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EFFECT OF OXIDANT EXPOSURE ON THIOL STATUS IN THE INTESTINAL MUCOSA

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Abstract—The intestinal epithelium is likely to be exposed to oxidants derived from diet, bacterial metabolic products or endogenously formed cellular metabolites. Glutathione, being the major intracellular thiol, provides protection against oxidative injury. The present study looks at the effect of luminal exposure to oxidants on intestinal thiol status. Different oxidants were placed in the lumen of anaesthetized rats for 30 min and the mucosal non-protein and protein-bound thiols were quantitated using HPLC. Oxidant exposure altered the thiol redox status and increased the oxidized glutathione (GSSG) level 6–7-fold and protein-bound reduced glutathione (GSH) 2-fold, whereas the non-protein GSH level remained the same or increased slightly. This could be due to either increased mucosal GSH synthesis or transport from circulation. After incubation with buthionine sulfoximine, a specific inhibitor of GSH synthesis, prior to oxidant exposure, the same increase in GSSG and GSH in the mucosa was seen. Oxidant exposure also decreased the plasma GSH level by 50%. The other cellular non-protein thiols, cysteine and cystine, did not change after luminal exposure to oxidants. These results suggest that oxidant exposure stimulates uptake of GSH from the circulation to maintain mucosal GSH.

Oxygen-derived free radical-mediated tissue damage is a well-known phenomenon, and the damage can be prevented by the presence of various protective mechanisms in the cell. Thiol compounds can protect the cell from oxidative stress and glutathione is the most abundant low molecular mass cellular thiol which has been implicated in the removal of electrophiles and oxidants by acting as a co-substrate for glutathione *S*-transferase [1] and reduced glutathione (GSH†)-dependent peroxidase [2]. Depletion of intracellular GSH could sensitize the cells to oxidative injury [3]. It has been shown that under conditions of oxidative stress, formation of oxidized glutathione (GSSG) increases many fold [4]. A linear correlation was observed between loss of cell viability and loss of critical-SH groups present in membranes and enzymes. Studies have suggested that cells can be protected from oxidative injury by supplementing the incubation medium with GSH [5, 6].

Intestinal epithelium is likely to be exposed to various toxic compounds originating from diet, bacterial metabolites, ingested drugs and oxidants formed during metabolism. These compounds can generate free radicals on their own or during reaction with other compounds. Like other tissues, the intestinal epithelium is also equipped with defense mechanisms to inactivate these reactive oxygen species. The presence of glutathione in the intestinal mucosal cells has been reported [7] and GSH depletion studies have indicated an important function for GSH in the intestinal epithelium [8]. *In vitro* studies with isolated epithelial cells have shown that glutathione supplementation can protect these cells from oxidative injury [5]. In the present study, the intestinal epithelium was exposed *in vivo* to a free radical generating system by instilling various pro-oxidants in the lumen, and the thiol status of the mucosal cells was assessed.

MATERIALS AND METHODS

Glutathione (oxidized and reduced forms) *t*-butyl hydroperoxide, menadione, xanthine, xanthine oxidase, buthionine sulfoximine (BSO), 1-fluoro-2,4-dinitrobenzene, Tris and bovine serum albumin were all purchased from the Sigma Chemical Co. All other chemicals used were analytical grade.

Luminal exposure of oxidants. Overnight-fasted Wistar rats weighing 180–200 g were anesthetized with pentobarbitone (50 mg/kg). The abdomen was opened with a midline incision, and a 30–40 cm segment of the small intestine was isolated and gently flushed with normal saline. The segment was cannulated proximally and distally, and returned to the abdomen, which was then closed. Iso-osmotic solution containing various oxidants in 0.9% NaCl was instilled in the intestinal segment which was clamped at both ends. The oxidants used were (all final concentrations) in 0.9% NaCl pH 7.0: (1) 1 mM H_2O_2, (2) 50 μM menadione (3) 100 μM *t*-butylhydroperoxide and (4) 1 mM xanthine + 10 mU xanthine oxidase. Control animals were also cannulated and the lumens were exposed to 0.9% NaCl. The body temperature was maintained with an overhead lamp. Following 30 min incubation, the luminal solution was flushed out, the intestine excised immediately and the mucosa scraped using a glass slide. For thiol estimation, the mucosa was immediately homogenized in 5% trichloroacetic acid (TCA) and centrifuged at 15,000 *g* for 30 min.

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† Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; BSO, buthionine sulfoximine; TCA, trichloroacetic acid.

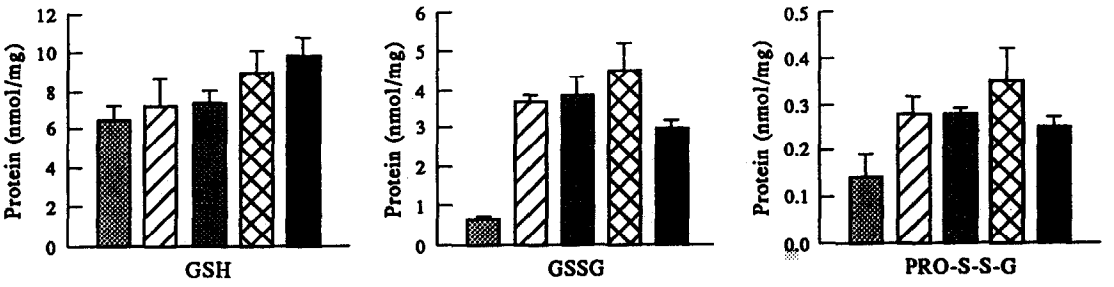

Fig. 1. Effect of luminal exposure of oxidants on intestinal mucosal levels of GSH, GSSG and protein-bound GSH (PRO-S-S-G). Details of the methodology are given in the text. ▨ control (0.9% saline); ▨ 1 mM hydrogen peroxide; ▨ 1 mM xanthine + 10 mU xanthine oxidase; ▨ 50 μM menadione; ▨ 100 μM *t*-butyl hydroperoxide. Each value represents mean ± SD of three separate estimations.

Mitochondria were isolated from mucosal homogenate prepared in 0.3 M sucrose adjusted to pH 7.4 [9], and the purity was judged by the marker enzyme, succinate dehydrogenase [10]. Mitochondrial thiols were determined after deproteination with TCA. Mucosal thiols were also assessed, after GSH depletion with BSO followed by oxidant exposure. This was done by preincubating the lumen with 1 mM BSO for 60 min followed by incubation with oxidants.

Glutathione estimation. Glutathione in the acid supernatant was quantitated using HPLC after derivatization as described [11]. The derivatization procedure included reaction of iodoacetic acid with thiols to form *S*-carboxymethyl derivatives followed by chromaphore derivatization (dinitrophenyl) of primary amines with 1-fluoro-2,4-dinitrobenzene. Dinitrophenyl derivatives were separated on a Ultrasil NH_2 column using a gradient of methanol and sodium acetate, and detected at 365 nm. For plasma GSH estimation, blood was collected in heparinized tubes from the orbital plexus, plasma was separated, proteins were precipitated with TCA and GSH in the acid supernatant was assayed by HPLC.

Assay of protein-bound glutathione. Protein pellet obtained from TCA precipitation was washed twice with absolute ethanol, suspended in 0.5 mL of 50 mM MOPS buffer pH 8.0 containing 25 mM dithiothreitol, sonicated for 30 sec and incubated for 60 min at 37° [12]. Proteins were precipitated with 20% TCA and GSH in the acid supernatant was quantitated by HPLC.

Estimation of cysteine and cystine. Cysteine and cystine were estimated colorimetrically using acid ninhydrin reagent [13]. Protein-bound cysteine was released by dithiothreitol and cysteine in the acid supernatant was estimated. Protein was estimated using bovine serum albumin as standard [14].

RESULTS

Exposure of intestinal lumen *in vivo* to various oxidants and pro-oxidants caused 5–7-fold increase in mucosal GSSG concentration as compared to the control (Fig. 1). Simultaneously, there was a 2-fold increase in the concentration of protein GSH mixed disulfide in the mucosa. There was no decrease in the mucosal GSH concentration on exposure to oxidants; in fact, a slight increase was observed. These alterations in thiol concentration resulted in a change in the thiol redox status of the mucosal cells. Cysteine and cystine are the other low molecular mass thiols present in the cell and there was no significant change in their concentration after exposure to oxidants (Fig. 2). A small percentage of the total cellular GSH is present in the mitochondria which has been shown to be resistant to alteration by chemical depleting agents. After exposure to H_2O_2 and menadione, there was a 70% decrease in GSH concentration with no corresponding increase in GSSG levels (Table 1) in the mitochondria. In contrast, the total cellular GSH and GSSG levels were both increased on exposure to oxidants.

The increase in GSH and GSSG in mucosal epithelium on exposure to oxidants could be due to either an increase in cellular GSH synthesis or transport of circulating GSH into mucosal cells followed by oxidation to GSSG. To test this, BSO, an inhibitor of GSH synthesis, was used. The lumen of the intestine was first exposed to BSO for 60 min followed by 30 min exposure to oxidants. Both tissue and plasma GSH were measured under these conditions. As shown in Fig. 3, BSO treatment alone decreased the GSH content of the mucosa by 50% as compared to untreated control, and this treatment did not increase GSSG or protein-bound GSH. On the other hand, H_2O_2 or menadione exposure after BSO treatment restored the GSH level to that of the control and an increase in GSSG and protein-bound GSH was also observed. There was no change in the level of cysteine or cystine on BSO treatment followed by oxidant exposure. An interesting observation was that H_2O_2 or menadione treatment reduced the plasma level of glutathione by 50%, suggesting possible transfer of plasma glutathione to the mucosal epithelium. Due to rapid oxidation of GSH to GSSG, we were able to detect plasma glutathione only as GSSG. It was earlier reported that rat plasma GSH is oxidized very rapidly even after taking many precautions [15]. In the present study, it was possible to obtain the blood and separate the plasma and acid precipitate within

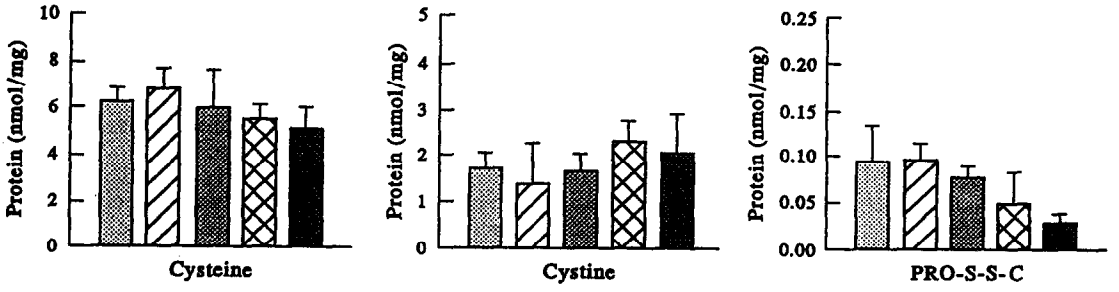

Fig. 2. Effect of luminal exposure of oxidants on intestinal mucosal levels of cysteine, cystine and protein-bound cysteine (PRO-S-S-C). ▨ control (0.9% saline); ▨ 1 mM hydrogen peroxide; ▩ 1 mM xanthine + 10 mU xanthine oxidase; ▩ 50 μM menadione; ■ 100 μM *t*-butyl hydroperoxide. Each value represents mean ± SD of three separate estimations.

Table 1. Effect of oxidants on intestinal mitochondrial glutathione

	Total cellular		Mitochondrial	
	GSH	GSSG	GSH	GSSG
	(nmol/mg protein)			
Control (saline)	6.48 ± 0.66	0.60 ± 0.04	0.160 ± 0.008	0.021 ± 0.002
H_2O_2 (1 mM)	7.22 ± 1.33	3.68 ± 0.16	0.051 ± 0.007	ND
Menadione (50 μM)	9.01 ± 1.07	4.56 ± 0.64	0.052 ± 0.004	ND

ND, not detectable.
Each value represents mean ± SD of three separate estimations.

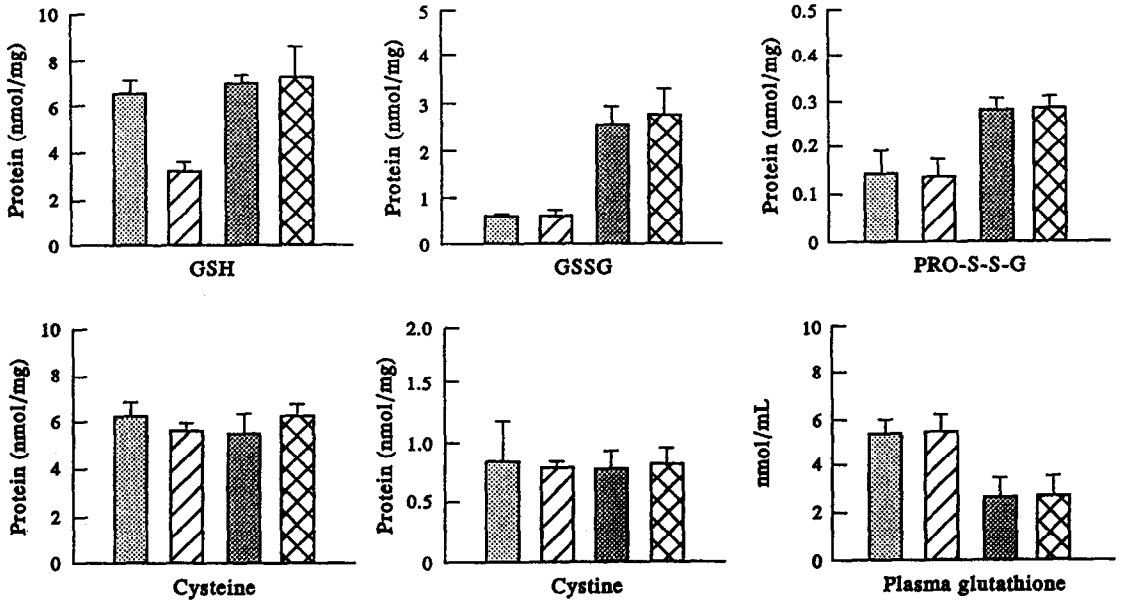

Fig. 3. Effect of oxidant exposure with prior treatment with BSO on the thiol status of intestinal mucosa and plasma glutathione levels. Intestinal lumen was incubated with 1 mM BSO for 60 min followed by oxidants H_2O_2 (1 mM) or menadione (50 μM) for 30 min. Thiols were quantitated as described in the text. ▨ control (0.9% saline); ▨ 1 mM BSO; ▩ 1 mM BSO + 1 mM hydrogen peroxide; ■ 1 mM BSO + 50 μM menadione. Each value represents mean ± SD of three separate estimations. PRO-S-S-G, protein-bound glutathione.

5 min, and still the plasma glutathione was seen only as GSSG even in control rats.

DISCUSSION

The intestinal epithelium is likely to be exposed to oxidants derived from diet, bacterial metabolic products or endogenously formed cellular metabolites. The presence of detoxifying enzymes such as GSH peroxidase, GSH transferase and superoxide dismutase in the intestinal epithelium might offer protection against these toxic compounds [16]. Glutathione is the most important non-protein thiol involved in the removal of oxidants and xenobiotics. This tripeptide plays an important role in protection against tissue damage induced by oxygen [17–19] and also against radiation, UV and photodynamic effects.

The present study looks at the effect of various oxidants on the status of mucosal glutathione and possible protection offered by circulating glutathione. Exposure of intestinal lumen to various oxidants showed an increase in the mucosal level of GSSG along with a small but significant increase in GSH. Formation of oxidants such as H_2O_2 *in vivo* is possible during oxidative metabolism and can be injurious to cells after conversion to hydroxyl radical. Menadione is a quinone which may be formed during bacterial metabolism and can deplete intracellular GSH by arylation [20]. Intestinal epithelium is known to have high xanthine oxidase activity which could generate free radicals [21]. In ischemia/reperfusion injury, xanthine oxidase has been implicated as the possible source of free radicals [22]. Increased oxidation of glutathione by these oxidants could increase the protein S-thiolation of cellular proteins [17]. Analysis of protein-bound GSH showed a 2-fold increase after exposure to oxidants. Protein GSH mixed disulfide formation may affect cellular metabolism. It is known that protein S-thiolation alters the activity of certain enzymes such as adenylate cyclase, phosphofructokinase and phosphorylase phosphatase [23].

Oxidant exposure did not affect the cellular content of other low molecular mass thiols, such as cysteine and cystine, indicating that these thiols may not be involved directly in protection against oxidants although it may be utilized for GSH synthesis. No change in protein-bound cysteine was observed after oxidant exposure.

Although the GSSG level increased nearly 7-fold after oxidant exposure, no concomitant decrease in mucosal GSH was observed. This resulted in alteration of the thiol redox status in the mucosa. The observed increase in mucosal GSH may be due to either increased cellular synthesis or transport from circulation. Inhibition of *de novo* synthesis of GSH by BSO showed a decrease in mucosal GSH. However, oxidant exposure after BSO treatment still caused an increase in mucosal GSH. This increase is probably brought about by uptake of GSH from circulation. Intestinal epithelium is known to receive GSH from the diet as well as biliary secretions via the transport system present in the brush border membranes [24, 25]. In the present study, the portion of the intestinal segment used was devoid of bile duct openings and the instillation medium was free of GSH, which excludes the possibility of GSH uptake from the lumen.

Studies have shown that the liver is the major organ involved in GSH synthesis and that this GSH is transported to extra-hepatic tissues [26]. It has been shown in isolated kidney cells that the Na^+-dependent uptake system present in the basolateral membranes is responsible for transport of extracellular GSH to support detoxication [27]. This transport is sensitive to probenecid, an organic anion transport inhibitor which inhibits the basolateral membrane transport system. Probenecid-inhibitable electrogenic and Na^+-dependent transport of GSH was also demonstrated in intestinal epithelial cells [5]. Incubation medium containing a physiological concentration of GSH protects cells from oxidative injury. This protection was eliminated in the presence of probenecid, indicating transport through basolateral membranes. In the present study, it was observed that luminal exposure of oxidants reduced the plasma GSH level by 50% with a simultaneous increase in mucosal GSH and GSSG, suggesting that GSH may be transported from the plasma in order to maintain the mucosal GSH level, in spite of increased oxidation to GSSG. Possibly, oxidant exposure stimulates this transport process. The relative importance of two sources of GSH depends upon the availability of GSH in the lumen and plasma. Under conditions where the luminal contents are free of GSH as in the post-absorptive state, uptake from plasma may take place.

A small percentage of cellular GSH is present in the mitochondria, which is relatively stable and is not altered during depletion with chemical agents [28]. It was seen here that the mitochondrial pool of GSH was susceptible to oxidant exposure and nearly 70% of GSH was utilized with no concomitant increase in GSSG. It is likely that the oxidized GSSG is transported out of the mitochondria as soon as it is formed and hence it was not detected in these organelles. In conclusion, this study shows that intestinal mucosal GSH is oxidized during oxidant stress and the GSSG generated forms mixed disulfide with the cellular proteins. Mucosal depletion of GSH in the cell is possibly replaced by uptake of circulating GSH.

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