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European Journal of Pharmacology 321 (1997) 113–119

ejp

# Antioxidant defenses of cultured colonic epithelial cells against reactive oxygen metabolites

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Received 12 September 1996; revised 12 November 1996; accepted 22 November 1996

**Abstract**

Reactive oxygen metabolites produce colonic epithelial cellular injury. The present study evaluated the protective role of cellular superoxide dismutase, catalase, and glutathione (GSH) redox cycle in cultured rabbit colonic cells. Cultured rabbit colonic epithelial cells were exposed to reactive oxygen metabolites generated by hypoxanthine (1 mM) and xanthine oxidase (1 mU/ml) for up to 5 h. Cytotoxicity was quantified by measuring $^{51}Cr$ release from prelabeled cells. Pretreatment with diethyldithiocarbamate (inhibitor of superoxide dismutase) reduced activity of cellular superoxide dismutase and increased $^{51}Cr$ release caused by hypoxanthine/xanthine oxidase from colonic cells. Pretreatment with diethyl maleate (covalently binds GSH as catalyzed by GSH transferase), or buthionine sulfoximine (inhibitor of γ-glutamylcysteine synthetase) decreased cellular GSH and enhanced reactive oxygen metabolites induced injury. Pretreatment with bis(chloroethyl)-nitrosourea (inhibitor of GSH reductase) inhibited activity of GSH reductase and increased $^{51}Cr$ release from colonic cells. Preincubation with aminotriazole (inhibitor of catalase) reduced cellular catalase, but did not affect cellular injury. Therefore, we concluded that both cellular superoxide dismutase and the GSH redox cycle appeared to play a role in detoxifying reactive oxygen metabolites and that cellular catalase may be less important in rabbit colonic epithelial cells.

*Keywords:* Oxygen metabolite, reactive; Superoxide dismutase; Glutathione redox cycle; Catalase; Colonic epithelial cell

## 1. Introduction

Reactive oxygen metabolites contribute to gastrointestinal injury in various pathological conditions such as ischemia-reperfusion injury (Granger et al., 1981, 1986; Kvietys et al., 1988) certain types of drug-induced gastroenteropathy (Szelenyi and Brune, 1988; Mutoh et al., 1990; Vaananen et al., 1991), necrotising enterocolitis (Clark et al., 1988), experimental colitis (Cueva and Hsueh, 1988; Von Ritter et al., 1989; Keshavarzian et al., 1990) and inflammatory bowel diseases (Craven et al., 1986; Tanner et al., 1984; Hermanowicz et al., 1985). Though reactive oxygen metabolites are involved in various pathological conditions in the colon, little is known about the mechanisms of how colonic cells detoxify reactive oxygen metabolites. The aim of the present study was to throw light upon the antioxidant mechanisms in colonic cells.

In some types of cells, cells may detoxify $H_2O_2$ by endogenous catalase and/or the glutathione redox cycle (Hiraishi et al., 1992, 1994). However, to date, the possible role of cellular superoxide dismutase, glutathione redox cycle, and catalase against reactive oxygen metabolites remains undetermined in colonic cells.

Diethyldithiocarbamate has been shown to inhibit the activity of superoxide dismutase (Heikkila, 1985; Heikkila et al., 1976). The activity of glutathione reductase can be selectively inhibited by 1,2-bis(chloroethyl)-1-nitrosourea (Nathan et al., 1980; Babson and Reed, 1978; Fischer and Ahmad, 1977). Cellular stores of GSH can be depleted by formation of a thioether conjugate with electrophilic agents such as diethyl maleate (Chasseaud, 1979) in a reaction catalyzed by endogenous glutathione S-transferase. GSH biosynthesis can be inhibited by buthionine sulfoximine, a selective inhibitor of γ-glutamylcysteine synthetase (Griffith and Meister, 1979; Meister and Anderson, 1983). In addition, another possible cellular antioxidant enzyme, catalase, may be inhibited by 3-amino-1,2,4-triazole

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*PII* S0014-2999(96)00929-6

(Margoliash and Novogrodsky, 1958). With the use of in vitro colonic culture model, we attempted to clarify the mechanism of colonic mucosal cells to protect themselves against oxidants. For this purpose, we investigated the effects of the above agents, which can inhibit intracellular superoxide dismutase, or disrupt the glutathione redox cycle, or inhibit endogenous catalase activity on oxygen metabolite-induced injury to cultured rabbit colonic epithelial cells.

## 2. Materials and methods

### 2.1. Animals and chemicals

Male New Zealand white rabbits weighing 2.5–3.0 kg (Charles River, Wilmington, DE, USA) were used. Basal medium Eagle (BME), *N*-2-hydroxyethyl piperadine-2-ethanesulfonic acid (Hepes) buffer, bovine serum albumin, hypoxanthine, xanthine oxidase (grade III, lot no. 88F-3837), 1,2-bis(chloroethyl)-1-nitrosourea, diethyl maleate, buthionine sulfoximine, 3-amino-1,2,4-triazole, diethyldithiocarbamate, NADPH, Triton X-100, superoxide dismutase, hydrogen peroxide (30% solution) and EDTA were purchased from Sigma (St. Louis, MO, USA). Coon's modified Ham's F-12 medium (F-12 medium), Hanks' balanced salt solution (HBSS) and Earle's balanced salt solution (EBSS) were obtained from GIBCO BRL Laboratories Life Technologies (Grand Island, NY, USA). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT, USA). Collagenase type B was from Boehringer-Mannheim (Indianapolis, IN, USA). $^{51}Cr$ (sodium chromate) was purchased from ICN Biomedicals (Irvine, CA, USA). Tissue culture plates were purchased from Costar (Cambridge, MA, USA).

### 2.2. Cell culture

Colonic epithelial cells were isolated and cultured as previously described with some modifications (Hata et al., 1993). In brief, colonic epithelial cells from distal colon of the adult rabbits were scraped and minced. The tissues were incubated in basal medium Eagle containing crude collagenase type B (1 mg/ml) and bovine serum albumin (2 mg/ml) in a shaker bath at 100 cycles/min for 40 min. After incubation, cells were washed 5 times with Hanks' balanced salt solution. The cells were cultured with F-12 medium supplemented with 10% heat-inactivated (at 56°C for 30 min) fetal bovine serum, 15 mM Hepes buffer, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml fungizone and inoculated onto 24-well tissue culture plates.

### 2.3. Cytotoxicity

Cytotoxicity was quantified by measuring $^{51}Cr$ release from prelabeled cells as described previously (Hiraishi et al., 1986, 1987). Culture medium containing 5 μCi/ml $^{51}Cr$ was added to confluent monolayers. After overnight prelabeling, cells were washed three times with HBSS and then incubated either with 1 ml EBSS alone (control) or with 1 ml EBSS containing test agents under culture condition. 0.5 ml of supernatant buffer was removed at intervals for determination of $^{51}Cr$ release. Maximum $^{51}Cr$ release per sample was determined by incubation in 1 N NaOH. The percentage of $^{51}Cr$ release per sample was expressed as follows: $(A - B/C - B) \times 100\%$, where $A$ represents the mean test $^{51}Cr$ cpm released, and $B$ the mean spontaneous $^{51}Cr$ cpm released, $C$ the maximum $^{51}Cr$ cpm released. $^{51}Cr$ radioactivity was counted with a gamma counting system, Beckman 7000 (Beckman).

### 2.4. Assay of superoxide dismutase activity

The superoxide dismutase activity was determined by 60-mm dishes of lyzing cells in 0.2% Triton X-100, 50 mM potassium phosphate buffer, 0.1 mM EDTA, pH 7.8. An aliquot of the lysate was then assayed for superoxide dismutase by monitoring the inhibition of the reduction of ferricytochrome c at 550 nm as described previously (Flohe and Otting, 1984). The enzyme activity was expressed as units/mg protein as determined by the method of Bradford (Bradford, 1976).

### 2.5. Assay of glutathione reductase and catalase

Assay of glutathione reductase and catalase. Cultured cells in 60-mm dishes were solubilized by incubation with 0.2% Triton X-100 at room temperature for 1 h, frozen at −20°C, and the supernatant was assayed for the activity of glutathione reductase and catalase (Beutler, 1975). Glutathione reductase activity was determined by the oxidation of 2 mM NADPH, monitored at 340 nm. Catalase activity was determined by disappearance of 10 mM hydrogen peroxide, monitored at 230 nm. The activity of these enzymes was expressed as (m)U/mg protein.

### 2.6. Assay of reduced glutathione (GSH)

Reduced soluble sulfhydryl content in 35 mm dishes of cultured cells was measured by the method of Beutler et al. (Beutler et al., 1963), as described previously (Hiraishi et al., 1987, 1991). Because reduced glutathione (GSH) accounts for the majority of soluble-reduced thiols in cells (Kosower and Kosower, 1978), GSH levels in colonic cells were determined by measuring total soluble-reduced thiol content and expressed as $nM/10^6$ cells.

### 2.7. Statistical analysis

Data are presented as means ± standard error of the mean (S.E.M.). Analysis of variance and Student's *t*-test were used to assess the significance of difference; $P < 0.05$ was considered significant.

## 3. Results

### 3.1. Cell culture

By the fourth day of culture, the colonic cells reached confluency. The cultured cells consisted of a homogeneous population of epithelial cells with large, oval nuclei, polyhedral shape, and organized sheet-like growth pattern (Fig. 1). More than 95% of the cultured cells were stained with anti-cytokeratin antibody (AE1/AE3, Boehringer-Mannheim) which is specific to epithelial cells (Hata et al., 1993).

### 3.2. Cytotoxicity of hypoxanthine / xanthine oxidase

Xanthine oxidase (1–5 mM) plus hypoxanthine (1 mU/ml) dose dependently increased $^{51}$Cr release for 5 h incubation from prelabeled cells (Table 1).

Table 1
Cytotoxicity of hypoxanthine (1 mM) plus xanthine oxidase (0–5.0 mU/ml)

| Xanthine oxidase (mU/ml) | $^{51}$Cr release (%) |
|---|---|
| 0 | 0.0 ± 2.1 |
| 1.0 | 2.0 ± 1.5 |
| 2.5 | 32.1 ± 3.3 |
| 5.0 | 47.5 ± 3.6 |

Values represent the means ± S.E.M. of 6 determinations.

### 3.3. Effect of pretreatment with diethyldithiocarbamate on activity of cellular superoxide dismutase and cytotoxicity by hypoxanthine / xanthine oxidase

Pretreatment with diethyldithiocarbamate (50 μM, 1 h) significantly ($P < 0.01$) reduced activity of cellular superoxide dismutase from 1.32 ± 0.23 (control) to 0.86 ± 0.23 U/mg protein (mean ± S.E.M. of 6 determinations). Pretreatment with diethyldithiocarbamate (0.5–50 μM, 1 h) increased $^{51}$Cr release caused by hypoxanthine (1 mM) and xanthine oxidase (1 mU/ml) for 5 h incubation from prelabeled cells (Fig. 2).

### 3.4. Effect of pretreatment with diethyl maleate (0.05–1 mM, 30 min) or buthionine sulfoximine (1–50 μM, 17 h) on activity of cellular GSH and cytotoxicity by hypoxanthine / xanthine oxidase

Pretreatment with diethyl maleate (1 mM, 30 min) significantly ($P < 0.01$) reduced cellular GSH from 45.23 ± 2.29 (control) to 24.34 ± 2.42 nmol/mg protein (mean ± S.E.M. of 6 determinations). Pretreatment with buthionine sulfoximine (50 μM, 17 h) significantly ($P < 0.01$) decreased cellular GSH from 50.83 ± 7.19 (control) to 30.71 ± 4.70 nmol/mg protein (mean ± S.E.M. of 6 determinations). Pretreatment with diethyl maleate (0.05–1 mM, 30 min) enhanced $^{51}$Cr release during 5 h incubation from prelabeled cells by hypoxanthine/xanthine oxidase (Fig. 3) dose dependently. Pretreatment with buthionine sulfoximine (1–50 μM, 17 h) enhanced $^{51}$Cr release during 5 h incubation from prelabeled cells by hypoxanthine/xanthine oxidase in a dose dependent manner (Fig. 4).

Fig. 1. Phase-contrast photograph of cultured rabbit colonic epithelial cells (×40).

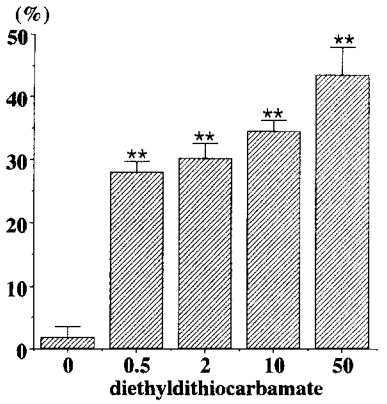


Fig. 2. Effect of diethyldithiocarbamate on cytotoxicity by hypoxanthine/xanthine oxidase. Pretreatment with diethyldithiocarbamate (0.5–50 μM, 1 h) increased $^{51}Cr$ release caused by hypoxanthine (1 mM) and xanthine oxidase(1 mU/ml) for 5 h incubation from prelabeled cells. ** $P < 0.01$, significant difference compared with control (diethyldithiocarbamate 0 μM) values.

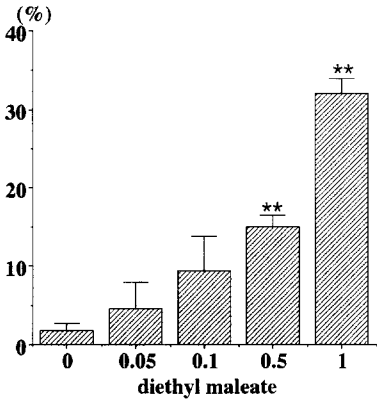


Fig. 3. Effect of diethyl maleate on cytotoxicity by hypoxanthine/xanthine oxidase. Pretreatment with diethyl maleate (0.05–1 mM, 30 min) enhanced $^{51}Cr$ release during 5 h incubation from prelabeled cells by hypoxanthine (1 mM) and xanthine oxidase(1 mU/ml). ** $P < 0.01$, significant difference compared with control (diethyl maleate 0 mM) values.

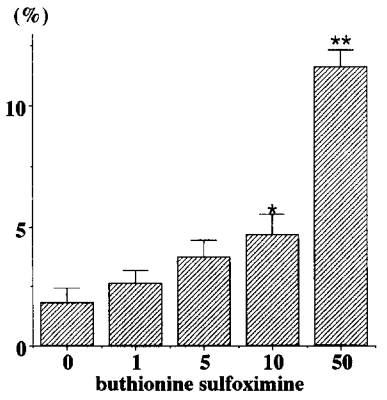


Fig. 4. Effect of buthionine sulfoximine on cytotoxicity by hypoxanthine/xanthine oxidase. Pretreatment with buthionine sulfoximine (1–50 μM, 17 h) enhanced $^{51}Cr$ release during 5 h incubation from prelabeled cells by hypoxanthine (1 mM) and xanthine oxidase (1 mU/ml). * $P < 0.05$; ** $P < 0.01$, significant differences compared with control (buthionine sulfoximine 0 μM) values.

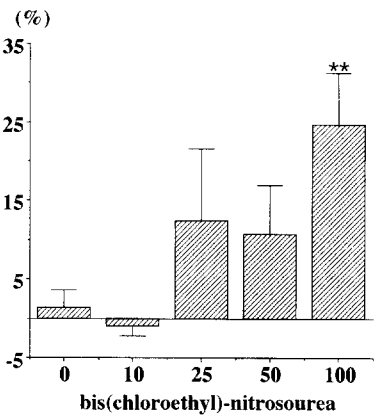


Fig. 5. Effect of bis(chloroethyl)-nitrosourea on cytotoxicity. Pretreatment with bis(chloroethyl)-nitrosourea (1–50 μg/ml, 15 min) enhanced $^{51}Cr$ release during 5 h incubation from prelabeled cells by hypoxanthine (1 mM) and xanthine oxidase (1 mU/ml). ** $P < 0.01$, significant difference compared with control (bis(chloroethyl)-nitrosourea 0 μg/ml) values.

### *3.5. Effect of pretreatment with bis(chloroethyl)-nitrosourea (1–50 μg/ml, 15 min) on activity of GSH reductase and cytotoxicity*

Pretreatment with bis(chloroethyl)-nitrosourea (50 μg/ml, 15 min) significantly ($P < 0.01$) inhibited activity of GSH reductase from 14.21 ± 1.11 (control) to 4.68 ± 0.51 IU/mg protein (mean ± S.E.M. of 6 determinations). Pretreatment with bis(chloroethyl)-nitrosourea (1–50 μg/ml, 15 min) enhanced $^{51}Cr$ release during 5 h incubation from prelabeled cells by hypoxanthine/xanthine oxidase (Fig. 5).

### *3.6. Effect of preincubation with aminotriazole (1–50 mM, 40 min) on cellular catalase and $^{51}Cr$ release*

Preincubation with aminotriazole (50 mM, 40 min) significantly ($P < 0.01$) reduced cellular catalase from 4.05 ± 1.04 (control) to 1.82 ± 0.75 U/mg protein (mean ± S.E.M. of 6 determinations), while preincubation with aminotriazole (1–50 mM, 40 min) did not affect $^{51}Cr$ release induced by hypoxanthine/xanthine oxidase (data not shown).

## 4. Discussion

Many studies suggest that reactive oxygen metabolites contribute to colonic epithelial cellular injury (Clark et al., 1988; Cueva and Hsueh, 1988; Von Ritter et al., 1989; Keshavarzian et al., 1990; Craven et al., 1986; Tanner et al., 1984; Hermanowicz et al., 1985). Little is known about the defense mechanism against reactive oxygen metabolites in these cells. We employed cultured rabbit colonic epithelial cells to exclude humoral, vascular, and neural factors. Hypoxanthine plus xanthine oxidase generates $H_2O_2$ and $O_2^-$. (Fridovich, 1970) as do activated neu-

trophils or macrophages in the in-vivo situation. These metabolites may be converted to $OH^{\cdot}$ which is more reactive and harmful. We reported in preliminary way that reactive oxygen metabolites generated by xanthine oxidase acting on hypoxanthine induce significant damage to cultured rabbit colonic cells in monolayer culture (Kawabe et al., 1992). Because this damage was prevented by catalase (an enzyme that detoxifies $H_2O_2$), $H_2O_2$ or its subsequent oxidizing species, such as $OH^{\cdot}$, has been hypothesized as the major mediator of oxygen metabolites-induced injury in this model.

Although superoxide dismutase is found intracellularly in almost all aerobes, its importance as an antioxidant is not certain. It has been reported that cultured endothelial cells with increased superoxide dismutase activity after incubation with native superoxide dismutase were more resistant to killing by $H_2O_2$ and activated neutrophils (Markey et al., 1990). In contrast, incubation of endothelial cells with polyethylene glycol-conjugated superoxide dismutase failed to protect cells from xanthine oxidase-induced $^{51}Cr$ release, although the manipulation increased cellular superoxide dismutase by fourfold (Beckman et al., 1988). Microinjection of superoxide dismutase into endothelial cells was also shown not to be protective against hyperoxia-induced toxicity (Michiels et al., 1990). In microorganisms, on the contrary, superoxide dismutase-rich *E. coli* was less resistant to paraquat (an intracellular $O_2 \cdot$ generator), hyperoxia, or radiation (Scott et al., 1987, 1989). Thus, it is possible that the protective role of cellular superoxide dismutase against oxidant may depend on cell types. Alternatively, the balance of antioxidant defense functions may be more important than simple adjustments of individual parts of the system (Scott et al., 1989). Diethyldithiocarbamate has been shown to inhibit Cu, Zn-superoxide dismutase activity by chelating copper ion, and active center of the enzyme both in vivo and in vitro (Heikkila, 1985; Heikkila et al., 1976). We too found that pretreatment with diethyldithiocarbamate significantly reduced activity of superoxide dismutase. We also found that pretreatment with diethyldithiocarbamate sensitized cells against reactive oxygen metabolites caused by hypoxanthine/xanthine oxidase. Therefore, intracellular superoxide dismutase may play a major role against reactive oxygen metabolites in cultured colonic epithelial cells, but the effect of diethyldithiocarbamate at differing concentrations on superoxide dismutase level needs to be determined to document the minimal level of superoxide dismutase required for normal defense.

The importance of the glutathione redox cycle as a potent antioxidant defense system has been described in a variety of tumor cells (Nathan et al., 1980; Arrick et al., 1982), endothelial cells (Suttorp et al., 1986), and epithelial cells (Hiraishi et al., 1991; Andreoli et al., 1992). In the present study, we have examined the relative importance of the glutathione redox cycle and cellular catalase as antioxidant defenses in cultured colonic epithelial cells.

Glutathione reductase was inhibited by pretreatment with bis(chloroethyl)-nitrosourea. And this agent rendered these cells more susceptible to cellular damage caused by hypoxanthine/xanthine oxidase. Pretreatment with diethyl maleate or buthionine sulfoximine decreased cellular GSH dose dependently. Diethyl maleate covalently binds GSH as catalyzed by endogenous glutathione *S*-transferase, and buthionine sulfoximine is a selective inhibitor of γ-glutamylcysteine synthetase. Pretreatment with both diethyl maleate and buthionine sulfoximine increased cytotoxicity caused by hypoxanthine/xanthine oxidase. Impairment of glutathione redox cycle at three sites independently increased cytotoxicity caused by hypoxanthine/xanthine oxidase. Thus, we suggested that glutathione redox cycle may play a role in cell defense against reactive metabolites.

Even though diethyl maleate at 1 mM and buthionine sulfoximine at 50 μM resulted in similar GSH depletion, 46% and 40% respectively, their effect on cytotoxicity was different. Diethyl maleate at 1 mM caused approximately 30% release of $^{51}Cr$, while buthionine sulfoximine at 50 μM caused only 12% release of $^{51}Cr$. These data suggest that GSH depletion may not be the only cause of increased cytotoxicity by pretreatment with diethyl maleate.

Pretreatment with aminotriazole reduced intracellular catalase by 45%. However, pretreatment with aminotriazole did not diminish the resistance of the colonic epithelial cells against hypoxanthine/xanthine oxidase. It is possible that 45% of normal catalase is enough to defend cells adequately. Thus, though the evidence is not conclusive, the present study suggests that endogenous catalase, an enzyme with a much higher $H_2O_2$-metabolizing capacity than the glutathione redox cycle (Chance et al., 1979), may not be involved in protecting colonic cells against reactive oxygen metabolites caused by hypoxanthine/xanthine oxidase. Our data are consistent with those of previous studies using renal cell lines or gastric cells. Disruption of glutathione redox cycle resulted in lytic injury to some types of renal epithelial cells, whereas inhibition of catalase did not affect the renal epithelial cells (Andreoli et al., 1992). Hiraishi et al. suggested that the GSH redox cycle, rather than endogenous catalase, plays a critical role in intracellular antioxidant defense in cultured gastric mucous cells (Hiraishi et al., 1991).

The reasons for the greater antioxidant effect of the glutathione redox cycle compared with that of endogenous catalase in cultured colonic epithelial cells remain undetermined. There may be several possibilities for this phenomenon. First, a compartmentalization within the cell is conceivable, with catalase and the glutathione redox cycle metabolizing the $H_2O_2$ of different sources in the cell, as has been shown for hepatocytes. In these cells, catalase is concentrated in peroxisomes, while the glutathione redox cycle is localized in the cytosol and mitochondria. Second, catalase displays a substantially higher $K_m$ for $H_2O_2$

(Flohe, 1979), suggesting that the glutathione redox cycle is operating at moderate intracellular $H_2O_2$ concentrations; when large amounts of $H_2O_2$ are added to colonic epithelial cells as bolus, intracellular catalase may very well gain an important defensive role, as has been shown experimentally for tumor cells (Arrick et al., 1982). Finally, the activity of endogenous catalase may vary in each cell type in vitro. In this study, cultured colonic epithelial cells contained 4.05 U/mg protein of catalase activity. In contrast, inhibition of catalase activity by aminotriazole in cultured hepatocytes, which contains much higher activity of catalase ( ~ 80 U/mg protein, unpublished observation), significantly enhanced oxygen metabolites-induced damage (Starke and Farber, 1985). Thus the cellular activity of catalase in each cell type may determine whether the enzyme can efficiently detoxify $H_2O_2$. Because reactive oxygen metabolites are involved in injury to the colon such as necrotising colitis or inflammatory bowel diseases, endogenous antioxidant defense mechanisms such as the GSH redox cycle may be critical in preventing oxidant-related colonic injury as a cellular antioxidant in vivo as well.

In conclusion, both cellular superoxide dismutase and the GSH redox cycle appear to play a role in detoxifying reactive oxygen metabolites. Cellular catalase may be less important in colonic epithelial cells.

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