Effect of Oxidant Exposure on Isolated Rat Colonocytes

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Background: The role of reactive oxygen species in injury to cells lining the gastrointestinal tract has gained importance in recent times, and their role in intestinal diseases has been studied. Methods: In this study colonic epithelial cells isolated from the rat colon were exposed in vitro to various oxidants such as menadione, xanthine-xanthine oxidase, hydrogen peroxide, cumene hydroperoxide, and tertiary butyl hydroperoxide, separately. Changes in cell viability, thiol status, and the antioxidant enzyme activities were measured. Results: Colonocytes were found to be sensitive to menadione and were not affected by various other oxidants. Decrease in cell viability, depletion of reduced glutathione and protein thiol, and change in antioxidant enzyme activities were observed when the cells were exposed to menadione. Conclusions: This study suggests that, unlike other cells, colonocytes are susceptible only to certain selective oxidants.

Key words: Antioxidants; colonocytes; oxidants; viability

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Gastrointestinal epithelium is likely to be exposed to various toxic compounds originating from food, bacterial metabolites, ingested drugs, and oxidants formed during metabolism. These compounds can generate free radicals on their own or during reaction with other compounds (1, 2). Oxygen-derived free radicals-mediated tissue damage is a well-known phenomenon. Colonic epithelial cells are in proximity to various free radical-generating systems, since the colonic lumen contains a large number of bacteria and their metabolic products; the presence of these bacteria attracts a large number of phagocytes, which, when activated, release superoxide radicals. The damage can be prevented by the presence of various protective mechanisms present in the cell. Like other tissues, the colonic epithelium is also equipped with defense mechanisms to inactivate these reactive oxygen species. Thiol compounds can protect the cells from oxidative stress, and glutathione is the most abundant low molecular weight cellular thiol. It has been shown that under conditions of oxidative stress, depletion of glutathione takes place, affecting the thiol redox status, and a linear correlation has been observed between loss of cell viability and loss of critical thiol groups present in membranes and enzymes (3, 4). Glutathione-associated enzymes such as reductase and peroxidase and the H2O2-metabolizing enzyme catalese are antioxidant enzymes that offer cells protection from the injurious effect of oxidants. Free radicals have been implicated in colonic disease such as ulcerative colitis, and the drug used for treatment, 5-aminosalicylic acid, is known to scavenge free radicals (5). In vitro studies using enterocytes have shown the damaging effect on exposure to oxidants (6, 7). In the present study isolated rat colonocytes were exposed to various oxidants, and cell viability and some of their protective components were studied.

MATERIALS AND METHODS
Reduced and oxidized glutathione, cysteine, cystine, bovine serum albumin (BSA), menadione, cumene hydroperoxide, tertiary butyl hydroperoxide, xanthine oxidase (XO), xanthine, 1-fluoro-2,4-dinitrobenzene (FDNB), nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), and 5,5’-dithiobis (2-nitrobenzoic acid) (DTNB) were all obtained from Sigma Chemical Co. All other chemicals used were of analytical grade.

Isolation of colonocytes
Overnight starved rats were killed by decapitation. Colon was removed and gently flushed with calcium-free Krebs-Henseleit (KH) buffer, pH 7.4. Colonocytes were isolated from everted colon as previously described (8). Isolated, washed cells were suspended in calcium-containing oxygenated KH buffer to a cell density of 2–3 × 10⁶ cells/ml. The viability of isolated cells was checked by means of release of lactate dehydrogenase (LDH), and the enzyme was assayed as described (9).

Oxidant exposure of isolated colonocytes
Exposure to oxidants was done by incubating colonocytes (2 × 10⁶ cells/ml) at 25°C with various concentrations of the following oxidants, separately: menadione (100 to 300 μM), H2O2 (0.1 mM to 1 mM), cumene hydroperoxide (50 to 200 μM), tertiary butyl hydroperoxide (50 to 200 μM), and XO (1 to 10 m unit with 1 mM xanthine) (all final con-
Centrations). Cell viability was assessed at different time intervals by LDH assay in both supernatant and cell suspension after centrifugation at 1000 g for 10 min. Total LDH activity of the cell suspension was measured by adding Triton X-100 to a final concentration of 5%, and the percentage of viable cells was calculated using the total activity.

**Estimation of thiols**

Colonocytes were exposed to different oxidants—menadione (100 μM), H₂O₂ (100 μM), X-XO (10 m unit XO + 1 mM xanthine)—separately for 30 min at 25°C. After incubation, proteins were precipitated by 5% trichloroacetic acid, and reduced glutathione (GSH) and oxidized glutathione (GSSG) in the acid supernatant were estimated by high-performance liquid chromatography (HPLC) after derivatization with FDNB on an aminoethyl column as previously described (10). Colonocytes exposed to menadione and H₂O₂ in the presence and absence of iron were used for measurement of protein-bound thiol groups. After incubation, proteins were precipitated with 10% trichloroacetic acid and centrifuged. After the pellet had been washed, the precipitate was dissolved in 100 mM Tris HCl buffer, pH 7.4, containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% sodium dodecyl sulphate (SDS), and the thiols were estimated by means of DTNB (11).

**Enzyme assays**

Antioxidant enzymes in the colonocytes were estimated after exposure to various oxidants and compared with those of control cells. Glutathione peroxidase (12), glutathione...
RESULTS

Freshly isolated colonocytes showed nearly 85-90% viability. Fig. 1 shows the percentage of viable cells when colonocytes were exposed in vitro to various oxidants at different time intervals. As can be seen, cell death followed a progressive time- and dose-dependent pattern when exposed to menadione. The viability of control cells also decreased slightly with time, but cell death occurred significantly after exposure to menadione (when treated samples were compared with control for 100 μM, 200 μM, and 300 μM menadione (p < 0.01)). There was no significant increase in cell death after exposure to H₂O₂, tertiary butyl hydroperoxide, cumene hydroperoxide, or X-XO systems as compared with control colonocytes. Fig. 2 shows the thiol status of the cells after exposure to different oxidants. There was no change in GSH level when cells without oxidants were incubated for 30 min. Exposure to 100 μM menadione for 30 min resulted in the depletion of nearly 60% of cellular GSH, and this was followed by a concomitant increase in GSSG level (p < 0.05 for menadione-treated samples when GSH and GSSG values were compared with control values). Exposure to X-XO or H₂O₂ did not alter the glutathione redox status of the cells. Protein-associated reduced thiols were decreased when exposed to either menadione or H₂O₂, and inclusion of a trace amount of iron did not enhance the decrease in protein-associated thiols. Fig. 3 shows the activity of certain cellular glutathione- and H₂O₂-utilizing enzymes after exposure to oxidants. Although menadione showed some decrease in catalase activity, it was not statistically significant, and other oxidants did not have any significant effect on these enzymes.

Fig. 2. Level of thiol compounds in isolated colonocytes in control and after exposure to various oxidants. Data represent mean ± SD of three separate experiments. Statistically significant (p < 0.05) change in reduced glutathione (GSH) and oxidized glutathione (GSSG) in menadione-treated groups. X = xanthine; XO = xanthine oxidase.

DISCUSSION

Colonic epithelial cells are likely to be exposed to free radicals generated within both the mucosa and the lumen. Infiltrating phagocytes in the mucosa during oxidative burst generate superoxide anion and H₂O₂, which in the presence of the neutrophil enzyme myeloperoxidase is converted to HOCl, and this compound is cytotoxic to the cells. In addition, mitochondrial oxidation releases a certain amount of superoxide radical, which can damage the intracellular constituents. Luminal contents, which are in constant contact with mucosal cells, contain drug and bacterial metabolites that form an important source of free radicals. Hence colonic cells are in proximity to these active species and are likely to be damaged by free radicals. Except for one report, in which it was shown that H₂O₂ exposure induces Cl⁻ secretion through prostaglandin stimulation, there is no detailed study on the effect of oxidants on colonocytes. Conditions were optimized for production of oxygen free radicals by selectively introducing different oxidant systems. Xanthine in the presence of xanthine oxidase undergoes oxidation to uric acid with the formation of superoxide radical, and superoxide dismutase converts superoxide to H₂O₂. The relative proportion of superoxide anion and H₂O₂ generated is dependent on pH and oxygen tension. H₂O₂ in the presence of transition metals is converted to highly active hydroxyl radicals. Menadione is a quinone that undergoes either one- or two-electron reduction, and in the presence of dioxygen the semiquinone radical can be reoxidized to the parent quinone with the concomitant formation of superoxide anion. tert-Butyl hydroperoxide is transformed in the cell to its allyloxy radical, which induces lipid peroxidation in the presence of metals (16). The oxidative stress induced by the oxidants results in the depletion of both soluble and protein
thiols and affects the antioxidant enzyme activities. A study on the antioxidant status gives the extent of injury caused by the free radicals produced by the various oxidant systems. In the present study isolated colonocytes were exposed in vitro to different oxidants, and their viability was tested along with alteration in some of the cellular protective systems.

Among the oxidants used only menadione showed cytotoxicity as judged by LDH release. Cells were more sensitive to 200 μM and 300 μM concentrations of menadione even at short time exposure. Luminal contents is known to contain quinones that are derived from bacterial metabolites. It has been shown that menadione mediates its cytotoxicity by altering adenosine triphosphatase (ATP)-generating pathways (17). It has been suggested that protein thiol loss disrupts the function of proteins critical to regulation of calcium homeostasis. This defect leads to increased levels of cytosolic calcium and the consequent loss of viability (18). Depletion of protein thiols leads to loss of viability, as shown in suspension of hepatocytes exposed to menadione (18), whereas change in protein thiols by H₂O₂ has been shown not to accompany the loss of viability (20). That oxidants are cytotoxic to various cells has been shown earlier (19, 20), but in the present study colonocytes were resistant to some of these oxidants. An earlier study has shown that although H₂O₂ exposure induced Cl⁻ secretion by the rat colon, it did not damage the colonocytes as assessed by LDH release and histology (21). A similar resistance to H₂O₂ was observed in this study.

Glutathione is the important cellular low molecular weight thiol that protects the cell from free radical-mediated damage. Glutathione may act either by protecting cells from lipid peroxidation or by protecting protein sulphydryl groups from becoming irreversibly oxidized after oxidant injury (22, 23). It was observed that only menadione exposure decreased the cellular glutathione; other oxidants did not have any significant effect. Superoxide radicals generated by menadione can oxidize GSH to GSSG, and an increase in GSSG level was observed after menadione exposure. A similar increase in oxidation of GSH was observed when enterocytes were exposed to menadione, and externally added GSH protected the cells from menadione toxicity (24). The GSSG formed can be reduced back to GSH by glutathione reductase, but the activity of this enzyme is regulated by the availability of NADPH. Oxidation of GSH is also likely to cause formation of mixed disulphide between glutathione and cellular proteins (25). A decrease in cellular protein thiol groups was observed on both menadione and H₂O₂ exposure, and the formation was not influenced by the presence of iron. Involvement of low molecular weight iron complex has been shown to catalyse cumene hydroperoxide-induced cell damage (26) and the effect of tertiary butyl hydroperoxide (27). In this study these two peroxides did not have any effect on colonocytes. This suggests that free iron possibly is not available for generation of active free radicals, and this is similar to our earlier observation of resistance of small-intestinal mucosal membranes to iron-induced lipid peroxidation (28, 29). It appears that the mech-
anion of protein thiol oxidation by menadione and H\textsubscript{2}O\textsubscript{2} is different. Menadione increases the formation of GSSG, which possibly forms mixed disulphide with protein thiol, thus reducing their level. On the other hand, H\textsubscript{2}O\textsubscript{2} may directly oxidize the protein-associated thiol groups without an increase in GSSG formation. Xanthine oxidase activity is very low in these cells and may not be the source for free radicals in the colon.

The activities of some of the enzymes involved in removing the oxygen-derived free radicals were measured in colonocytes after exposure to various oxidants. These oxidants did not significantly alter the activities of catalase and other glutathione-metabolizing enzymes in the cell. The present study has suggested that, unlike other cells, colonocytes are susceptible only to certain selective oxidants. This is physiologically significant since these cells are likely to be exposed to many oxidants.

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