Lipid peroxidation of colonocyte membranes

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Received 25 July 1994; revised 16 January 1995

Effects of various oxidants on the colonic membrane lipid peroxidation have been studied in rats. 2,2'-Azobis (2-aminopropane) dihydrochloride (ABAP), which generates free radicals by thermal decomposition, induced peroxidation as judged by the formation of conjugated diene, malondialdehyde (MDA), and depletion of arachidonic acid. Exposure to other oxidants which require free iron for peroxidation was ineffective. Alpha tocoherol level was not altered on exposure to various oxidants except with ABAP which depleted its level in these membranes. Exposure of the membranes to both ABAP and xanthine-xanthine oxidase (X-XO) decreased total protein thiols, whereas other oxidants had no significant effect. Isolated colonocyte membranes were found to contain considerable amount of nonesterified fatty acids as part of the total lipids and removal of free fatty acids from the membrane using fatty acid-free albumin made the membranes susceptible to iron-induced free radical generation and lipid peroxidation. These studies suggest that colonocytes are possibly protected from lipid peroxidation by the free fatty acids associated with the membrane.

Cells lining the gastrointestinal tract, particularly in the large intestine, are likely to be exposed to oxygen-derived free radicals generated both within the mucosa and in the lumen. Potential sources of free radicals in the lumen include unabsorbed dietary material which is likely to contain oxidants such as iron, ascorbic acid, bile acids, and peroxidized lipids along with contents of the desquamated cells. Number of bacteria colonize the large intestine and their metabolites also form a source of free radicals. Due to the presence of bacteria and their metabolic products, a large number of phagocytes are attracted towards the mucosa which during oxidative burst release free radicals. Involvement of free radicals in the pathophysiology of gut mucosal inflammatory conditions such as ulcerative colitis has been suggested and the drug, 5-aminosalicylic acid used for treatment has been shown to scavenge free radicals.

One of the mechanisms by which free radicals damage the cells is through peroxidation of membrane lipids and presence of free iron facilitates the induction of peroxidation. We have earlier shown that due to the presence of high level of nonesterified fatty acids (NEFA), small intestinal mucosal membranes are resistant to iron-induced lipid peroxidation. Very little is known on the damaging mechanism of free radicals on colonic mucosa, although these reactive species have been implicated in the pathophysiology of this tissue. Hence the present study looks at the effect of various oxidants on the colonic membrane lipid peroxidation.

Materials and Methods
Alpha tocopherol, authentic fatty acids, xanthine, xanthine oxidase, cumene hydroperoxide, thiobarbituric acid, 1,1',3,3'-tetramethoxy propane, bovine serum albumin, Tris and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. 2,2'-Azobis (2-aminopropane) dihydrochloride (ABAP) was obtained from Polysciences Inc., USA. All the solvents were redistilled before use and for HPLC, special grade solvents were used. All other chemicals used were of analytical grade.

Total membrane preparation—Overnight fasted adult rats weighing around 200 g were killed by decapitation. Colon was removed, washed with ice-cold Krebs-Henseleit (KH) buffer (pH 7.4) and colonocytes were isolated from everted colon using EDTA chelation method. The isolated cells were suspended in oxygenated calcium containing KH buffer. More than 75% of the cells obtained were viable as judged by trypan blue ex-
clusion. Total, cellular membranes were prepared from isolated colonocytes as follows. Suspended colonocytes in KH buffer were homogenized using Porter-Elvehjem homogenizer, centrifuged at 100,000 × g for 60 min and the pelleted membranes were suspended in normal saline (pH adjusted to 7.4). This was considered as the total membrane preparation and was used for further studies. Due to the low yield of isolated cells, colonocyte total membranes were used rather than any specific membrane fraction. Moreover preliminary results showed similarity of different subcellular fractions in their susceptibility towards various oxidants.

Peroxidation studies—Colonocyte membranes were exposed in vitro to different oxidants to induce lipid peroxidation. Cell membranes (1 mg protein/ml) in 0.1M tris. HCl buffer (pH 7.4) were incubated for 30 min at 37°C with each of the following free radical generating systems: (a) ascorbate (500 μM) + ferrous sulphate (5 μM), (b) cumene hydroperoxide (1 mM), (c) H2O2 (10 μM) + ferrous sulphate (10 μM), (d) xanthine (1 mM) + xanthine oxidase (10 μmin) + ferrous sulphate (10 μM), (e) ABAP (50 mM) [all final concentration]. Control had only membrane and buffer. After incubation, the reaction was stopped with trichloroacetic acid and malondialdehyde (MDA) in the protein-free supernatant was measured using thiobarbituric acid (TBA). MDA was calculated from a standard curve prepared using 1,1,3,3-tetramethoxy propane. Arachidonic acid and conjugated diene were also measured after exposure of membranes to oxidants. Conjugated diene content was obtained in the extracted total lipid by measuring the absorbance at 233 nm, and the amount calculated using the molar extinction coefficient of 2.52×104. Total arachidonic acid was measured in the extracted total lipids after hydrolysis and methylation using methanolic HCl and quantitation after separation by gas chromatography. Membrane tocopherol was extracted as described for liver microsomes, separated and quantitated by HPLC using silica column. Membrane associated protein-bound thiol groups were assayed after protein precipitation by trichloroacetic acid and quantitation using DTNB. Protein was measured using BSA as standard.

Lipid extraction and analysis—Total lipids were extracted from the membrane by Bligh & Dyer’s method and lipid analysis was carried out. Total cholesterol was estimated as described by Zlatkis et al. Total phospholipids were quantitated after acid digestion and the liberated phosphate was estimated as described. Individual phospholipids were separated on silica gel H thin layer plates using the solvent system, chloroform—methanol—acetic acid—water (25:15:4:2, v/v) and the separated phospholipids were identified by iodine exposure with corresponding standards, scraped from the plates, eluted with a solution containing chloroform—methanol—water (5:5:1, v/v) and quantitated after digestion followed by phosphate estimation. An aliquot of the total lipids was separated on silica gel G thin layer plates using the solvent system, hexane—diethyl ether—acetic acid (80:20:1, v/v) and the spot corresponding to nonesterified fatty acids was extracted and methylated with methanolic HCl. Fatty acid methyl esters were separated and quantitated by gas chromatography using Pye Unicam 4550 gas chromatograph equipped with flame ionization detector fitted with a 5% EGSS-X column. Heptadecanoic acid was used as internal standard. Individual fatty acids were identified and quantitated using authentic fatty acid methyl esters. TLC separated triglycerides and diglycerides were extracted and quantitated as described. Fatty acids of total lipid were also quantitated using gas chromatography after direct hydrolysis and methylation.

Removal of free fatty acids from colonocyte membrane—Non-esterified fatty acids associated with the membrane were removed by treatment with fatty acid-free albumin. Colonocyte membranes were divided in to two portions and one portion was treated with fatty acid-free albumin corresponding to 15 times the concentration of membrane protein to remove the free fatty acids from the membrane. The other untreated portion was taken as control. They were incubated at 4°C for 30 min with occasional mixing and centrifuged at 105000 × g for 60 min. The pellet was washed twice with KH buffer and the final membranes were suspended in normal saline.

Results
Colonocyte membranes were exposed to different oxidants and lipid peroxidation products, viz. formation of conjugated diene and malondialdehyde and disappearance of arachidonic acid and tocopherol, were measured. As shown in Fig. 1, MDA formation was observed on exposure to ABAP and to a lesser extent with H2O2 whereas conjugated diene formation was seen only with ABAP. About 75% of arachidonic acid depletion was observed on exposure of membranes to ABAP. Although these parameters represent the extent of peroxidation, their formation depends on the type of oxidant used for inducing peroxidation.
and possibly the biological material which undergoes peroxidation. Fig. 2 shows the level of tocopherol and protein associated thiols after exposure to different oxidants. After ABAP exposure, 90% decrease in tocopherol content was observed and protein thiol was decreased with ABAP and X-Xo treatment. Analysis of the lipid composition of the isolated colonocyte membrane showed that in addition to normally seen membrane lipids, considerable amounts of NEFA forming nearly 15% of the total lipids were also present (Table 1). Fatty acid compositions of total lipids and the NEFA fraction were analysed. It showed the commonly seen long chain fatty acids (Fig. 3) including polyunsaturated fatty acids. There was no alteration in the lipid constituents of the membrane after exposure to various oxidants (data not shown).

Colonocyte membranes when incubated with fatty acid-free albumin removed nearly 80% of free fatty acids associated with the membrane without any change in other membrane lipids. Native membranes had 59 nmole free fatty acids/mg protein which decreased to 11 nmole/mg protein after albumin treatment. Albumin-treated membranes, when incubated with oxidants requiring iron for induction of peroxidation, showed increased peroxidation as judged by MDA and conjugated diene formation (Figs 4, 5). Ascorbate/iron system showed the maximum increase in the MDA and conjugated diene formation after the removal of free fatty acid from the membrane. On the other hand, same amounts of conjugated diene and MDA were formed when native or free

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Table 1—Lipid composition of colonocyte membranes

<table>
<thead>
<tr>
<th>Lipid</th>
<th>mmoles/mg protein</th>
<th>Percentage</th>
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<tbody>
<tr>
<td>Total cholesterol</td>
<td>38.5 ± 2.6</td>
<td>11.59</td>
</tr>
<tr>
<td>Esterified cholesterol</td>
<td>2.2 ± 0.6</td>
<td>0.65</td>
</tr>
<tr>
<td>Nonesterified fatty acids</td>
<td>50.3 ± 8.4</td>
<td>15.14</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>72.8 ± 3.3</td>
<td>21.87</td>
</tr>
<tr>
<td>Diglyceride</td>
<td>33.0 ± 3.3</td>
<td>9.93</td>
</tr>
<tr>
<td>Total phospholipids</td>
<td>135.6 ± 0.7</td>
<td>40.81</td>
</tr>
<tr>
<td>Phosphatidyl choline</td>
<td>37.4 ± 4.4</td>
<td>32.01*</td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine</td>
<td>28.9 ± 2.9</td>
<td>24.81*</td>
</tr>
<tr>
<td>Spingomyelin</td>
<td>32.2 ± 8.2</td>
<td>27.59*</td>
</tr>
<tr>
<td>Phosphatidyl inositol +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidyl serine</td>
<td>18.7 ± 0.6</td>
<td>15.59*</td>
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*Represents the percentage of total phospholipids.

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Fig. 3—Total and nonesterified fatty acid composition of the colonocyte membranes (Each value represents mean ± SD of three separate estimations)
Fig. 4—Effect of oxidant exposure on formation of malondialdehyde by free fatty acid depleted colonocyte membranes. [Each value represents mean ±SD of three separate estimations. The figure shows control membrane, membrane incubated for 30 min without oxidants, ascorbate + ferrous sulphate, H₂O₂ + ferrous sulphate, ABAP, cumene hydroperoxide, xanthine + XO + ferrous sulphate]

Fig. 5.—Effect of oxidant exposure on conjugated diene formation by free fatty acid depleted colonocyte membranes. [Each value represents mean ±SD of three separate estimations. The figure shows control membrane, membranes incubated for 30 min without oxidants, ascorbate + ferrous sulphate, H₂O₂ + ferrous sulphate, ABAP, cumene hydroperoxide, xanthine + XO + ferrous sulphate]

fatty acid depleted membranes were exposed to ABAP. It appears that presence of free fatty acid in the membrane does not influence free radical generation from ABAP.

Discussion
Oxygen-derived free radicals may act as potential mediators of damage in the colonic mucosa, since there are numbers of sources for free radicals both in the lumen and in the mucosa. Resident bacteria in the lumen may aid in the generation of free radicals in two ways: (1) Many colonic bacteria are catalase negative and can generate considerable amount of H₂O₂ which may be present in the lumen. Presence of dietary free iron in the lumen facilitates the formation of active hydroxyl radicals on reacting with H₂O₂. (2) Presence of bacteria and their metabolic products can attract phagocytes to the tissue which during oxidative burst can release free radicals and thereby damage the mucosal cells. Peroxidation of membrane lipids is one mechanism by which free radicals damage cells resulting in structural and functional alterations.

Lipid peroxidation normally occurs in presence of trace amount of free iron which facilitate the generation of active hydroxyl radicals. In the present study, colonocyte membranes were exposed to various free radical generating systems and different parameters of lipid peroxidation were studied. It was seen that colonocyte membranes were resistant to lipid peroxidation when exposed to oxidants requiring iron for free radical generation. On the other hand, exposure of membranes to ABAP resulted in peroxidation. Compounds such as ABAP generate free radicals by thermal decomposition and do not require free iron. This is similar to our earlier observation on the small intestinal mucosal membranes which were also resistant to iron-induced lipid peroxidation. This seems to be unique to gastrointestinal mucosal membranes, since other tissues such as liver, brain and kidney are known to undergo lipid peroxidation in presence of trace amount of iron. It is possible that the organisation and composition of lipids might influence the susceptibility of membranes to peroxidation.

Composition of the lipids especially the unsaturated fatty acid content and the presence of antioxidants determine the susceptibility of membranes to peroxidation. Polyunsaturated fatty acid is not a limiting factor in these membranes since fatty acid analysis of colonocyte membrane lipids showed the presence of considerable amount of polyunsaturated fatty acids. Lipid analysis revealed that NEFA constituted a high proportion of the total lipids. Trace amounts of NEFA may be present in biological membranes but concentrations as seen in gut mucosal membranes are not normally present. Earlier studies with small intestinal mucosal membranes have suggested that NEFA are inherently present in these membranes.

Presence of NEFA in the membrane might influence the course of lipid peroxidation. We have earlier suggested that possibly NEFA forms a complex with iron and the iron-fatty acid complex is unable to induce peroxidation, unlike certain other iron complexes. Hence, only ABAP, which generates free radicals independent of iron, induced peroxidation of colonocyte membranes.
whereas other oxidants did not show any significant peroxidation. This was further supported by experiments in which removal of free fatty acids associated with the membrane made these membranes susceptible to iron-induced lipid peroxidation. Formation of iron-fatty acid complex and its role in iron transport by the intestinal brush border membranes have been suggested 28, 29.

Tocopherol is the membrane associated antioxidant which protects membranes from free radical damage and an alteration in its level is an indication of the extent of peroxidation damage in the membrane. Similarly free radicals might oxidize protein thiol groups leading to inactivation of certain enzymes and an elevation of protein thiol groups has been used as an indicator of oxidant mediated damage 30. Tocopherol and protein thiol levels in colonocyte membranes after exposure to different oxidants indicated that ABAP exposure completely depleted tocopherol and considerably reduced protein thiol levels whereas other oxidants did not have significant effect on these parameters. This further suggests that free radical generation and lipid peroxidation may not occur in presence of colonocyte membranes when oxidants requiring iron are used. MDA was measured as an index of lipid peroxidation in experimentally induced rat colon tumours and human colorectal cancer tissue 31, 32. These studies have shown that total amount of MDA measured and the changes in its level observed as an index of lipid peroxidation were very minimal as compared to other tissues known to undergo peroxidation. This further suggests that possibly under in vivo conditions too very minimal peroxidation might occur in colonic mucosa. In addition to lipid peroxidation, toxicity of free radicals is manifested in the form of protein modification or DNA damage. It has been shown that hypochlorite, an oxidant generated by the action of myeloperoxidase on $\text{H}_2\text{O}_2$ produced by neutrophils, damages the lysine and tryptophan residues of LDL proteins rather than peroxidizing lipids 33. In inflammatory conditions of the colon, large infiltration of phagocytes and the formation of free radicals have been established 4. The present study suggests that under these conditions possibly free fatty acids associated with the membrane might protect these membranes from lipid peroxidation.

Acknowledgement

The Wellcome Research Unit is supported by The Wellcome Trust, London. Financial assistance from the Department of Science & Technology, and Indian Council of Medical Research, Government of India is acknowledged. The authors thank Prof. VI. Mathan for his keen interest in this work.

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