from 2 to 3 pmoles L-leucine/mg protein under experimental conditions.

TABLE I

Fluorescence polarization studies on monkey intestinal brush-border membrane vesicles using DPH and pyrene

r_0, the limiting anisotropy for the probe. r_0 for DPH is 0.362.

	DPH		Pyrene
	Anisotropy (r)	$\left(\frac{r_0}{r}-1\right)$	excimer/monomer ratio
Control	0.251 ± 0.005	2.256 ± 0.14	0.354 ± 0.09
Asc-Fe^{2+}	0.255 ± 0.002	2.374 ± 0.08	0.345 ± 0.08
ABAP	0.277 ± 0.005	3.251 ± 0.25	0.313 ± 0.06
X-XO	0.253 ± 0.003	2.314 ± 0.11	0.347 ± 0.11
H_2O_2	0.254 ± 0.003	2.370 ± 0.08	0.317 ± 0.08

± 0.11 μM whereas the V_{max} was reduced from 480 pmol/mg protein for control to 165 pmol/mg protein for the cumene hydroperoxide exposed BBMV.

Membrane fluidity

To evaluate the effect of free radical exposure on the physical state of BBMV lipids, fluorescence anisotropy was measured using lipid soluble fluorophores DPH and pyrene. As shown in Table I there was a decrease in fluidity (increased molecular order) when BBMV were exposed to ABAP. Studies with DPH, which is a probe for rotational mobility, showed that only ABAP exposure could alter fluorescence anisotropy while with pyrene, which is a probe for lateral mobility, both ABAP and H_2O_2 exposure could decrease fluidity.

Peroxidation of free fatty acid depleted BBMV

Treatment of BBMV with fatty acid free albumin removed nearly 80% of free fatty acids associated with the membrane. Albumin treatment reduced the free fatty acid concentration from 127 ± 32 nmol/mg protein for normal membranes to 25 ± 6 nmol/mg protein for albumin-treated membranes. No significant decrease in the cholesterol and phospholipid concentrations were observed after albumin treatment. These membranes showed peroxidation when exposed to iron dependent and independent peroxidation systems as

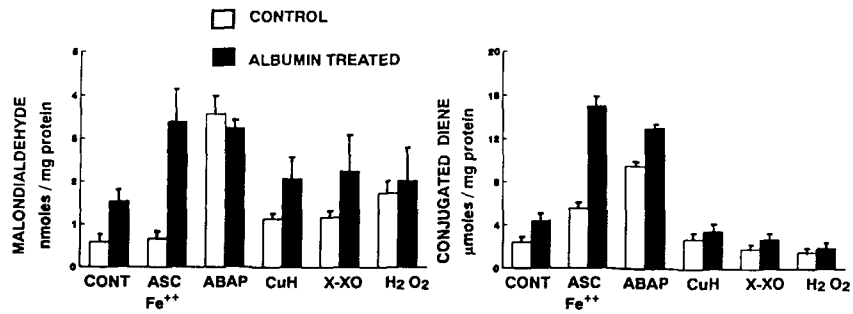

Fig. 5. Effect of oxidant exposure on formation of malonaldehyde and conjugated diene by free fatty acid depleted BBMV. Each value represents mean ± S.D of three separate experiments. Experimental details are given in the text.

judged by malonaldehyde production and conjugated diene formation (Fig. 5). This was quite apparent with iron/ascorbate system which showed negligible peroxidation with native membranes.

Discussion

Cellular membranes are the main targets of oxygen-derived free radicals which results in peroxidation of their lipids. Biologically important oxygen-derived free radicals are superoxide anion and hydroxyl radicals of which the latter species is very reactive and unstable. Transition metals such as iron, facilitate the formation of hydroxyl radical through Fenton reaction. Iron complex with protein such as ferritin or transferrin is unable to participate in Fenton reaction and very little free iron is normally present in tissues. An exception to this is the lumen and possibly mucosa of the gastrointestinal tract where dietary iron is present in free form which might facilitate the formation of these reactive species.

Earlier studies using rat intestinal microsomes and mitochondria have shown that these organelles are resistant to in vitro lipid peroxidation as measured by malonaldehyde production [3–5]. In the present work, a detailed study was undertaken to assess the extent of peroxidation of BBMV isolated from monkey intestine after exposure to various oxidants by studying different parameters. Free radicals were generated using different oxidants which included iron dependent and independent systems. Among the oxidants used, ABAP generates free radicals by thermal decomposition and does not require iron [24]. X-Xo generates superoxide and possibly hydroxyl radicals and controversy exists regarding the requirement of iron for this reaction [25,26]. All other systems used in the present experiment require iron as an essential component for free radical generation. This study has shown that only ABAP is capable of inducing lipid peroxidation on monkey intestinal BBMV as judged by production of MDA, conjugated diene and depletion of arachidonic acid. BBMV was resistant to peroxidation using all other iron-dependent systems.

Tocopherol can scavenge free radicals and during this process it is depleted in the membrane. Tocopherol content of BBMV was decreased considerably only after exposure to ABAP whereas other oxidants did not alter its content in the membrane. Cellular proteins contain thiol groups which are known to be oxidized when exposed to free radicals [27]. A measure of the content of protein thiol group is an indication of damage to the system on exposure to free radicals. In the present study, BBMV associated protein thiol groups were decreased after exposure to ABAP and to a lesser extent with cumene hydroperoxide whereas none of the other oxidants had any significant effect. This once again supports the observation that BBMV only susceptible to iron-independent free radical damage. Earlier studies on the interaction of flurogenic thiol reagents to BBMV proteins showed that the binding was decreased in peroxidized membranes which was attributed to changes in protein conformation due to peroxidation leading to masking of protein thiol groups rather than decrease in total thiol groups in the membrane proteins [7]. These studies have used ascorbic acid/Fe^{2+}/tBuOOH to peroxidize the porcine intestinal BBMV. Our results also show that iron-dependent peroxidation did not alter protein associated thiol groups.

Peroxidation of membrane lipids might result in structural and functional alteration of the membrane and decrease in membrane fluidity has been shown. In the present study, fluorescence polarization was used to measure the fluidity of the membrane after exposure to free radicals. Fluorescence anisotropy measurements were made using two different lipid soluble probes, DPH and pyrene. In bilayer membranes, measurement of fluorescence polarization of DPH provides an estimate of environmental resistance to the rotational mobility of this probe, whereas the intensity of the excimer fluorescence of pyrene molecule is dependent on the rate constant for formation of dimer. The rate constant is diffusion controlled which is limited by the rate of the lateral diffusion of the pyrene molecules and therefore the fluidity of the membrane lipid. Hence the measurement of fluorescence with these probes is a direct measure of the fluidity of the membrane. ABAP exposure resulted in decrease in fluidity of BBMV using both these probes and H_2O_2 treatment with pyrene as probe revealed a decrease in fluidity whereas other systems did not have any effect. This suggests that free radicals alter the physical state of the membrane lipid and only iron-independent free radical generation can alter BBMV fluidity. It has been previously demonstrated that lipid peroxidation of the porcine intestinal brush-border membrane is dependent on the membrane surface charge [28] and the inhibitory effect of α-tocopherol on lipid peroxidation is closely related to its stabilizing effect on membrane lipids [29].

Alteration in the physical state of the lipids and proteins can alter the absorptive function of the membrane. To test this, transport of sugars and amino acids were studied using BBMV after exposure to different free radical generating systems. Incubation with ABAP or cumene hydroperoxide resulted in alteration of glucose and amino acid transport by BBMV whereas other treatments had no effect. Inclusion of superoxide dismutase along with the oxidants failed to protect the membranes. Equilibrium uptake studies using ABAP exposed BBMV showed a complete loss of uptake suggesting that the decrease in transport is not due to inhibition of the Na^+/D-glucose cotransporter but may

be due to the complete damage to the membrane. This reveal the role of iron-independent peroxidation in damaging BBMV. Even though a decrease in transport by cumene hydroperoxide exposed BBMV was observed, equilibrium uptake studies suggested no damage to the membrane and kinetic analysis showed no alteration in K_m but a decrease in V_{max}. This observation may be due to alteration of membrane associated thiol groups, since thiol groups have been shown to be important for transport [30,31]. It has been shown that cyclosporin A, a nephrotoxic agent can induce lipid peroxidation in kidney BBMV resulting in increased permeability and altered glucose transport [32]. Mitomycin C, an antitumour drug also induces lipid peroxidation and decrease glucose and alanine transport across the rat intestinal BBMV [33].

The present study has clearly shown that the intestinal BBMV are resistant to iron-dependent lipid peroxidation as assessed by quantitation of various products of lipid peroxidation after exposure to different oxidants. This was not due to lack of polyunsaturated fatty acids in the membrane lipids since enterocyte membranes had similar PUFA content as other known peroxidising tissues such as liver. BBMV had high levels of NEFA as part of total membrane lipids and similar observation had been reported by others [34]. The resistance of BBMV to peroxidation may be due to the presence of high levels of NEFA and possibly free fatty acids forms a complex with iron which is incapable of inducing peroxidation. A role for fatty acid-iron complex in iron transport into epithelial cells has been suggested [35,36]. Formation of fatty acid-iron complex has been observed by us (unpublished observation) and a similar observation has been reported recently on the complex formation between free fatty acid associated with cigarette smoke and iron [37]. Further it was shown in the present study that free fatty acid depleted BBMV was susceptible to iron induced peroxidation supporting the hypothesis that the formation of free fatty acid-iron complex is responsible for the resistance of BBMV to peroxidation. This observation is significant since in vivo epithelial cells lining the gastrointestinal tract are continuously exposed to dietary free iron and presence of protective mechanisms in these cells might prevent their damage due to lipid peroxidation.

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